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Synthesis, Spectral, Antimicrobial and *In vitro* Antitumorous Studies of Copper based mulltidentate thiosemicarbazones

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ABSTRACT

This is a report of the studies conducted on the synthesis, spectral, biological viz. antibacterial and antitumorous activities of some copper complexes synthesized from a multidentate thiosemicarbazone ligand, HL^2. This is based on a reaction between a secondary amine viz.N-phenyl piperazine and an N-substituted heterocyclic ketone viz. 2-Benzoylpyridine. The ligand exhibits a multidentate mode of coordination through N,N,S donor atoms in the copper complexes. The electron paramagnetic resonance studies of the complexes(solution spectra) at LNT in DMF shows typical axial spectra with distinct g-values, $g_{||} > g_{\perp}$ indicating a slightly distorted four coordinated planar geometry. The biological studies viz. antibacterial and antitumorous studies suggest their use as competent antibacterial and antitumor agents.

Keyword: Copper complexes, Multidentate, EPR spectra, g-values, Antitumorous studies.

INTRODUCTION

The metal complexes derived from the thiosemicarbazone ligands has gained large focus and become a major subject of research for many chemists. Their remarkable chelating ability bonding through sulphur and azomethine nitrogen atoms with transition elements belonging to first row has attracted many researchers.[1-3] The most striking biological properties include anti tumorous, antioxidant, antibacterial, antifungal, anticancerogenic,besides showing insulinmimeticeffects.[4-12] thiosemicarbazones exist in thione and thienol forms both in solution and solid state, most strikingly thione form stays in solution phase.

The biological activity of thiosemicarbazones with heterocyclic bases is proven to exhibit a N,N,S tridentate planar system of coordination enhancing the biological activity.[13,14] The biological activity is rather well influenced by the point of attachment of thiosemicarbazone moiety with the heterocyclic ring system. The highest activity arises when it gets attached through the 2nd position and subsequently diminishes towards to positions 3rd or 4th. This work involves the synthesis, characterization, antibacterial and short term antitumorous properties of the copper complexes. Obtained from the thiosemicarbazone ligand, HL^2.

MATERIALS AND METHODS

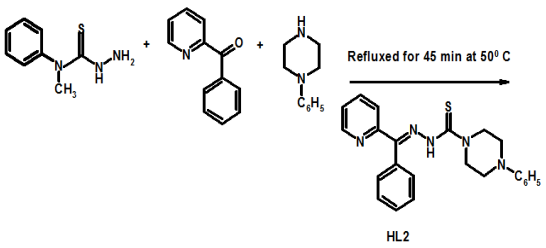
Materials

Analytical grade pure chemicals and solvents

were used in this study withoutfurtherpurification. The copper salts used were $Cu(NO_3)_2 \bullet xH_2O$, $CuCl_2.2H_2O$, $CuSO_4.5H_2O$, $Cu(CO_2CH_3)_2.H_2O$, besides NaN_3 and KSCN. Elemental C,H,N analyses was done using Elementar Vario ELIII analyzer. FTIR spectra was recorded as KBr pellets in the range 4000-400 cm^{-1} on a Thermo Nicolet Avatar FTIR spectrometer. The UV-Vis spectra were recorded using Varian Carry 5000 UV-VIS-NIR spectrophotometer in the range 900-250 nm. ^{1}H-NMR spectra was recorded in $CDCl_3$ solvent using TMS as internal reference in a Bruker 400 AvanceIII spectrometer. The ESR-JEOL Japan instrument was used to record solution phase EPR spectra (X-Band) in frozen DMF at LNT(77K).

Synthesis of thiosemicarbazone ligand(HL^2)

The already reported procedure was used to prepare compounds 4-methyl-4-phenyl-3-thiosemicarbazide and carboxymethyl-N-methyl-N-phenyl dithiocarbamate.[15,16] Equimolar amounts of 4-methyl-4-phenyl-3-thiosemicarbazide (1 g, 5.52 mmol) dissolved in 5 mL hot methanol along with 2-benzoylpyridine (1.011 g, 5.52 mmol) and N-phenyl piperazine (0.84 mL, 5.52 mmol) separately dissolved in 10 mL methanol (99.9%) were refluxed for 45 min at 50°C. The resulting solution was left to chill which yielded 40% (0.160 g)yellow shining microcrystals of HL^2. The product so obtained was filtered, washed and recrystallized using methanol and kept in dessicator with P4O10 to remove all the moisture content. The synthesis of the ligand is as shown.



Scheme 1. Synthesis of ligand, HL^2

Synthetic route of copper complexes

The methanolic solutions of appropriate copper salts and ligand, HL^2 were refluxed. Chloro, nitrato and sulphato complexes were synthesized by refluxing solutions of respective hydrated copper (II) salts and ligand in 1:1 molar ratio for three hours. The mixture was allowed for slow evaporation at room temperature. Dark green colored microcrystals of copper(II) complexes obtained were filtered, washed with water and ether and dried over P_4O_{10} in *vacuo*.

The azido and thiocyanato complexes were synthesized by refluxing equimolar mixtures of ligand, HL^2 and copper(II) acetate salt first followed by the addition of equimolar amounts of methanolic solution of sodium azide and potassium thiocyanate, respectively to the hot mixture and refluxed for next 3 hours. The mixture was left to cool at room temperature that gave rise to dark green microcrystals of azido and thiocyanato copper complexes. The complexes were filtered, washed with ether and dried over P_4O_{10} in *vacuo*.

Bio-Assay

Antibacterial studies

The antibacterial studies the ligand, HL^2 and synthesized copper complexes were done using agar well diffusion technique using MHA (Mueller Hinton Agar) medium and streptomycin positive control at two different concentrations.

Antitumorous Studies

The antitumorous studies of the copper complexes were done by Trypan blue dye exclusion method on DLA ("Daltons lymphoma ascites") tumour cells derived from tumour bearing mice at different concentrations.

RESULTS AND DISCUSSION

The C,H,N results of the complexes are in good agreement with the anticipated values as per the empirical formula [M(L)X], where M=Cu(II), L=deprotonated ligand, HL^2 and X=Cl, NO_3, N_3, SCN and $[M_2(L)_2X]$ where X=SO_4. The low molar conductivity values of the copper (II) complexes at room temperature in 10^{-3} M solution of DMF indicates their non-electrolytic nature suggesting the absence of counter ions outside the coordination sphere.[17] The magnetic moment values of copper complexes in the range 1.7-2.2 B. M. are characteristic of a d^9 planar system in all the synthesized complexes.

Table 1. shows colour, stoichiometries, elemental C,H,N data and magnetic moments of the copper(II) complexes of ligand HL^2.

Table 1.: Colour, elemental analyses data and magnetic moments of ligand HL^2 and its copper(II) complexes

Compound	Formulae[a]	Colour	Composition%\Found /(Calcd)			μ[b](BM)
			Carbon(%)	Hydrogen(%)	Nitrogen(%)	
HL^2	$C_{23}H_{23}N_5S.$	Yellow	67.88 (67.32)	6.00 (5.85)	17.70 (17.61)	-
$[Cu(L_2)Cl]$	$CuC_{23}H_{22}N_5SCl$	Green	55.24 (55.30)	4.13 (4.39)	13.91 (14.06)	1.55
$[Cu(L_2)(NO_3)]$	$CuC_{23}H_{22}N_6SO_3$	Green	52.42 (52.51)	4.10 (4.19)	16.23 (15.98)	1.73
$[Cu_2(L_2)_2(SO_4)].H_2O$	$Cu_2C_{46}H_{46}N_{10}S_3O_5.$	Green	53.12 (53.01)	4.20 (4.41)	13.40 (13.44)	1.82
$[Cu(L_2)N_3]$	$CuC_{23}H_{22}N_8S$	Green	54.91 (54.60)	4.50 (4.34)	22.02 (22.15)	1.93
$[Cu(L_2)(NCS)]$	$CuC_{24}H_{22}N_6S_2$	Green	55.70(55.22)	4.41 (4.21)	16.41 (16.10)	1.96

[a]Empirical formulae [b]Magnetic Susceptibility

Spectral characterization

Infrared spectral studies

The positions of bands due to ν(C=C) and ν(C=N) vibrational modes vary from those of the ligand spectrum constituting bands in the 1600-1350 cm^{-1} region in infrared spectra. This could be because the newly formed (N=C) bond overlaps with the ν(C=N) bond. Consequently the complexes shift the azomethine group stretching ν(C=N)str, thiosemicarbazone moiety band at 1592 cm^{-1} in the ligand HL^2, showing coordination through azomethine nitrogen. The (N-N) band shift at 1014 cm^{-1} of thiosemicarbazone moiety in complexes further confirms coordination through azomethine nitrogen. The bands due to thiolate sulphur stretching, ν(C=S)str at 1339 cm^{-1} and thiolate sulphur bending, δ(C=S) at 870 cm^{-1} in HL^2 shifts to lower frequencies attributing to coordination via the thiolate sulphur.[17] This implies substantial electron-delocalisation and a change of bond order occuring during chelation.The coordination through pyridyl nitrogen is confirmed by plane bending vibrations of pyridine ring, δpy(o.p) at 650 cm^{-1} in free ligand shifting to higher frequencies.[17-19] Bands at 1430 cm^{-1} and 1297 cm^{-1} with a spacing of ~130 cm^{-1} corresponding to 1 and 4 nitrato group vibrational modes are seen in the nitrato complex. This confirms terminally N-coordinated monodentate nitrato group coordination in the synthesized complex.[17,19]

The spectrum of synthesized "sulpato complex" to complex contains two moderate intensity regions with bands at 923 cm^{-1} and 463cm^{-1} due to ν_1 and ν_2 vibrational modes, correspondingly. The ν_3 vibrational mode splits into three weak bands at 1021 cm^{-1}, 1112 cm^{-1}, 1226 cm^{-1}. The spectrum fails to show bands at 500, 575, 825 cm^{-1} indicating water molecules to be present as lattice water.[22] These spectral results indicate that in the synthesized complex, sulphato anion adopts a bridging bidentate form of coordination.[18,22]

The synthesized azido complex exhibits strong bands at 2043 cm^{-1} due to the coordinated azido group asymmetric, a(NNN) vibration,and a strong band at1386 cm^{-1} from its symmetric, s(NNN) vibration. The (N-N-N) azido group vibrational modes are represented by the medium band at 648 cm^{-1}. These spectral data suggests a non-linear Cu-(NNN) bond in the synthesized azido complex.[18] The synthesized thiocy an to complex of ligand HL^2 demonstrates a very strong band at 2095 cm^{-1} characteristic of (C=N) thiocyanato group vibrations. The bands at 787 cm^{-1} and 463 cm^{-1} may be allocated to (C=S)andⅡ(NCS)vibrations, of the thiocyanato complex. These facts confirm the thiocyanate anion to be N-coordinated to the copper(II)ion.

Table 2. shows some of the most important tentative IR spectral assignments of the ligand and its complexes.

Table 2: Infrared spectral assignments (cm^{-1}) of ligand, HL^2 and its copper(II) complexes

Compound	Ⅱν(C=N) & (N=C)	Ⅱ ν(N-N)	Ⅱ (C=S) & δ(C=S)	Ⅱpy δ(o.p)
HL^2	1593 m	1014 w	1339 m, 788 m	650 w
$[Cu(L_2)Cl]$	1598 m	1123 w	1305 m, 763 m	698 w
$[Cu(L_2)(NO_3)]$	1597 m	1122 w	1299 m, 759 m	695 w
$[Cu_2(L_2)_2(SO_4)].H_2O$	1598 m	1112 w	1307 m, 784 m	684 m
$[Cu(L_2)N_3]$	1597 m	1112 w	1304 m, 779 m	699 w
$[Cu(L_2)(NCS)]$	1599 m	1122w	1301 m, 787m	689 w

Electronic spectral studies

The band at 282 nm (35,460 cm^{-1})resulting from the pyridylring $\pi \rightarrow \pi^*$ transitions shows a red shift because of >C=S bond weakening and strengthening of the conjugated system. The band at 341 nm (29, 325 cm^{-1}) caused by the pyridyl nitrogen $n \rightarrow \pi^*$ transition in the free ligand, HL^2 changes to shorter wavelengths or displays a blue shift and significantly loses intensity in complexes, indicating that pyridyl nitrogen serves as a coordinating molecule.[28] The synthesized copper complexes exhibits two ligand-metal charge transfer, at about 380 nm (26,000 cm^{-1}) & 430 nm (23,000 cm^{-1}). The band at higher wavenumber may be assigned to sulphur to copper(II) viz. ligand to metal charge transfer, where as the second intense band in the range can be due to the combination of sulphur and pyridyl nitrogen combined charge transfer from ligand to copper(II) in the synthesized complexes.[29,30] The synthesized complexes exhibit d-d bands as weak shoulders in the in the 500-625 nm (16,000-20,000 cm^{-1}) range which is typical of a square planar geometry.[28] Table 3. shows the tentative electronic spectral assignments of the ligand HL^2 and its copper complexes recorded in the region 200-800 nm.

Table 3: Electronic spectral assignments (nm) of ligand HL^2 and its copper(II) complexes

Complex	d-d bands	Charge-transfer bands	$n \rightarrow \varpi^*$	$\pi \rightarrow \pi^*$
HL^2	-	-	341,439 (sh)	282
$[Cu(L_2)Cl]$	570 (sh)	383 (sh), 432 (sh)	337	286
$[Cu(L_2)(NO_3)]$	527 (sh)	375 (sh), 432 (sh)	332	289
$[Cu_2(L_2)_2(SO_4)].H_2O$	569 (sh)	377 (sh), 421 (sh)	335	288
$[Cu(L_2)N_3]$	543 (sh)	378 (sh), 432 (sh)	332	283
$[Cu(L_2)(NCS)]$	615 (sh)	376 (sh), 429 (sh)	322	285

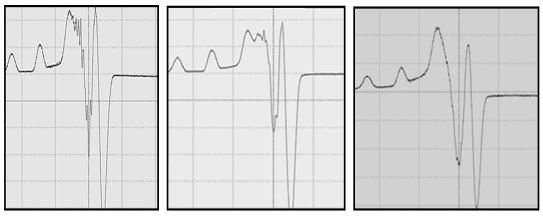
EPR Spectral studies

EPRspectra of the synthesized copper complexes were recorded in DMF at 77K, (LNT).The EPR spectra (axial type) of chloride, nitrate and azide complexes in DMF at 77K, (LNT) is shown in Fig .1 (a,b,c) correspondingly. The spectra represents a typical axial pattern with distinct g || and g $\perp$ values varying for each complex. The G-values less than 4 in the range 3.5-4, are evident of appreciable exchange interaction. The synthesized copper(II) complexes show $g_{||} > g_{\perp} > 2$ coherent to a $d_{x^2-y^2}$ ground state characteristic of a planar geometry. The synthesized complexes exhibit orbital reduction variables $K_{||}$ and $K_{\perp}$, that are consistent with the trend, $K < K_{\perp}$ indicating π-bonding. Both in-plane σ and π bonding are consistent with the bonding parameters that were assessed.[24] Three of the four hyperfine lines in the $g_{||}$ area of the chloro and nitrato complexes, of ligand HL^2 are clearly visible, while the fourth hyperfine line overlaps with the $g_{\perp}$ || component leading to the observation of five super-hyperfine lines in the perpendicular component. Four hyperfine lines as well as seven super-hyperfine splitting can be seen in the $g_{||}$ region of the azido and thiocyanato complex because of the electron spin coupling with the nitrogen atoms nuclear spinning in the azide as well as thiocyanate anions which are coordinating to the copper(II) ions. The assessed bonding parameters agree with both the in-plane σ and π bonding.[24-30] Table 4. displays the spin Hamiltonian and bonding characteristics of the synthesized copper complexes.

Bio assay

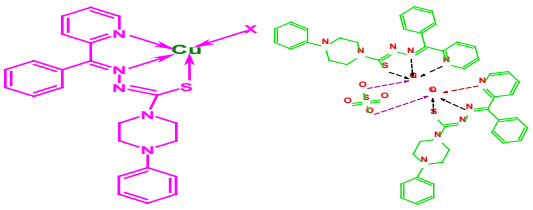
Table 4: Spin Hamiltonian and Bonding parameters of ligand HL^2 and its copper complexes

DMF Soln(77K)	$[Cu(L^2)Cl]$	$[Cu(L^2)(NO_3)]$	$[Cu_2(L^2)_2(SO_4)].H_2O$	$[Cu(L_2)N_3]$	$[Cu(L_2)(NCS)]$
$g_{||}$ ||	2.034	2.057	2.046	2.031	2.030
g$\perp$ ||	2.125	2.199	2.170	2.114	2.110
g_{av}	2.0783	2.1267	2.092	2.072	2.082
$A_{||}$(mT)	20.30	13.6	16.2	19.0	17.1
A$\perp$(mT)	2.87	18.8	2.50	2.69	2.62
G	3.8392	3.6629	3.7517	3.9268	3.8881
α^2	0.7337	0.6469	0.6822	0.6855	0.6733
β^2	0.7453	1.0795	0.9491	0.7710	0.7674
γ^2	0.761	1.128	0.9805	0.7783	0.7784
$K_{||}$ ||	0.547	0.6983	0.6472	0.5287	0.5167
K$\perp$ ||	0.5584	0.7297	0.6684	0.5335	0.5240



(a) (b) (c)

Fig. 1. EPR spectra of $[Cu(L_2)Cl]$, $[Cu(L_2)(NO_3)]$, $[Cu(L_2)N_3]$ complexes in DMF at 77K, LNT



(a) X=Cl, NO_3, N_3, SCN (b) X=SO_4

Fig. 2. Proposed structures of the synthesized copper(II) complexes

Antibacterial studies

The copper(II) complexes were tested for antibacterial property using Mueller Hinton Agar(MHA) medium and streptomycin as positive control against two *Gram positive* and two *Gram negative* strains of bacteriae at two sample concentrations.[31] The inhibition zones produced by complexes against selected bacterial strains are shown in Fig. 3. *Gram positive and Gram negatives* trains of bacteria chosen were; *S. aureus* (ATCC25923), *S. mutans* (MTCC890), *E. coli* (ATCC25922) and *P. aeruginosa* (ATCC27853). These copper(II) complexes exhibit antibacterial activities superior to the ligand HL^2 showing inhibition zones above 1.0 cm. The highest activities have been recorded by $[Cu(L_2)N_3]$ and $[Cu(L_2)(NCS)]$ complexes, producing inhibition zones of 2.2 cm against *S.aureus* (1000 µg/mL) and *S. mutans* (1000 µg/mL), respectively. In view of the chelation action which lowers the polarity of the metal ion one may hypothesize a potential route of toxicity.[32,33] In addition to chelation, metal ion participation in normal cell activity might be responsible for the increased toxicity of the metal complexes.[34] The most ideal location for metal ion interactions are in the walls of the cells and membranes with a lipophilic character. A subsequent reduction in the polarity strengthens the chelate's lipophilic character which facilitates the metal ion interaction with the lipid. This causes the permeability barrier of the cell to break down, disrupting regular cell activities. Because all of the structures induce a range of functional groups that might serve as metal binding agents, metal ions interacting with binding molecules.[34] The addition of anion coordination and polar substituents further enhance antibacterial activity. Table 5 shows the antibacterial screening results of copper(II) complexes of ligand HL^2 by agar well diffusion method.

Table 5: The antibacterial results (inhibition zone in cm's) of copper complexes of ligand HL^2 following agar-well diffusion method

Complexes	Inhibition Zone (in cm's) Bacterial strains							
	S. aureus		*S. mutans*		*P. aeruginosa*		*E. coli*	
	C(I)	C(II)	C(I)	C(II)	C(I)	C(II)	C(I)	C(II)
HL^2	1.6	1.9	-	1.1	-	-	-	1.3
$[Cu(L^2)Cl]$	1.1	1.4	1.6	2.1	-	1.3	-	1.3
$[Cu(L^2)(NO_3)]$	1.7	2.0	1.7	2.1	1.0	1.4	-	1.6
$[Cu_2(L^2)_2(SO_4)].H_2O$	1.7	2.1	1.3	1.9	-	1.8	-	1.4
$[Cu(L^2)N_3]$	1.9	2.2	1.4	2.0	-	1.1	-	1.7
$[Cu(L^2)(NCS)]$	1.2	1.5	1.4	2.2	-	1.0	-	1.1

C(I)=250 µg/mL, C(II)=1000 µg/mL

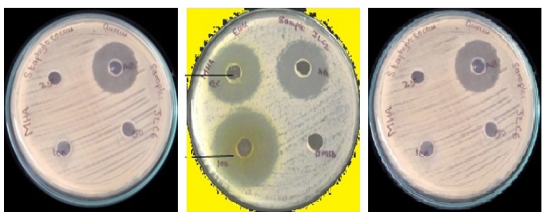


Fig. 3. Petriplates showing inhibition zones produced by synthesized copper(II) complexes

Antitumorous studies

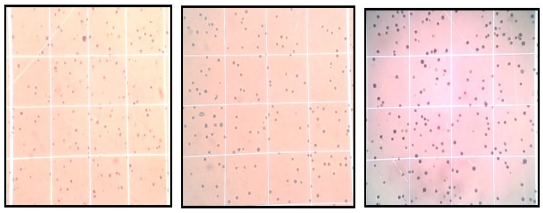
Using (DLA) tumor cells aspirated from the peritoneal cavity of tumour bearing mice copper(II) complexes were investigated for anti tumorous studies following the trypan blue dye exclusion approach. The photographs of cytotoxic screening results of the complexes are shown in Fig. 4(a),(b)&(c). The cytotoxicity findings show extremely high activities ranging from 6% to 100% with the highest activity of

100% recorded for [Cu(L_2)NCS], complex at 200 µg/mL concentration. These results suggest their use as effective anti tumorous agents. This could be due to the fact that whereas healthy live cells are unstained because the stain is blocked from entering the cell due to a tough cell membrane, tumor affected cells with compensated cell membrane integrity allow the entrance of the trypan blue dye and appear blue. Table 6. Shows the short term *In vitro* antitumorous results of the copper(II) complexes of HL^2.

$$\% \text{ cytotoxicity} = \frac{\text{No.of dead cells}}{\text{No.of Live cells + No. of Dead cells}} \times 100$$

Table 6: The short term antitumorous screening results of the copper complexes of HL^2

Complex concentration (µg/mL)	%cytotoxicity of complexes				
	[Cu(L_2)Cl]	[Cu(L_2)NO_3]	[Cu(L_2)$_2SO_4$].H_2O	[Cu(L_2)N_3]	[Cu(L_2)NCS]
10	20	6	15	20	40
20	48	12	20	38	52
50	58	22	26	50	75
100	70	36	35	63	88
200	82	58	46	84	100



(a) [Cu(L^2)NO_3]58% (b) [Cu(L^2)N_3]84% (c) [Cu(L^2)NCS]100%

Fig. 4. Photographs showing antitumorous effects of the synthesized copper complexes against tumor cells at 200 (*µ*g/mL) concentration

CONCLUSION

In this analysis five complexes of thiosemicarbazone ligand (HL^2) were well characterized after physico-chemical and spectroscopic methods. The studies reveal a tridentate mode of coordination by ligand HL^2 to the copper(II) ions in the complexes using thiolate sulphur, pyridylnitrogen and azomethine nitrogen, holding the 1:1:1 metal:ligand:anion coordination stoichiometry. Antibacterial screening results of copper complexes are superior to that of the ligand against selected strains of bacteriae suggesting their use as potent antibacterials. The short term in vitro anti tumorous studies indicate their ability to show highly evolved activities against the tumour cells suggesting their use as competent antitumor agents.

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Conflicts of Interest

There are no conflict of interest.

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