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Synthesis, characterization and *In-Vitro* cytotoxic study of novel ligands and their metal complexes

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ABSTRACT

A series of Cu(II) and Zn(II) complexes of Mannich bases L^1 , L^2 , L^3 and L^4 have been synthesized and characterized by spectroscopic methods such as UV, IR, ^1H NMR, ^{13}C NMR , Mass spectra, ESR spectra and physiochemical methods. The ligands act as a bidentate, coordinating through the oxygen/sulphur atom of urea/thiourea moiety and nitrogen atom of N-methyl piperazine/piperazine. The spectral studies confirm a six coordinate environment around the metal ion. The low molar conductance value in DMF indicates that the metal complexes are non-electrolytic nature. The magnetic moments and electronic spectral data suggest octahedral geometry for the Cu(II) and Zn(II) complexes. All the complexes have the general composition of $[M(L)_2(X)_2]$ (M = Cu(II) and Zn(II); L = Mannich base and X = Cl). All the Cu(II) complexes are paramagnetic and Zn(II) complexes are diamagnetic in nature. These compounds were also tested for in-vitro cytotoxic effect against human lung cancer cell (A549), colon cancer cell (HCT15) and normal cell lines (VERO).

Keywords: Mannich base metal complexes, Thiophene-2-carbaldehyde, Anticancer activity of metal complexes and ESR.

INTRODUCTION

Mannich bases play a vital role in the development of synthetic organic and medicinal chemistry. It is well known that the compounds contain amide moiety are found to exhibit a wide range of biological activities such as antimicrobial, anticancer, anti-inflammatory, analgesic and anticonvulsant activities**[1 - 16]**. Studies on DNA interactions of organic and inorganic small molecules are important in understanding cellular processes, such as mutagenesis and cancer. Some molecules have been developed as cancer therapeutics or molecular biology tools **[17–23]**. Metal complexes of Mannich bases can bind to DNA in non-covalent modes such as electrostatic, intercalative, and groove binding. Many potential applications of such complexes require that the complexes bind to DNA through an intercalative mode **[24-25]**.

Cancer is the second most frequent cause of death in the world, which influences the abnormal growth of the normal cells **[26]**. Cancer is caused by abnormalities in the genetic material of the affected cells. On the way to tumorigenesis, there occurs an accumulation of successive mutations in proto-oncogenes and suppressor genes that deregulates the cell cycle. The events key to tumorigenesis are for instance point mutations in DNA sequences, chromosomal aberrations such as translocations or deletions and changes that affect the chromatin structure such as methylation of DNA or acetylation of histones. An estimated amount of 12 million death rates due to cancer may surpass in 2030. Cancer therapy is based largely on surgery, radiotherapy, hormone and chemotherapy. However, the clinical results are often only a short prolongation in patient survival **[27]**. The development of a new anticancer agent is a multi-stage process which includes synthesis, characterization, study of biological activity, pre-clinical

and clinical screenings. Testing for the biological activity requires the measurement of the biological effect in *in-vitro* (in the cancer cell lines) and in vivo (in animals) screens **[28]**. The discovery of antitumor activity of cisplatin began a search for other metal complexes with cytotoxic properties against cancer cells.

One of the transition metals, whose complexes are extensively tested for antitumor application, is copper. Copper is a trace element essential for the survival and betterment of human life. It is a building element of several important enzymes such as superoxide dismutase, cytochrome oxidase, tyrosinase and it regulates the intracellular redox potential, while its complexes possess antibacterial, antifungal, antiviral, anti-inflammatory and anticancer properties. Copper is a part of many redox-active metalloenzymes and it is an important metal involved in the endogenous oxidative DNA damage associated with aging and cancer **[24-25]**. Cu(II) complexes have been extensively studied in this context **[29]**. For the past several decades, great effort has been devoted to binding studies of copper complexes with DNA **[30–32]**. Copper(II) has been shown to bind to the DNA bases adenine, guanine and cytosine at the N(7) of purines and N(1) of pyrimidines **[33]**. As being the potential anticancer drugs, there is a wide range of studies going on with regard to the complexes of copper (II). There are only a few complexes of copper (I) in the literature, whereas they also show a very strong cytotoxic activity against tumor cells *in-vitro* **[34]**.

Anticancer activity of copper complexes are related to their ability to produce Reactive Oxygen Species (ROS). Copper (I) ions can reduce hydrogen peroxide to hydroxyl radical. Copper (II) ions may in turn be reduced to Cu (I) by superoxide anion($O_2^{\bullet-}$), or glutathione. Therefore, it can be concluded that the production of reactive oxygen species such as $OH^{\bullet}$ are driven by the copper, regardless the form in which it is initially introduced into the body. Superoxide anion ($O_2^{\bullet-}$) is the product of reduction of molecular oxygen that occurs in many biological processes. It is converted into hydrogen peroxide through dismutation. Both of these forms of ROS lead to the formation of another type of Reactive Oxygen Species, the hydroxyl radical ($OH^{\bullet}$). It occurs in a reaction catalyzed by copper (or iron) ions. This radical is believed to be the main factor causing DNA damage in cells under oxidative stress **[35]**.

Copper (I) compounds may become an alternative for cisplatin, which possesses a few drawbacks but is still more popular. Copper, as an essential element for human life is supposed to be less toxic than other metals, like platinum or ruthenium, is analysed for medical application. Both copper ions, Cu^{+} and Cu^{2+} can induce oxidation stress via catalysis and production of Reactive Oxygen Species. Superiority of copper(I) compounds over copper(II) compounds results from nuclease activity of Cu^{+} complexes and selectivity exhibited by human copper transporters (hCtr) in introduction of Cu^{+} ions into the cells **[36]**. The foremost target of most research groups was to find a convenient anticancer drug that can be used efficiently for the treatment of human cancer. This study gives an emphasis on the new strategies used in the development of anticancer agents from newly synthesized novel Mannich bases and their Cu(II) and Zn(II) complexes by *in-vitro* cytotoxic assay using human lung cancer (A549) and Colon cancer (HCT 15) cell lines.

MATERIALS AND METHODS

2.1. Reactants

Analytical grade solvents and reactants were used. N- Methyl piperazine, Piperazine and thiophene-2-carbaldehyde were purchased from Merck Products and used as such.

2.2. Measurements

Elemental analyses and characterization studies were carried out at Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology - Madras - Chennai, Tamil Nadu, India. Melting points of synthesized compounds were measured on electric melting point apparatus SMP1. ^{1}H NMR spectra of the samples were recorded on 300 MHz Shimadzu spectrometer using DMSO-d_6 with TMS as the internal standard. The homogeneity of the compounds was monitored by Thin Layer Chromatography (TLC) Silica-Gel coated on glass plate and visualized by iodine vapour. The absorption in the UV-Vis region was recorded for the compounds by a Perkin Elmer Lambda 35 Spectrophotometer using DMF/DMSO as solvents. IR spectra were recorded on KBr pellets with a Nicolet model Impact 470 FTIR spectrophotometer in the range 4000 - 400 cm^{-1}. C, H and N elemental analysis were performed on a model Perkin-Elmer 240 elemental analyzer. Molar conductance of complexes in DMF at a concentration of 10^{-3} M solution was measured at room temperature using Conductivity Bridge with a tip type cell having a cell constant of 1.03. Gouy's method was employed for getting magnetic measurement values. The magnetic susceptibilities of the solid complexes were recorded on Magnetic Susceptibility Balance. The thermogravimetric analyses were carried out in a nitrogen atmosphere with a heating rate of $25^{o}C$ min^{-1} using a Shimadzu TGA-50H. ESR spectra of the copper complexes were recorded on a Varian E112 X- band spectrometer at the Regional Sophisticated Instrumentation Centre, Indian Institute of Technology, Chennai, Tamil Nadu, India,

using tetracyanoethylene (TCNE) as the internal standard. Anti-cancer and cytotoxic studies were carried out at Royal Bio Research Centre, Velachery, Chennai, Tamil Nadu, India.

2.3. Anticancer (cytotoxic) evaluation

The anticancer activity of the synthesized ligands, and their metal complexes were studied against human lung cancer (A549) cell and colon cancer (HCT15) cell lines. The cancer cell lines were obtained from the National Centre for cell sciences (NCCS), Pune, India. The cell lines were cultured in minimum essential media fetal bovine serum supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 µg/mL) and streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37 ^oC. The experiment was carried out at Royal Bio Research Centre, Velachery, Chennai, Tamil Nadu, India. Anticancer activity of synthesized ligands at various concentrations were assessed using trypsin, methylthiazol, diphenyl- tetrazolium bromide (MTT) (Sigma) assay, as described by Mosmann, but with minor modification, followed by 72 h of incubation. Assay plates were assessed using spectrophotometer at 520 nm. Data generated were used to plot a dose–response curve, from which the concentration of test compounds required to kill 50% of the cell population (IC_{50}) **[37]**. The effect of the samples on the proliferation of A549, HCT15 & normal(VERO) cells were expressed in % cell viability.

2.4. Experimental

2.4.1. Synthesis of Mannich base

1-((4-methylpiperazin-1-yl)(thiophene-2-yl)methyl)urea (MPTMU), was prepared by reacting urea (0.05 mol), N-methyl piperazine (0.05 mol) and thiophene-2-carbaldehyde (0.05 mol) in 1:1:1 mol ratio. N- methyl piperazine was added to the methanolic solution of urea, followed by liq.NH_3. This reaction mixture was stirred well using magnetic stirrer. After few minutes, thiophene-2- carbaldehyde was added in dropwise to the reaction mixture. Then, the reaction mixture was allowed to stand at 5 ^oC for 5 h, colourless solid formed was separated, washed with water several times and finally with the 1:1 mixture (V / V) of acetone and petroleum ether, filtered and dried in vacuum. The same procedure was employed for the synthesis of the L^2 , L^3 and L^4. The structures of the synthesized compounds are shown in scheme 1&2.

Table 1: Physico-chemical characterization of ligands and their metal complexes.

Sl. No.	Compound (M.F.)	M.P. (^0C)	Yield (%)	Elemental analysis, [Found% (Calcd.%)]							
				C	H	N	O	S	Cl	Zn	Cu
1.	L^1 ($C_{11}H_{18}N_4OS$)	146	52	51.23 (51.94)	7.12 (7.13)	22.03 (22.03)	6.19 (6.29)	12.11 (12.61)	--- ---	--- ---	--- ---
2.	L^2 ($C_{10}H_{16}N_4OS$)	148	54	49.46 (49.98)	6.11 (6.71)	23.28 (23.31)	6.28 (6.66)	13.27 (13.34)	--- ---	--- ---	--- ---
3.	L^3 ($C_{11}H_{18}N_4S_2$)	150	55	48.46 (48.86)	6.11 (6.71)	20.28 (20.72)	--- ---	23.72 (23.72)	--- ---	--- ---	--- ---
4.	L^4 ($C_{10}H_{16}N_4S_2$)	152	56	46.64 (46.85)	6.11 (6.29)	21.28 (21.89)	--- ---	25.07 (25.01)	--- ---	--- ---	--- ---
5.	$[Zn(L^1)_2Cl_2]$ ($C_{22}H_{44}Cl_2N_8O_2S_2Zn$)	350	52	40.31 (40.46)	6.16 (6.79)	17.13 (17.16)	4.80 (4.90)	9.45 (9.82)	10.56 (10.86)	10.01 (10.72)	--- ---
6.	$[Cu(L^1)_2Cl_2]$ ($C_{22}H_{44}Cl_2CuN_8O_2S_2$)	260	60	40.12 (40.98)	6.16 (6.81)	17.13 (17.21)	4.72 (4.91)	9.22 (9.82)	10.43 (10.89)	--- ---	9.23 (9.76)
7.	$[Zn(L^2)_2Cl_2]$ ($C_{20}H_{40}Cl_2N_8O_2S_2Zn$)	360	61	38.43 (38.43)	6.16 (6.45)	17.13 (17.93)	5.03 (5.12)	10.02 (10.26)	11.23 (11.34)	11.13 (11.23)	--- ---
8.	$[Cu(L^2)_2Cl_2]$ ($C_{20}H_{40}Cl_2CuN_8O_2S_2$)	280	53	38.14 (38.55)	6.16 (6.47)	17.13 (17.98)	5.05 (5.13)	11.00 (11.05)	11.38 (11.38)	--- ---	10.23 (10.20)
9.	$[Zn(L^3)_2Cl_2]$ ($C_{22}H_{44}Cl_2N_8S_4Zn$)	360	60	38.01 (38.56)	6.16 (6.47)	16.13 (16.35)	--- ---	18.01 (18.72)	10.34 (10.35)	9.46 (9.54)	--- ---
10.	$[Cu(L^3)_2Cl_2]$ ($C_{22}H_{44}Cl_2CuN_8S_4$)	285	55	38.14 (38.67)	6.16 (6.49)	16.13 (16.40)	--- ---	18.01 (18.77)	10.23 (10.38)	--- ---	9.33 9.30
11.	$[Zn(L^4)_2Cl_2]$ ($C_{20}H_{40}Cl_2N_8S_4Zn$)	370	54	36.00 (36.55)	6.16 (6.14)	17.13 (17.05)	--- ---	19.01 (19.52)	10.92 (10.79)	9.02 (9.95)	--- ---
12.	$[Cu(L^4)_2Cl_2]$ ($C_{20}H_{40}Cl_2CuN_8S_4$)	280	57	36.14 (36.66)	6.16 (6.15)	17.13 (17.10)	--- ---	19.01 (19.57)	10.23 (10.82)	--- ---	9.45 (9.70)

2.4.2. Preparation of complexes

Methanolic solution of each ligand (L^1, L^2, L^3 & L^4) (Fig. 1, 2, 5 & 6) was added to the each MCl_2 (M = Cu(II) and Zn(II)) dissolved in mixture of $CHCl_3$ and CH_3OH (1:2) V / V solution. The resulting mixture was stirred well using on a magnetic stirrer for 2 h, during which solid separated out was collected by filtration, washed with methanol followed by diethyl ether and dried in vacuum.

Scheme 1: Synthesis of compounds (L^1, L^2 and their Metal (II) Complexes)

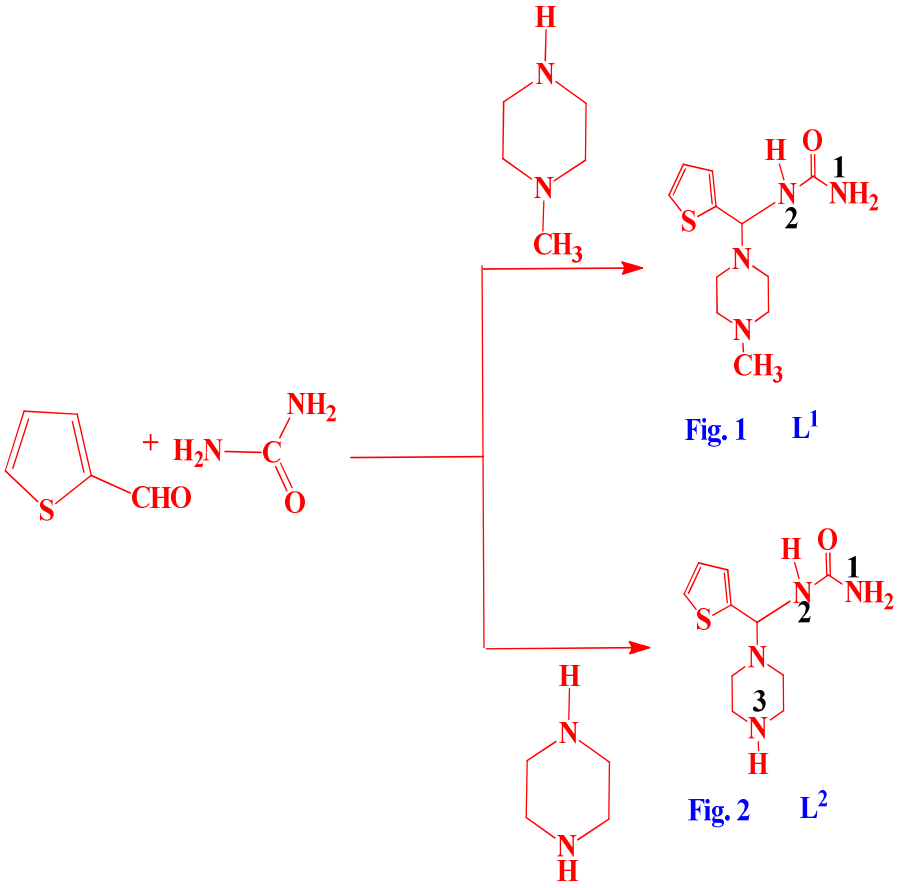


Fig. 1 L^1

Fig. 2 L^2

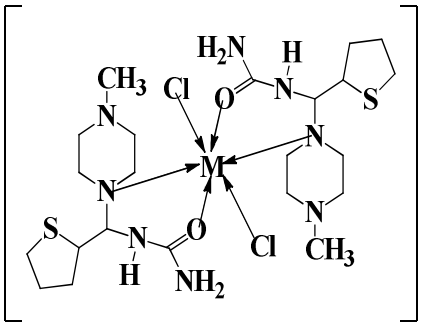


Fig. 3 $[M(L^1)_2Cl_2]$

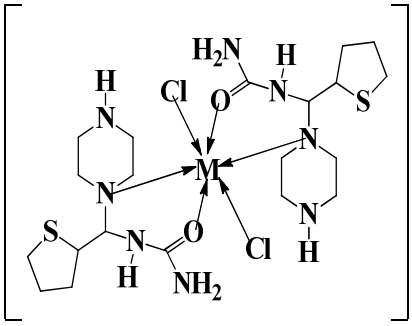


Fig. 4 $[M(L^2)_2Cl_2]$

Where $M = Cu^{2+}$ or Zn^{2+}

L^1 = MPTMU and L^2 = PTMU

Scheme 2: Synthesis of compounds (L^3, L^4 and their Metal (II) Complexes)

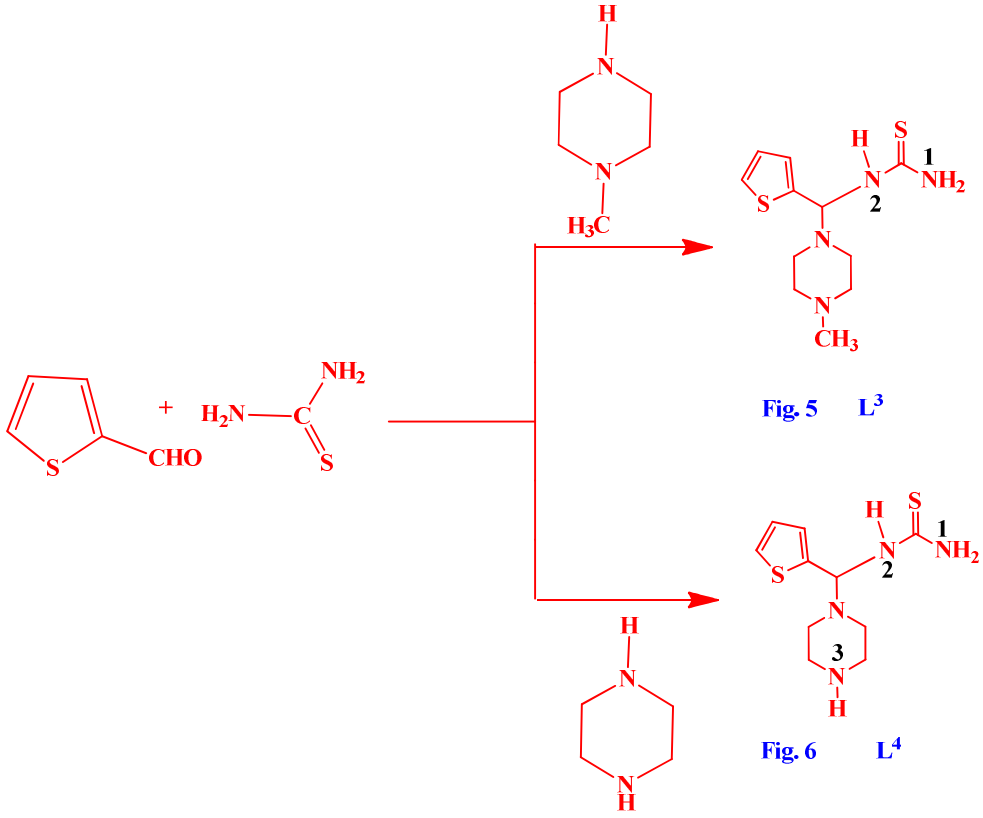


Fig. 5 L^3

Fig. 6 L^4

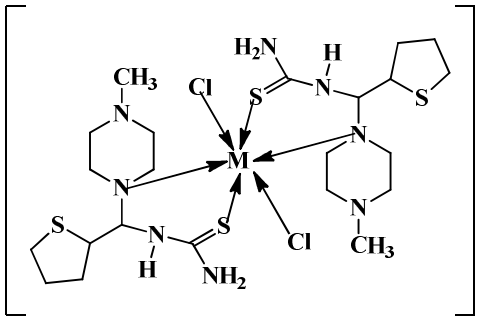


Fig. 7 $[M(L^3)_2Cl_2]$

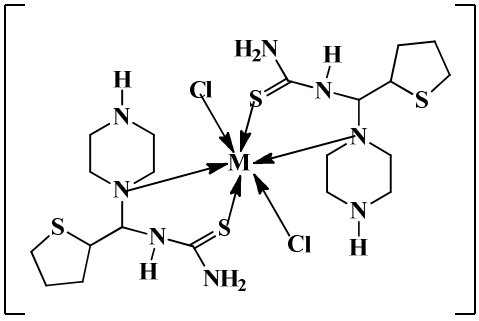


Fig. 8 $[M(L^4)_2Cl_2]$

Where M = Cu^{2+} or Zn^{2+}

L^3 = MPTMU & L^4 = PTMU

RESULTS AND DISCUSSION

The purity of each ligand and its metal complexes was determined by TLC. The physiochemical parameters are shown in **Table - 1** and **Table - 2**. The results of elemental analysis show that the metal to ligand ratio is 1: 2 for all the complexes. The complexes are powdery solids, coloured and nonhygroscopic in nature. The magnetic susceptibility data of the complexes in the solid state show that all the Cu(II) complexes are paramagnetic and Zn(II) are diamagnetic at room temperature. The molar conductance values of the complexes measured (in 10^{-3}M DMF) at room temperature lie in the range from 40.4 – 51.2 Ω^{-1} mol^{-1} cm^2, which suggest that these complexes are non-electrolytes.

Table 2: Molar conductance and Magnetic susceptibility data of Complexes.

Sl. No.	Compound/Complex	Colour	Molar Conductance (Ω^{-1} mol^{-1} cm^2)	μeff.(BM)
1.	$[Cu(L^1)_2Cl_2]$	Dark green	51.2	1.80
2.	$[Zn(L^1)_2Cl_2]$	Colourless	40.4	----
3.	$[Cu(L^2)_2Cl_2]$	Green	48.9	1.82
4.	$[Zn(L^2)_2Cl_2]$	Colourless	45.8	----
5.	$[Cu(L^3)_2Cl_2]$	Dark green	58.5	1.82
6.	$[Zn(L^3)_2Cl_2]$	Colourless	48.6	----
7.	$[Cu(L^4)_2Cl_2]$	Green	50.7	1.87
8.	$[Zn(L^4)_2Cl_2]$	Colourless	47.9	----

3.1. *^{1}H NMR and ^{13}C NMR Spectra*

To confirm the mode of binding and geometry of the complexes, ^{1}H NMR spectrum of Zn(II) complex of each ligand was recorded using DMSO-d_6 and the results are compared with the ^{1}H NMR spectrum of their free ligands and the spectra are shown in **Fig. 9.** In the spectra of the free ligands, signals observed at δ 7.23-7.22 (L^1), 7.21 (L^2) are assigned to the (NH(C=O)) protons. In the case of (L^3) and (L^4) signals occurred at δ 8.21-8.19 and 8.0 for (NH(C=S)) protons. The signals due to -$CONH_2$ /-$CSNH_2$ protons have been found shifted slightly towards downfield in the spectra of complexes. This shifting spectral value indicate, oxygen atom of carbonyl / sulphur atom of thiocarbonyl are involved in the binding with the metal ion. The absence of signal in the region of δ 10-11 ruled out the existence of keto - enol form **[38].** The presence of doublet at δ 5.41, 6.0, 5.4 and 5.4 corresponds to (CH(NH)) proton. The N- methyl piperazine/ piperazine proton signal values have been found shifted slightly towards downfield in the spectra of all complexes. The shifting spectral value indicate, nitrogen atom which involved in the metal formation. This supports the participation of N atom of N- methyl piperazine / piperazine of the ligand in the complex formation.

The ^{13}C NMR spectral data of Mannich bases L^1, L^2, L^3, and L^4 and their Zn (II) complexes are mentioned below and the spectra are shown in **Fig. 10.** The Mass spectral data of Mannich bases L^1, L^2, L^3, and L^4 and their Zn (II) complexes are mentioned below and the spectra are shown in **Fig.11.**

Fig.9. ^{1}H NMR SPECTRUM OF THE LIGANDS

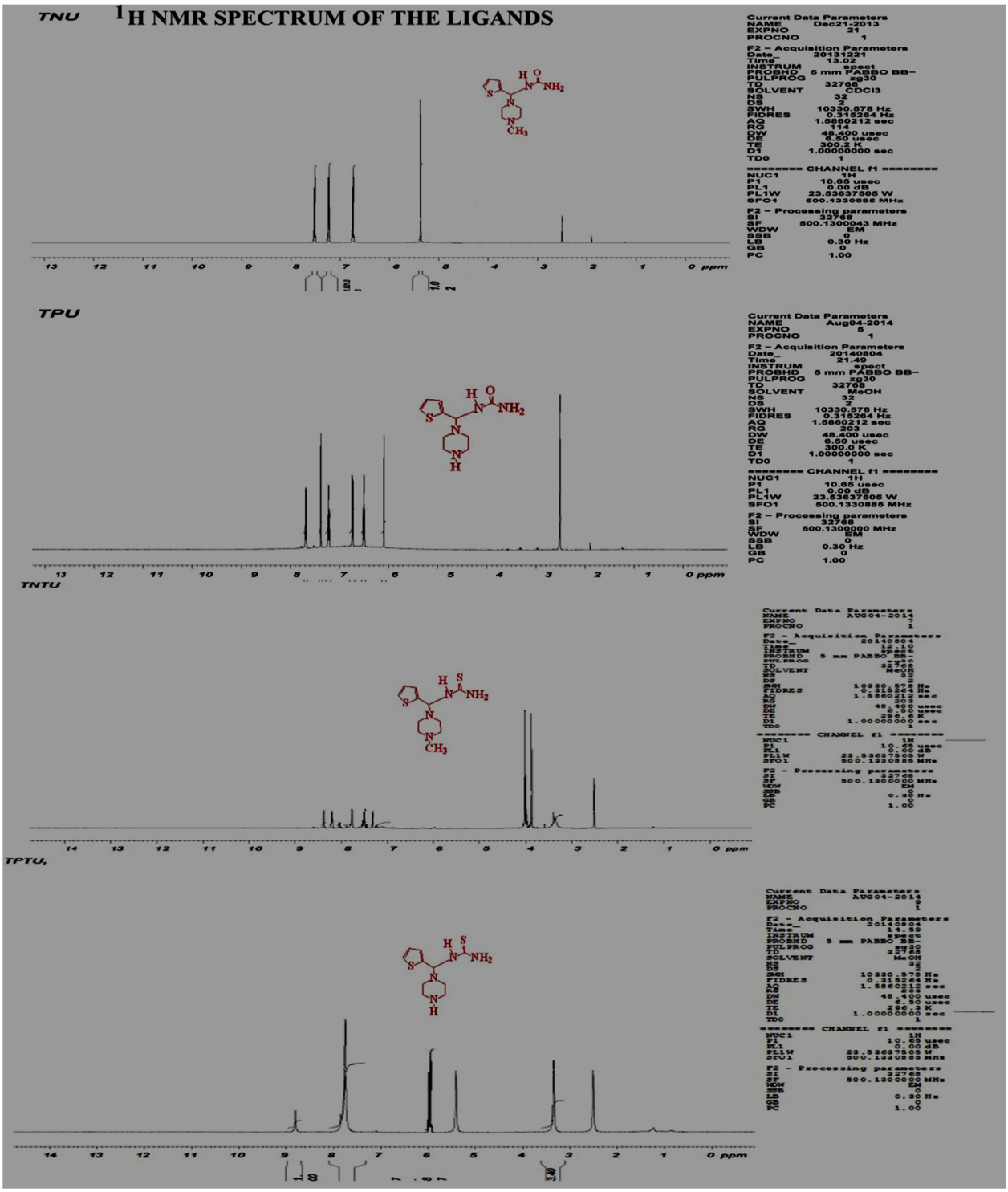


Fig.10. ^{13}C NMR SPECTRUM OF THE LIGANDS

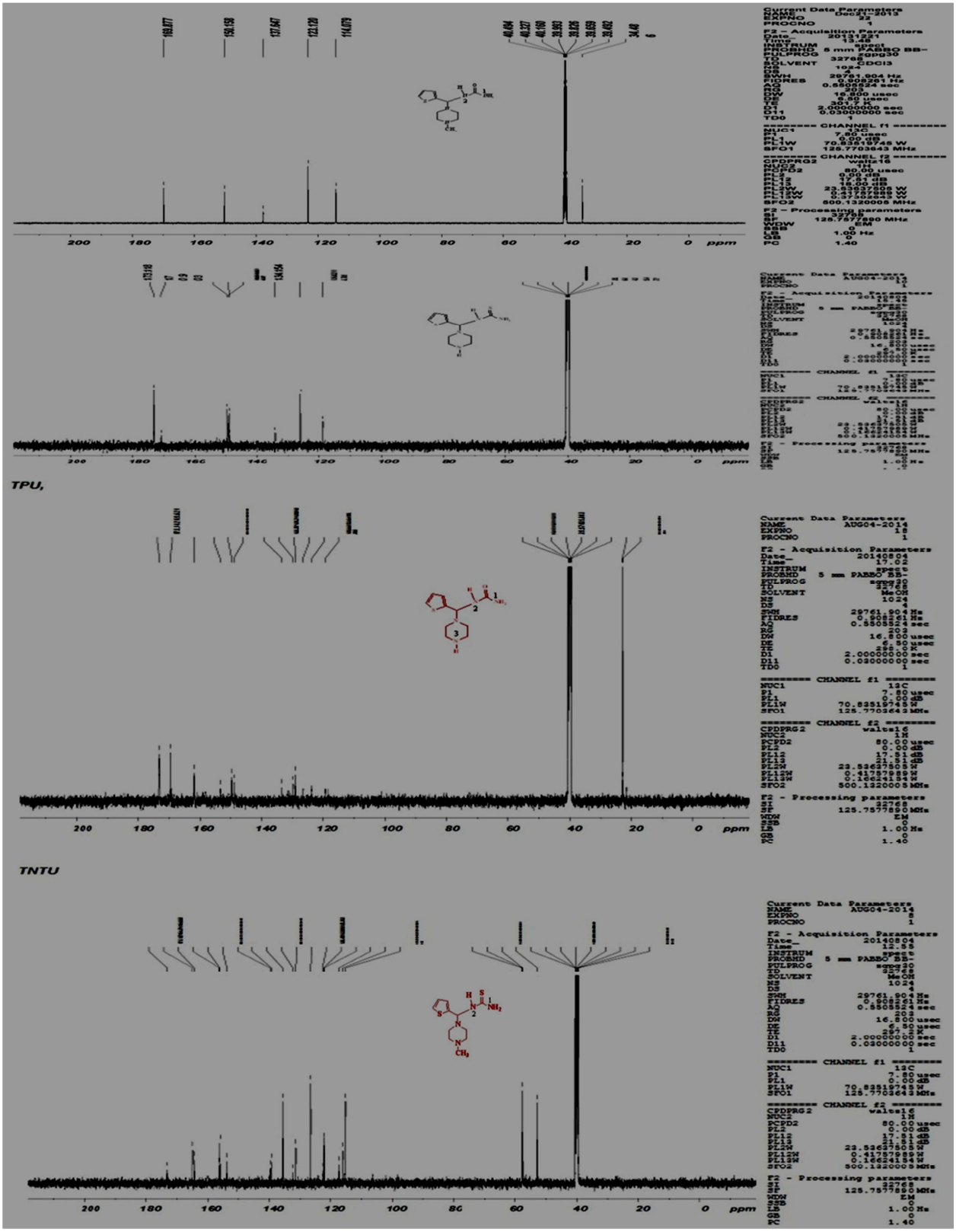
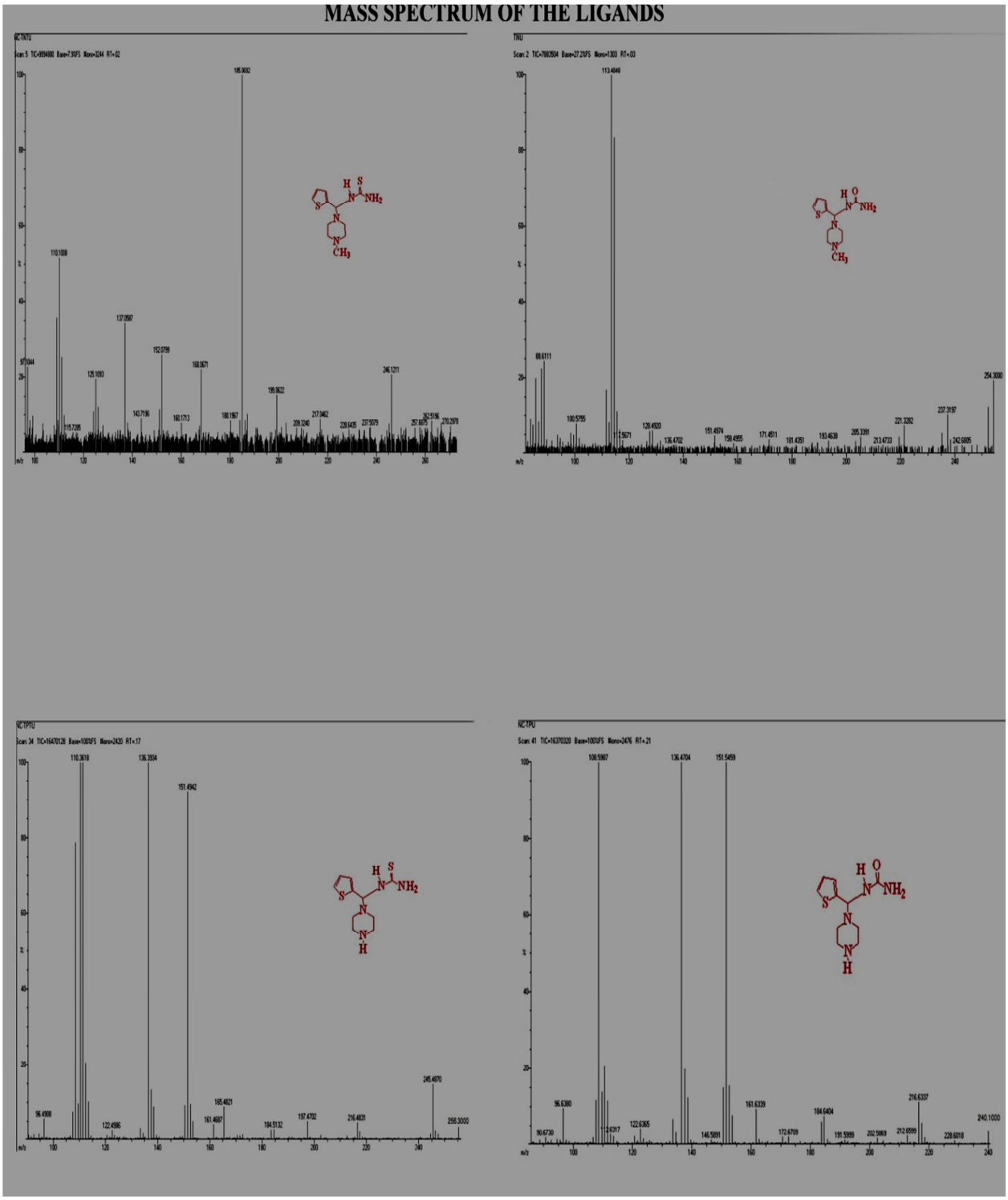


Fig.11. MASS SPECTRUM OF THE LIGANDS



3.2. 1-((4-methylpiperazin-1-yl)(thiophen-2-yl)methyl)urea (L^1) (Fig.1)

^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) 7.52 (s, 2H, NH_2), 7.23-7.22 (d, H, NH(C=O)), 6.80 (m, 3H, Thiophene), 5.41 (d, CH,(NH)), 2.50 (m, 8H, (piperazine)), 1.90 (s, 1H, CH_3 (piperazine)). **^{13}C NMR (300 MHz, DMSO-d_6, δ ppm)** 169.87 (N=C-OH, urea), 137.64 (C-S, thiophene), 123.12 (Ar-CH), 114.07 (CH(NH)), 40.49 - 40.16 (Aliphatic - CH), 39.99 (N-CH_3). The mass spectrum of the compound (L^1) exhibits a molecular ion peak[M] at m/Z 254.3000 which corresponds to its molecular weight. The fragmentation peaks at m/z 254.30, 237.31, 193.46, 171.45, 158.49, 100.57 indicate the cleavage of $C_{11}H_{18}N_4OS$, $C_{11}H_{18}N_3OS$, $C_{10}H_{16}N_2S$, $C_7H_{16}N_4O$, $C_6H_9N_2OS$ and $C_5H_{12}N_2$ fragments respectively.

3.3. Bis(1-((4-methylpiperazin-1-yl)(thiophen-2-yl)methyl)urea)dichloro Zinc(II). [M(L^1)$_2$Cl$_2$](Fig.3)
^{1}H NMR (300 MHz, DMSO-d$_6$, δ ppm) 7.52 (s, 2H, NH_2), 7.00 (d, H, NH(C=O)), 6.80 (m, 3H, Thiophene), 5.41 (d, CH,(NH)), 2.00 (m, 8H, (piperazine)), 1.90 (s, 1H, CH_3 (piperazine)). **^{13}C NMR (300 MHz, DMSO-d$_6$, δ ppm)** 169.87 (N=C-OH, urea), 137.64 (C-S, thiophene), 123.12 (Ar-CH), 114.07 (CH(NH)), 40.49 - 40.16 (Aliphatic - CH), 39.99 (N-CH_3). ESI-MS: Mass spectra of the complex [M(L^1)$_2$Cl$_2$](Fig.3) give peaks at m/Z: 651.02 is assigned to[M+H]$^+$.

3.4. 1-(piperazin-1-yl(thiophen-2-yl)methyl)urea (L^2) (Fig.2)
^{1}H NMR (300 MHz, DMSO-d$_6$, δ ppm) 7.81 (s, 2H, NH_2), 7.21 (d, H, NH(C=O)), 6.80 (m, 3H, Thiophene), 6.00 (d, CH,(NH)), 2.50 (m, 8H, (piperazine)), 1.90 (s, 1H, NH of piperazine). **^{13}C NMR (300 MHz, DMSO-d$_6$, δ ppm)** 161.86 (N=C-OH, urea), 133.31 (C-S, thiophene), 126.58 (Ar-CH), 119.28 (CH(NH)), 40.47 - 39.97 (Aliphatic - CH). The mass spectrum of the compound (L^2) exhibits a molecular ion peak[M] at m/Z 240.1000 which corresponds to its molecular weight. The fragmentation peaks at m/z 240.10, 228.60, 202.58, 184.64, 161.63 and 112.63 indicate the cleavage of $C_{10}H_{16}N_4OS$, $C_{10}H_{16}N_3OS$, $C_9H_{15}N_3S$, $C_9H_{15}N_2S$, $C_6H_{14}N_4O$ and C_5H_4OS, fragments respectively.

3.5. Bis(1-(piperazin-1-yl(thiophen-2-yl)methyl)urea)dichloroZinc(II). [M(L^2)$_2$Cl$_2$] (Fig.4)
^{1}H NMR (300 MHz, DMSO-d$_6$, δ ppm) 7.81 (s, 2H, NH_2), 7.01 (d, H, NH(C=O)), 6.80 (m, 3H, Thiophene), 6.00 (d, CH,(NH)), 2.01 (m, 8H, (piperazine)), 1.90 (s, 1H, NH of piperazine). **^{13}C NMR (300 MHz, DMSO-d$_6$, δ ppm)** 161.86 (N=C-OH, urea), 133.31 (C-S, thiophene), 126.58 (Ar-CH), 119.28 (CH(NH)), 40.47 - 39.97 (Aliphatic - CH). The ESI-MS: Mass spectra of the complex [M(L^2)$_2$Cl$_2$] (Fig.4) give peaks at m/Z 651.18 is assigned to[M+H]$^+$.

3.6. *1-((4-methylpiperazin-1-yl)(thiophen-2-yl)methyl)thiourea (L^3) (Fig.5)* **^{1}H NMR (300 MHz, DMSO-d$_6$, δ ppm)** 8.37 (s, 2H, NH_2), 8.21-8.19 (d, H, NH(C=S)), 7.54-7.46 (m, 3H, Thiophene), 4.22 (d, CH,(NH)), 5.40 (m, 8H, (piperazine)), 2.60 (s, 1H, CH_3 (piperazine)). **^{13}C NMR (300 MHz, DMSO-d$_6$, δ ppm)** 173.12 (-C=S, thiourea), 139.33 (C-S, thiophene), 126.49 (Ar-CH), 122.12 (CH(NH)), 57.73 (Aliphatic - CH), 40.47 (N-CH_3). The mass spectrum of the compound (L^3) exhibits a molecular ion peak[M] at m/Z 270.2978 which corresponds to its molecular weight. The fragmentation peaks at m/z 257.68, 211.11,199.06,199, 185.069, 168.06, 115.72 indicate the cleavage of $C_{11}H_{17}N_3S_2$, $C_{10}H_{17}N_3S$, $C_{10}H_{16}N_2S$, $C_{10}H_{17}N_2S$, $C_7H_{16}N_4S$, $C_6H_8N_2S_2$ and C_5H_4OS fragments respectively.

3.7. *Bis(1-((4-methylpiperazin-1-yl)(thiophen-2-yl)methyl)thiourea)dichloro Zinc(II). [M(L^3)$_2$Cl$_2$] (Fig.7)*
^{1}H NMR (300 MHz, DMSO-d$_6$, δ ppm) 8.37 (s, 2H, NH_2), 8.00 (d, H, NH(C=S)), 7.54-7.46 (m, 3H, Thiophene), 4.22 (d, CH,(NH)), 3.40 (m, 8H, (piperazine)), 2.60 (s, 1H, CH_3 (piperazine)). **^{13}C NMR (300 MHz, DMSO-d$_6$, δ ppm)** 173.12 (-C=S, thiourea), 139.33 (C-S, thiophene), 126.49 (Ar-CH), 122.12 (CH(NH)), 57.73 (Aliphatic - CH), 40.47 (N-CH_3). ESI-MS: Mass spectra of the complex [M(L^3)$_2$Cl$_2$] (Fig.7) give peaks at m/Z 683.12 is assigned to[M+H]$^+$.

3.8. 1-(piperazin-1-yl(thiophen-2-yl)methyl)thiourea (L^4) (Fig.6)
^{1}H NMR (300 MHz, DMSO-d$_6$, δ ppm) 8.90 (s, 2H, NH_2), 8.00 (d, H, NH(C=S)), 6.00 (m, 3H, Thiophene), 5.41 (d, CH,(NH)), 3.40 (m, 8H, (piperazine)), 2.50 (s, 1H, NH of piperazine). **^{13}C NMR (300 MHz, DMSO-d$_6$, δ ppm)** 173.11 (-C=S, thiourea), 134.15 (C-S, thiophene), 126.02 (Ar-CH), 122.12 (CH(NH)), 57.73 (Aliphatic - CH). The mass spectrum of the compound (L^4) exhibits a molecular ion peak[M] at m/Z 256.3000 which corresponds to its molecular weight. The fragmentation peaks at m/z 245.48, 197.47, 184.51 indicate the cleavage of $C_{10}H_{15}N_3S_2$, $C_9H_{15}N_3S$ and $C_9H_{15}N_2S$, fragments respectively.

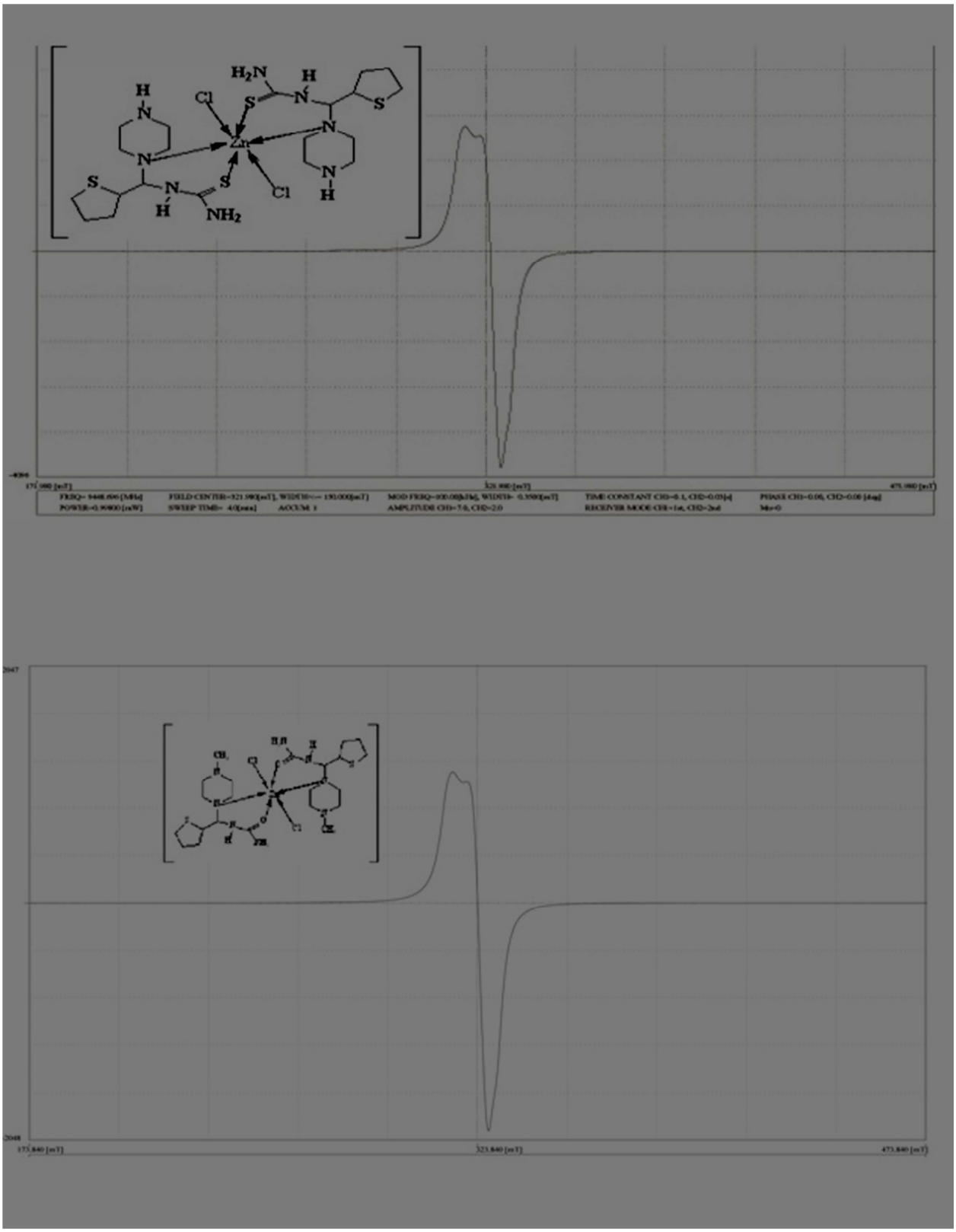
3.9. Bis(1-(piperazin-1-yl(thiophen-2-yl)methyl)thiourea)dichloroZinc(II). [M(L^4)$_2$Cl$_2$] (Fig.8)
^{1}H NMR (300 MHz, DMSO-d$_6$, δ ppm) 8.90 (s, 2H, NH_2), 7.01 (d, H, NH(C=S)), 6.00 (m, 3H, Thiophene), 5.41 (d, CH,(NH)), 3.42 (m, 8H, (piperazine)), 2.50 (s, 1H, NH of piperazine). **^{13}C NMR (300 MHz, DMSO-d$_6$, δ ppm)** 173.11 (-C=S, thiourea), 134.15 (C-S, thiophene), 126.02 (Ar-CH), 122.12 (CH(NH)), 57.73 (Aliphatic - CH). ESI-MS: Mass spectra of the complex [M(L^4)$_2$Cl$_2$] (Fig.8) give peaks at m/Z: 655.01 is assigned to[M+H]$^+$.

4.0. ESR SPECTRA
The ESR spectra of the complexes were recorded in the solid state at room temperature. The **g_{iso}**, **g_{II}** and **$g_\perp$** values are presented in **Table -3** and shown in **Fig. 12.** From the observed g values, it is clear that the unpaired electron lies predominantly on $d_{x^2-y^2}$. According Kivelson and Nieman, the **g_{II}** value of complexes greater than 2.3 are usually ionic in nature and less than 2.3 are covalent in nature**[39].** In the present study, the g values of all the complexes are found to be less than 2.3 indicating the covalent nature of the complexes.

Table 3: Solid State EPR spectral parameters of the Cu(II) complexes of Ligands (L^1, L^2, L^3 and L^4).

Sl. No.	Compound/Complex	g_{II}	$g_{\perp}$	$g_{average}$/ g_{iso}
1.	$[Cu(L^1)_2Cl_2]$	2.150548	2.084279	2.1284
2.	$[Cu(L^2)_2Cl_2]$	2.150924	2.084397	2.1287
3.	$[Cu(L^3)_2Cl_2]$	2.180040	2.017017	2.1256
4.	$[Cu(L^4)_2Cl_2]$	2.252644	2.079209	2.1948

Fig. 12. ESR SPECTRA OF Cu(II) COMPLEXES



4.1. IR SPECTRA AND MODE OF BONDING

The IR spectral data of ligands and their metal complexes are presented in **Table – 4.** IR spectral study of both the ligands and complexes help us to identify the coordination sites of the ligand. IR spectra of the ligands show ν(N^1H) in the range between 3464 and 3414 cm^{-1} whereas the values of ν(N^2H) lie in the range from 3310 to 3296 cm^{-1}. In the spectra of the complexes, ν(C=S) mode of the free ligand is not disturbed which indicates the absence of enolisation. The band due to ν(C=N) is not appeared, eventhough C=O and C=S bands appeared at 1606, 1661 , 951 and 967 cm^{-1} respectively. The bands due to ν(C=O) and ν(C=S) were found shifted to lower wave numbers in the spectra of the complexes indicating the involvement of oxygen/sulphur atom of carbonyl and thiocarbonyl in binding with the metal ion. The bands due to $\nu_{(NH)}$ and $\nu_{(C-O)}$ are found shifted to lower frequency by 20 – 30 cm^{-1} in the spectrum of each complex corroborating the coordination of nitrogen atom of N-methyl piperazine/piperazine with the metal ion. The stretching ν(N^1H), ν(N^2H) and ν(CH) of the ligands were not much altered in the spectra of the complexes which indicate the non- participation of groups . Hence it is concluded that the compound L^1, L^2, L^3 and L^4 act as a neutral bidentate ligand. Further, all the complexes exhibit bands around 559 – 553 and 484 – 464 cm^{-1} which are assignable to ν(M – O) and ν(M – S) respectively.

Table 4: IR Spectral data of Ligands (L^1, L^2, L^3, L^4) and their metal (M = Cu(II) and Zn(II)) complexes.

Sl. No.	Compound	$\nu_{(N^1H2)}$	$\nu_{(N^2H)}$	$\nu_{(CH)}$	$\nu_{(C-O)}$	$\nu_{(C=S)}$	$\nu_{(C-S-C)}$	$\nu_{(M-O)}$	$\nu_{(M-N)}$	$\nu_{(M-S)}$
1.	L^1	3424	3306	2977	1606	---	822	---	---	---
2.	$[M(L^1)_2Cl_2]$	3414	3300	2970	1598	---	828	553	484	---
3.	L^2	3453	3302	2932	1661	---	856	---	---	---
4.	$[M(L^2)_2Cl_2]$	3443	3301	2931	1655	---	816	559	484	---
5.	L^3	3464	3310	2930	---	951	854	---	---	---
6.	$[M(L^3)_2Cl_2]$	3426	3301	2922	---	942	832	---	675	464
7.	L^4	3458	3302	2928	---	967	844	---	---	---
8.	$[M(L^4)_2Cl_2]$	3450	3296	2919	---	952	817	---	677	484

4.2. UV-VIS SPECTROSCOPY

Electronic spectra of complexes were recorded in DMSO solution and are presented in **Table - 5.** For$[Cu(L^1)_2Cl_2]$ complex, an absorption band exhibited at 16000 cm^{-1} is assigned to $2B_{1g} \rightarrow 2A_{1g}$ transition. This corresponds to octahedral geometry of the complex. The absorption band exhibited at 17793 cm^{-1} for $[Cu(L^2)_2Cl_2]$ complex is assigned to $2B_{1g} \rightarrow 2B_{2g}$ transition, favouring octahedral geometry. $[Cu(L^3)_2Cl_2]$ complex shows two absorption bands at 13966 cm^{-1} and 12500 cm^{-1} respectively. These absorptions are assigned to $^2E_g \rightarrow {}^2T_{2g}$ and $^3A_{2g} \rightarrow {}^3T_{1g}$ transitions respectively, suggesting the distorted octahedral geometry. In the case of $[Cu(L^4)_2Cl_2]$ complex, two absorption bands are exhibited, one at 13927 cm^{-1} and another at 12019 cm^{-1} which are assigned to $^2E_g \rightarrow {}^2T_{2g}$ and $^3A_{2g} \rightarrow {}^3T_{1g}$ respectively. These transitions favour distorted octahedral geometry. The diamagnetic Zn(II) complex did not show any d-d bands and its spectrum is subjected only by a charge transfer band due to distribution of electrons between metal and ligands.

Table 5: UV Spectral data of metal complexes ($[Cu(L^1)_2Cl_2]$, $[Cu(L^2)_2Cl_2]$,$[Cu(L^3)_2Cl_2]$ and $[Cu(L^4)_2Cl_2]$).

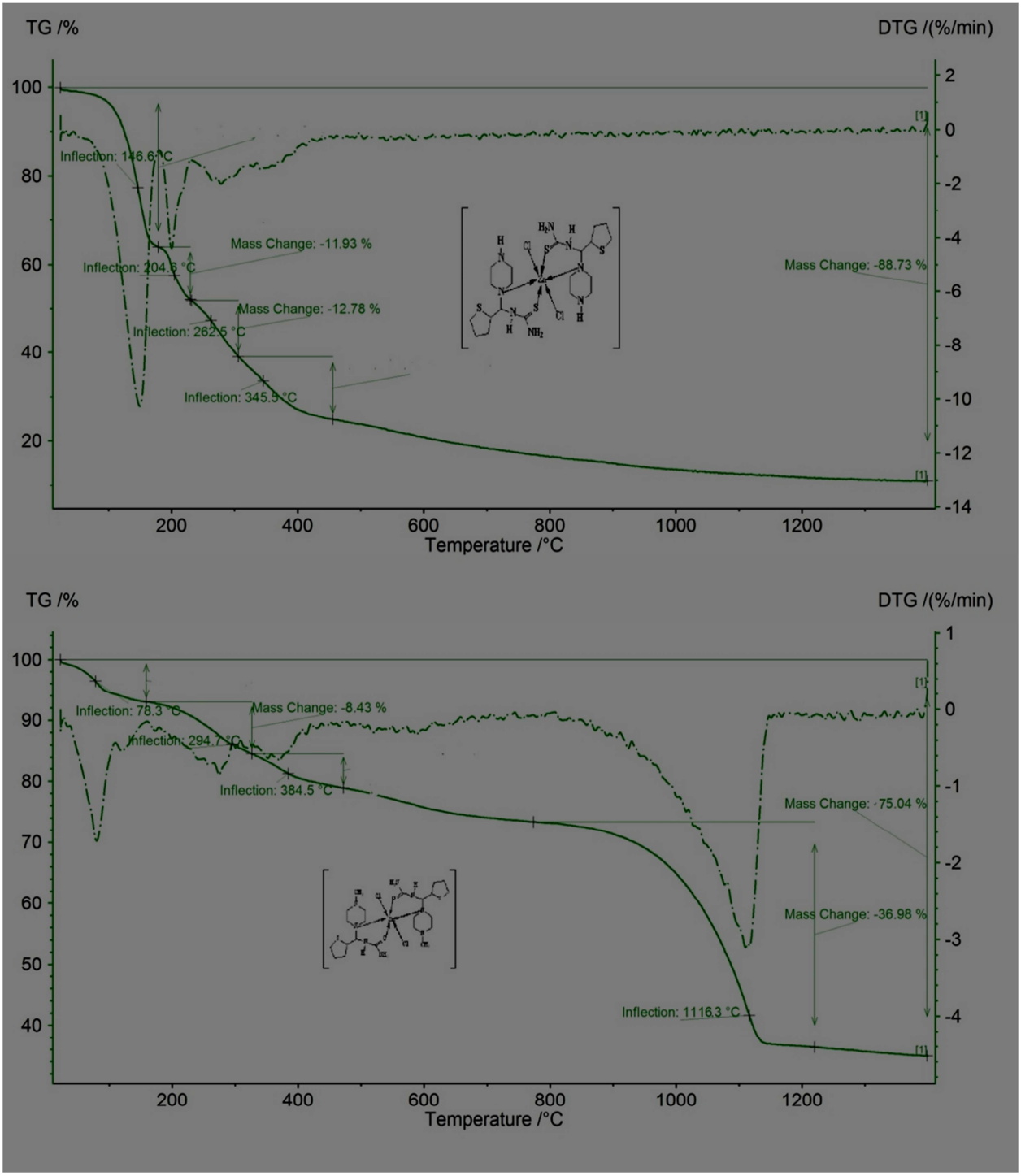
Sl. No.	Compound/Complex	Observed nm (cm^{-1})	Transitions
1.	$[Cu(L^1)_2Cl_2]$	625(16000)	$2B_{1g} \rightarrow 2A_{1g}$
2.	$[Cu(L^2)_2Cl_2]$	562(17793)	$2B_{1g} \rightarrow 2B_{2g}$
3.	$[Cu(L^3)_2Cl_2]$	716, 800(13966, 12500)	$^2E_g \rightarrow {}^2T_{2g}$, $^3A_{2g} \rightarrow {}^3T_{1g}$
4.	$[Cu(L^4)_2Cl_2]$	718, 832(13927,12019)	$^2E_g \rightarrow {}^2T_{2g}$, $^3A_{2g} \rightarrow {}^3T_{1g}$

4.3. THERMAL ANALYSIS

The nature of thermal stability and the molecules that are desorbed or buried in the complexes with respect to temperature was studied by TGA and compared with the literature values and thermogram are shown in **Fig. 13.** The complexes $[Cu(L^1)_2Cl_2]$ and $[Cu(L^2)_2Cl_2]$ were heated in nitrogen atmosphere with a heating rate of 25°C min^{-1}. The thermogram shows Three stages. The first mass change 8.43% (Calculated mass loss 10.9%) at 190 °C indicates the loss of Chlorine molecules. The second stage between at 480- 770°C with the mass change of 75.04% (Calculated mass loss 79.53%) corresponds to the loss of ligand moieties. The third stage between at 770- 1220°C with mass change 36.98% (Calculated mass loss 39.76%). Further, decomposition of metals takes place at 1220°C and remains stable upto1400°C.

The complexes $[Cu(L^3)_2Cl_2]$ and $[Cu(L^4)_2Cl_2]$ (Fig. 7) were heated in nitrogen atmosphere with a heating rate of 25°C min^{-1}. The thermogram shows three different regions. The first weight loss 11.93% (Calculated mass loss 10.85%) at 146.6 -180°C indicates the loss of two chlorine molecules. This is further supported by the color change due to the change of co-ordination from 6 to 4. The region between 210 - 310°C corresponds to the loss of coordinated organic moieties. The third region between at 310-450°C and stays stable up to 1400°C and the weight loss found to be about 88.73% (Calculated mass loss 80.49%) for this step. The residue of metal atom remains behind because under nitrogen atmosphere no metal oxide will be formed.

Fig.13. TGA AND DTA CURVE OF COPPER(II) COMPLEXES



4.4. ANTICANCER ACTIVITY

The *in-vitro* anticancer activity of synthesized compounds L^1 and L^3 was studied against human lung cancer (A549) and colon cancer (HCT15) cell lines along with normal (VERO) cell lines. The results of the study are presented in **Table - 6**. The results revealed that both the compounds exhibited good anticancer potential. Compounds L^1 and L^3 are found to possess potent anticancer activity (IC_{50} = 12.5% for L^1 and 12.2% for L^3) and (IC_{50} = 11.6% for L^1 and 9.7% for L^3) in 1000 µg/mL against human lung cancer (A549) and colon cancer (HCT15) cell lines respectively. It may be taken as a new lead for the development of novel anticancer agents. At the same time, these two compounds show 55.5% of cell viability of normal cell line (VERO). Among the two compounds, higher cytotoxic activity was observed for L^3 against colon cancer cell (HCT15).

Table 6: Cytotoxic activity of synthesized Mannich bases L^1 and L^3 against normal (VERO), human lung (A549) and colon (HCT15) cancer cell lines.

Sl. No.	Cell Viability%						
	Concentration µg/ml	Normal Cell (VERO)		Lung Cancer (A549)		Colon Cancer (HCT15)	
		L^1	L^3	L^1	L^3	L^1	L^3
1	1000	55.5	54.5	12.5	12.2	11.6	9.7
2	500	60.3	59.5	20.4	21.6	17.6	14.1
3	250	67.1	66.9	26.6	25.0	24.5	21.3
4	125	71.6	71.2	44.0	42.7	41.8	40.9
5	62.5	78.2	77.0	48.3	46.5	46.5	45.4
6	31.2	89.3	87.5	62.1	60.6	61.1	59.7
7	15.6	93.5	90.7	81.1	80.3	79.5	77.3
8	7.8	98.4	92.3	89.5	89.4	90.1	88.9
9	Control	100	100	100.0	100	100.0	100

CONCLUSION

Mannich bases L^1, L^2, L^3 and L^4 were prepared for studying the anticancer activity. The results of physical and chemical methods reveal that the ligands act as bidentate ligands. From the spectral and physical studies, Octahedral geometries have been proposed for Cu(II) and Zn(II) complexes of L^1, L^2, L^3 and L^4. The coordination with metal ion takes place through O and S atoms. The results of molar conductivity and magnetic susceptibility measurements reveal the non-electrolytic nature of the metal complexes. EPR and TG-DTA studies also support the other spectral data. Invitro anticancer study shows that both L^1 and L^3 have moderate activity against the selected cell lines. Further, these synthesized compounds have less toxic and side effects when compared to the other commercially used anticancer drugs. In the near future, we can also work on the cell line culture to be generated and use it for remedy of other types of cancer.

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