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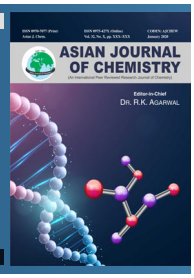

Equilibrium, DNA Cleavage and Antimicrobial Studies of Cu(II) and Zn(II) Complexes with Novel 1-Propionyl-4-methyl-3-thiosemicarbazide

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Cu(II) and Zn(II) metal complexes with novel Schiff base 1-propionyl-4-methyl-3-thiosemicarbazide (PMTSC) were synthesized and characterized by LC-MS, IR, ^{1}H NMR (D_2O exchangeable), ^{13}C NMR and UV-visible spectra, ESR, TGA, molar conductance and magnetic susceptibility measurements. The spectro-analytical studies revealed the composition of complexes as ML_2 for Cu(II)-PMTSC and Zn(II)-PMTSC complex. The proton-ligand dissociation constant and metal-ligand formation constants of PMTSC with Zn(II) have been determined in 70 % (v/v) DMF-water medium at 0.1M (KNO_3) ionic strength and 303 K using potentiometric Irving-Rossotti titration technique. The results revealed that PMTSC acts as monobasic ligand by releasing proton from amide *via* enol form and forms 1:2 (Zn-L) complex in solution. The cleavage of plasmid pBR322 DNA without any additives was monitored by gel electrophoresis and these complexes exhibited hydrolytic cleavage of plasmid DNA. The antibacterial activity of the Schiff base and its Cu(II) and Zn(II) complexes were tested against Gram-positive and Gram-negative bacteria.

Keywords: Schiff base, Copper(II), Zinc(II), Complexes, Thiosemicarbazide.

INTRODUCTION

Acylthiosemicarbazides represent versatile synthons for various syntheses of nitrogen heterocycles. Because of the acylthiosemicarbazide moiety, cyclocondensations and addition-cyclization reactions can be performed. The reaction products of acylthiosemicarbazides, such as triazoles [1,2], thiazolidinones [3] and imidazolidinediones [4], are all highly active pharmaceuticals [1-7]. Novel thiosemicarbazide derivatives were synthesized and evaluated for their anticonvulsant activity and neurotoxicity [8].

Equilibrium studies assist in the determination of dissociation constant of ligands and stability constants of their metal complexes in solution. Recently, studies on metal complexes in the solid and solution states are gaining importance because the complexes exhibit a variety of properties and vast applications in different fields of biology and chemistry. Nucleic acid cleavage may be considered an enzymatic reaction that consists of multiple biological processes and it is highly useful in the biotechnological manipulation of genetic material [9]. Designing new metal complexes that specifically bind to DNA and cause its cleavage are crucial in the development of new anti-tumor agents [10,11]. In particular, reagents that cleave nucleic acids are gaining considerable scientific interest in the field of artificial metallonucleases [12,13]. Cu(II) complexes exhibit higher reactivity in DNA cleavage than other transition metal complexes. Consequently, they have attracted the greatest scientific interest and multiple Cu(II) complexes have been synthesized as artificial nucleases [14-17].

EXPERIMENTAL

All the chemicals and solvents were of AnalaR grade and used without further purification. Metal chloride salts [MCl_2 M = Cu(II) and Zn(II)] were used for the synthesis of complexes. Conductance of the metal complexes was determined in DMSO (1×10^{-3} M) using Digisun digital conductivity meter model D1 909. LCMS of all the compounds were recorded on LCMS 2010 A, Shimadzu spectrometer. IR spectra were recorded in KBr phase (4000 cm^{-1} to 250 cm^{-1}) on Shimadzu IR prestige-21 FTIR spectrophotometer. ^{1}H NMR, ^{13}C NMR and D_2O exchangeable ^{1}H NMR spectra were taken on Bruker 400 mHz NMR spectrophotometer. UV spectra were obtained from Shimadzu

UV2450 spectrophotometer with in the range of 200-1000 nm. ESR spectrum was obtained from EMX-PLUS-BRUKER X-band RT spectrometer. Magnetic susceptibilities were measured at room temperature on Faraday balance model 7550. Thermo gravimetric analyses of the complexes were carried out on TA model DTG 60 H Shimadzu in temperature range of 0-1100 ºC with a ramp of 20 ºC/min. A digital Elico (L1-120) pH meter with a combined glass and calomel electrode was used for equilibrium studies. DNA cleavage experiments were performed with the help of Biotech electrophoresis system supported by Genei power supply over a potential range of 50-500 V, visualized and photographed by Biotech Transilluminator system.

Synthesis of 1-propionyl-4-methyl-3-thiosemicarbazide (PMTSC): To 2.10 g (20 mmol) of 4-methyl-3-thiosemicarbazide, 1.49 mL of propionic acid (20 mmol) and 2.5 mL of propionic anhydride (20 mmol) were added and refluxed for 1 h in water-bath. The progress of the reaction was monitored by TLC. On cooling after 20 min, white crystalline solid of PMTSC started separating out, which was recrystallized using ethanol and water in 3:1 ratio (**Scheme-I**). The compound was soluble in methanol, ethanol and DMF (m.p.: 162-164 ºC).

Synthesis of metal complexes: Aqueous metal salt solution (Cu(II) 0.26 g, 1.55 mmol, Zn(II) 0.21 g, 1.55 mmol) was added to hot methanolic solution of PMTSC (0.5 g, 3.10 mmol) [MCl_2 M = Cu(II) and Zn(II)] in 1:2 (M:L) molar ratio and the mixture was refluxed for 5-10 h. The pH of the solution was adjusted by addition of few drops of methanolic ammonium hydroxide. The dark green coloured Cu(II)-PMTSC complex and whitish coloured Zn(II)-PMTSC complex were separated. The solid complexes formed were filtered in hot condition, washed with hot methanol and double distilled water to remove unreacted ligand and metal salts respectively, then washed with petroleum ether and finally dried in vacuum.

Equilibrium studies: To understand the chelation properties of PMTSC in solution, an attempt is made to study its potential donor sites that bind with metal ions. Irving-Rossotti pH titration technique [18-20] was employed for the determination of dissociation constant of PMTSC and its stability constants with Zn(II) ions in 70 % v/v DMF-water medium at 303 K and 0.1M KNO_3 ionic strength [21-25].

DNA cleavage studies: pBR322 plasmid DNA dissolved in buffer containing 10 mM *tris*-HCl (pH 7.5), 1 mM EDTA and 0.1% sodium azide was used. TAE buffer (pH 8.0; 40 mM tris base, 20 mM acetic acid and 1 mM Na_2EDTA) was used for gel electrophoresis. 3 µL of Super coiled pBR322 DNA (100 ng/µL) was treated with the complexes (15-45 µM), incubated for 1 h at 37 ºC and loaded onto a 0.8 % agarose gel after addition of 1 µL loading buffer (0.25 % bromophenol blue). This was subjected to electrophoresis at 60 V for 2 h until bromophenol blue had travelled through 75 % of gel. Later, gel was stained with ethidium bromide (EB) and then destained in sterile distilled water. The plasmid bands were visualized under trans illuminator and photographed.

Biological activity: Kirby-Bauer disc diffusion method was followed to test the biological activity of all the synthesized compounds in 0.10 mL of test bacteria [(*Staphylococcus aureus*, *Bacillus subtilis* (Gram-positive) and *Klebsiella pneumoniae*, *Escherchia coli* (Gram-negative)]. Bacterial solutions were spread evenly on the surface of nutrient agar. Sterile discs of 5 mm diameter with capacity of 5 mL dipped in sample solutions are placed on the surface equidistance. Potency of all the samples tested was 1250 µg/disc. All the petriplates were incubated for 24 h at 37 ºC. All these tests were run in triplicates.

RESULTS AND DISCUSSION

Characterization of PMTSC

LC-MS: Liquid chromatogram (Fig. 1) of PMTSC showed a single peak with retention time of 0.923 min indicating its purity.

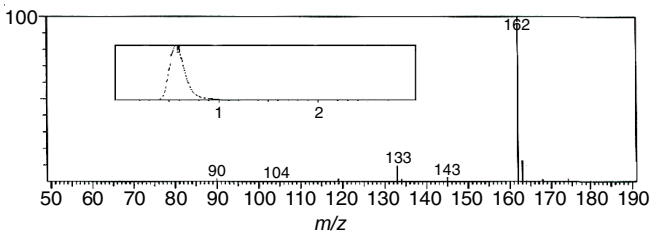
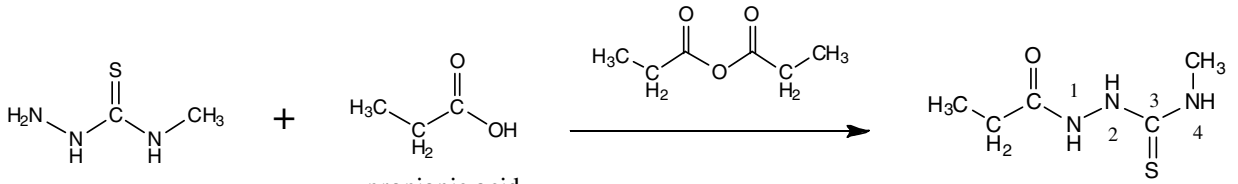


Fig. 1. LC-mass spectrum of PMTSC

Mass: Mass spectrum of PMTSC revealed molecular ion peak $[M+1]^+$ at *m/z* 162. Other fragmentation peaks were observed at *m/z* 145 $[C_4H_7N_3OS]^+$, *m/z* 133 $[C_3H_6N_3OS]^+$, *m/z* 104 $[C_2H_6N_3S]^+$, *m/z* 90 $[C_2H_5N_2S]^+$ and at *m/z* 72 $[C_3H_6NO]^+$.

IR: IR spectrum of PMTSC showed characteristic bands at 3300 cm^{-1} ν(N_4-H), 3193 cm^{-1} ν(NH amide), 3039 cm^{-1} ν(N-H thioamide), 2979 cm^{-1} ν(CH_2), 2942 cm^{-1} ν(CH_3), 1685 cm^{-1} ν(C=O), 1284 cm^{-1} ν(C=S), 1066 cm^{-1} ν(C-C) and 935 cm^{-1} ν(N-N) [26,27].

^{1}H NMR: ^{1}H NMR spectrum of PMTSC exhibited peaks at δ 9.59 ppm (s, 1H, OH), δ 9.11 ppm (s, 1H, NH), δ 4.13 ppm (s, 1H, NH), δ 3.61 ppm (s, 1H, SH), δ 2.85-2.88 ppm (s, 3H, $NHCH_3$), δ 2.17 ppm (s, 2H, CH_2), δ 2.09 ppm (s, 1H, NH), δ 0.97-1.19 ppm (s, 3H, CH_3). The integration in the spectrum indicated the number of protons as eleven in PMTSC. However, two protons were found to be present in two forms. More number of peaks observed as against expected from number of hydrogens present can be interpreted by keto as well as enol forms. Chemical shifts of NH and NH_2 protons were supported by their ready exchange with D_2O.



Scheme-I: Synthesis of 1-propionyl-4-methyl-3-thiosemicarbazide (PMTSC)

^{13}C NMR: ^{13}C NMR analysis of PMTSC revealed peaks at δ 9.67 ppm (CH_3), δ 26.4 ppm (CH_2), δ 30 ppm ($NHCH_3$), δ 173-178 ppm (C=O), δ 181-182 ppm (C=S) each signal of C=O and C=S splits into two indicating keto-enol tautomerism. ^{1}H and ^{13}C NMR studies indicated keto-enol tautomerism in PMTSC.

UV-Visible: The UV-visible spectrum of PMTSC showed (Fig. 2) bands at 269 nm (37,174 cm^{-1}) corresponds to C=O (n→π*) and 207nm (48,309 cm^{-1}) corresponds to C=S (n→π*).

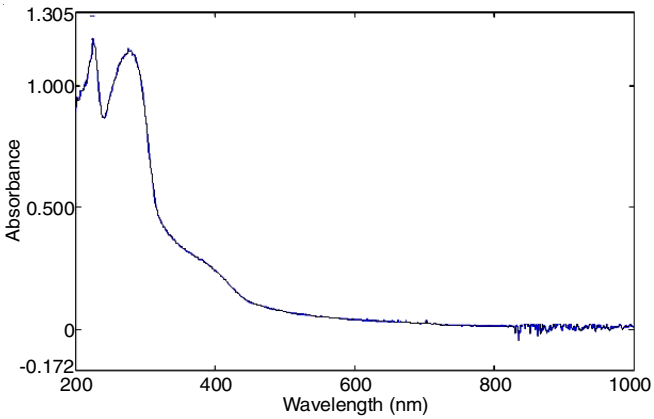


Fig. 2. UV-visible spectrum of PMTSC

Equilibrium studies: From the titration data (Table-1), a dissociation constant has been calculated by plotting linear graphs of log $(1 - \bar{n}_A)/(\bar{n}_A)$ *vs.* pH. The results indicated the presence of one dissociable proton corresponding to amide (-NH) proton *via* enol formation (pK_a = 10.44). Values of $\bar{n}$ ranges from 0.2 to 1.9 (Table-2), indicating the formation of 1:1 and 1:2 complexes in solution. The stability constant of binary complex was calculated from linear plots of log $[(1 - \bar{n})/\bar{n}]$ *vs.* pL and log $(2 - \bar{n})/(\bar{n} - 1)$ *vs.* pL. The metal ligand complex formation complexes were obtained as log k_1 = 7.98, log k_2 = 7.17. These values were further refined by using MINIQUAD program [26].

TABLE-1
DISSOCIATION CONSTANT VALUES OF
1-PROPIONYL-4-METHYL-3-THIOSEMICARBAZIDE

pH	$\bar{n}_A$	$\log(1-\bar{n}_A)/(\bar{n}_A)$
10.2	0.6216	-0.2155
10.3	0.5818	-0.1433
10.4	0.5222	-0.0385
10.5	0.4824	0.0305
10.6	0.4228	0.1351
10.7	0.3633	0.2436
10.8	0.3136	0.3402

Characterization of metal complexes of PMTSC: Cu(II) and Zn(II) complexes of PMTSC were quite stable to air and moisture, amorphous and also soluble in DMF and DMSO solvents. Molar conductivities recorded in DMSO were in the range of 6-8 $ohm^{-1}cm^{-1}mol^{-1}$ suggesting the non-electrolytic nature of the complexes.

LC: Liquid chromatograms of Cu(II) and Zn(II)-PMTSC showed a single peak with retention time at 0.773 min and 0.765 min respectively indicating the purity of the complexes.

TABLE-2
STABILITY CONSTANT VALUES OF
Zn(II)-PMTSC IN 70 % v/v DMF-WATER MEDIUM
AT 303 K AND 0.1 M IONIC STRENGTH

$\bar{n}$	$\log(1-\bar{n})/(\bar{n})$	PL	$\bar{n}$	$\log(2-\bar{n})/(\bar{n}-1)$	PL
0.25	0.4771	8.27	1.16	0.7201	7.45
0.35	0.2688	8.15	1.33	0.3075	7.30
0.45	0.0871	8.04	1.59	-0.1580	7.12
0.56	-0.1047	7.93	1.66	-0.2880	7.06
0.60	-0.1761	7.88	1.73	-0.4319	7.02
0.70	-0.3680	7.76	1.80	-0.6020	6.94
0.75	-0.4771	7.70	1.91	-1.0048	6.81
0.85	-0.7535	7.54	–	–	–

Mass: Mass spectrum of Cu(II)-PMTSC recorded at APCI positive mode showed molecular ion peak at *m/z* 420.5 $[ML_2 2H_2O]^+$, at *m/z* 402.5 $[ML_2{\cdot}H_2O]^+$, *m/z* 384.5 $[ML_2]^+$ and at *m/z* 225 $[ML+1]^+$. Similarly, APCI positive mode mass spectrum of Zn(II)-PMTSC showed a molecular ion peak at *m/z* 440.38 $[ML_2 3H_2O)]^+$, *m/z* 422.38 $[ML_2{\cdot}2H_2O)]$, *m/z* 386.38 $[ML_2]$, peak at *m/z* 227 [ML+1].

Thermal analysis: TG curve of Cu(II)-PMTSC (Fig. 3a) indicated the decomposition pattern of the complex in four steps. The loss of weight of 8.7 % upto 250 ºC indicated the loss of two coordinated water molecules. Weight loss of 57.54 %, between 250-1070 ºC in two sudden steps and then gradual decomposition was due to decomposition of the complex. 33.76 % of residue left at 1070 ºC indicates the partial decomposition of complex.

Thermogram of Zn(II)-PMTSC (Fig. 3b) showed decomposition of Zn-complex in four steps. 4.2 % of weight loss up to 180 ºC indicated the loss of one mole of lattice water. Loss of 8.4 % mass up to 300 ºC indicated loss of two moles of coordinated water. Sudden weight loss of 41.53 % upto 487 ºC, and gradual loss of weight (20.59 %) from 487-1100 ºC indicates the decomposition of complex moiety. The percentage of residue left over at 1100 ºC was 25.28 %, indicating partial decomposition of the complex.

IR analysis: In the IR spectrum of PMTSC, the $\nu NH_{(N1)}$ peak observed at 3193 cm^{-1} was absent in Cu(II)-PMTSC complex indicating the participation of 'N'(1) in the bonding by the dissociation of hydrogen atom. The $\nu NH_{(N2)}$ peak observed in the IR spectrum of PMTSC at 3039 cm^{-1} has been shifted to higher frequency region of 3118 cm^{-1} in complex. The ν(C=O) peak observed at 1685 cm^{-1} in the IR spectrum of PMTSC shifted to 1724 cm^{-1} in the complex supporting the participation of 'N'(1) in the bonding. The ν(C=S) peak observed at 1284 cm^{-1} in the IR spectrum of ligand has been shifted to lower frequency region 1246 cm^{-1} in the complex indicating the participation of 'S' in the bonding in thione form. Thus, ligand binds with Cu(II) ion forming a five membered chelate.

The $\nu NH_{(N1)}$ peak observed at 3193 cm^{-1} in the ligand IR spectrum was absent in spectrum of Zn(II)-PMTSC complex and an extra peak corresponds to ν(C=N) was observed at 1533 cm^{-1}. The ν(C=O) peak observed at 1685 cm^{-1} in the IR spectrum of PMTSC was absent in the complex and an extra peak corresponds to ν(C-O) has been observed at 1095 cm^{-1} indicating deprotonation of amide proton through oxygen *via* enol form and indicate the participation of oxygen in the bonding. The $\nu NH_{(N2)}$ peak observed in the IR spectrum of PMTSC at 3039

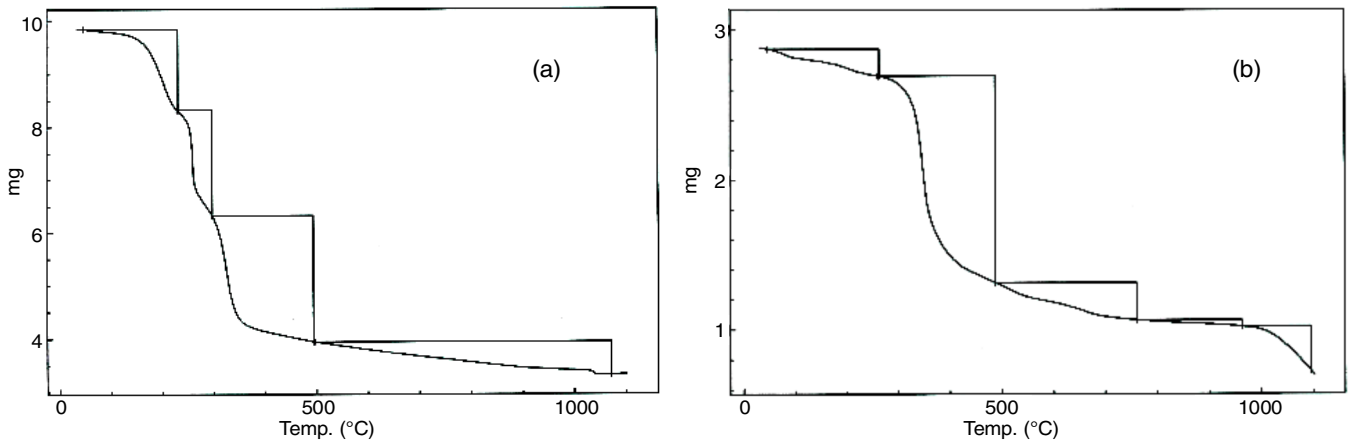


Fig. 3. Thermograms of Cu(II)-PMTSC (a) and Zn(II)-PMTSC (b)

cm^{-1} has been shifted to higher frequency region at 3099 cm^{-1} in the complex indicating the participation of 'N'(2) in the bonding without deprotonation. Oxygen in enol form and 'N'(2) binds to Zn(II) ion and forms a five membered ring.

In Far-IR region of complexes, the presence of νM-N (485-435 cm^{-1}) [28], ν(M-O) (430-400 cm^{-1}), ν(M-S) (395-355 cm^{-1}) and ν(M-OH_2) (440-400 cm^{-1}) peaks were evident, however it is difficult to predict the specific peaks.

UV-visible: The UV-visible spectrum of Cu(II)-PMTSC (Fig. 4) showed an intense peak at 12,065 cm^{-1} due to $^2E_g \rightarrow {}^2T_g$, while other intense peak at 44,100 cm^{-1} may be charge transfer transition along with ligand chromophore at 38,167 cm^{-1}.

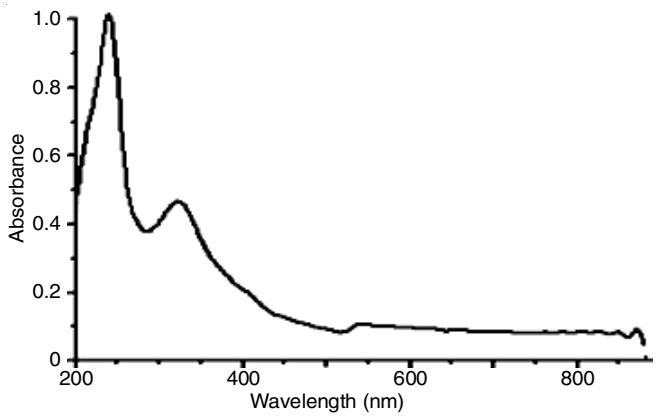


Fig. 4. UV-visible spectrum of Cu(II)-PMTSC

ESR spectrum and magnetic susceptibility measurements: Cu(II)-PMTSC (I = 3/2) ESR spectrum (Fig. 5) showed three g values. The g_x, g_y and g_z values 2.1365, 2.1041 and 2.0422, respectively indicating distorted octahedral geometry. The magnetic moment value of Cu(II)-PMTSC is 1.74 BM indicating presence of one unpaired electron in the complex.

From the above discussions based on all the analytical, spectral techniques employed and equilibrium studies the following tentative structures (Fig. 6) for the metal complexes have been proposed.

DNA cleavage studies: Super coiled (SC) plasmid DNA, commonly seen in bacteria cells, is a cyclic super coiled double strand made up of several thousand base pairs. This is an important substrate for hydrolytic cleavage. Metal ions in complexes serve as Lewis acids to activate the phospho-diester links for

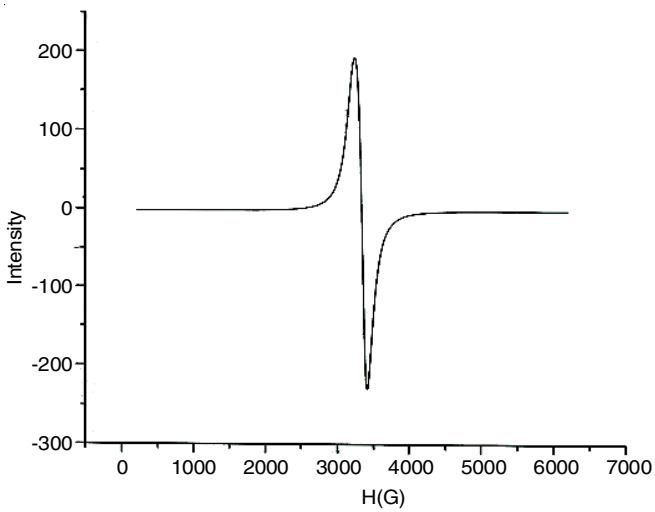


Fig. 5. ESR spectrum of Cu(II)-PMTSC

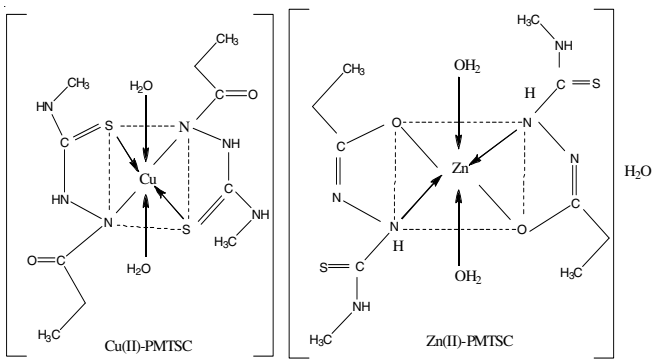


Fig. 6. Tentative structures of Cu(II)-PMTSC and Zn(II)-PMTSC

nucleophilic attack and metal coordinated water species acts as a nucleophile. When DNA is subjected to electrophoresis, the intact super coiled form migrates faster. When scission occurs due to action of complex, super coiled (SC) form will relax to nicked (NC) form that migrates slowly. Cleavage of both types of strands leads to linear form which migrates between SC and NC forms. This is because shorter molecules migrate more easily through the pores of gel [9]. In the present investigation, it is observed (Fig. 7) that Cu(II) and Zn(II)-PMTSC complexes promote hydrolytic cleavage of plasmid pBR322 to certain extent due to scission in SC forms of DNA to NC forms.

TABLE-3
ANTIBACTERIAL STUDIES OF SYNTHESIZED LIGAND (PMTSC) AND ITS METAL(II) COMPLEXES

Compound	Gram-positive		Gram-negative	
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumoniae*
PMTSC	12 mm	6 mm	13 mm	10 mm
Cu(II)-PMTSC	16 mm	8 mm	6 mm	7 mm
Zn(II)-PMTSC	NIL	NIL	8 mm	NIL

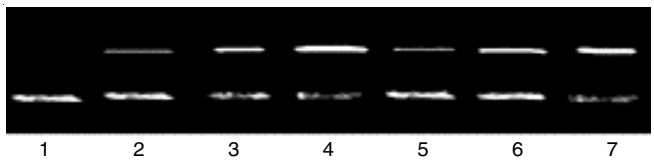


Fig. 7. Agarose gel electrophoresis pattern for the cleavage of supercoiled pBR 322 DNA by complexes. Lane 1, DNA control, Lane 2-4 DNA+ Cu(II) (20, 40, 60 µM resp.), Lane 5-7 DNA+ Zn(II) (20, 40, 60 µM resp.) of PMTSC

Antibacterial studies: The PMTSC and Cu(II)-PMTSC were found to inhibit the growth of Gram-positive and Gram-negative bacteria. Cu(II)-PMTSC showed more activity on Gram-positive bacteria compared to PMTSC. However, Zn(II)-PMTSC is found to inhibit the growth of Gram-negative bacteria (Table-3). This can be attributed to the chelating capacity of ligand with metal ions. Metal atom partially shares its positive charge with the donor atoms of the ligand. This leads to delocalization of π-electron cloud over the chelating ring. Due to this the lipophilic character of metal gets enhanced and favours its permeability into bacterial cell membranes and inhibits the growth of bacteria.

Conclusion

Spectral and analytical studies indicate that PMTSC forms distorted octahedral complexes in 1:2 (M:L) composition with copper(II) and zinc(II) ions. Equilibrium studies revealed that PMTSC acts as monobasic ligand and forms stable 1:1 and 1:2 (M-L) complex with zinc(II) ions in solution. DNA cleavage studies revealed that both complexes can cleave super coiled (SC) form of plasmid DNA. Both the solid complexes showed good antibacterial activity against Gram-positive and Gram-negative bacteria.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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