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Original article

Synthesis and antitumor studies on novel Co(II), Ni(II) and Cu(II) metal complexes of bis(3-acetylcoumarin)thiocarbohydrazone

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Abstract

The synthesis, structure, physico-chemical investigation and biological studies of some metal complexes of thiocarbohydrazone ligands are described. The ligand is obtained by condensation of *N*,*N*′-thiocarbohydrazide with 3-acetylcoumarin. The metal complexes of Co(II), Ni(II) and Cu(II) with bis(3-acetylcoumarin)thiocarbohydrazone were synthesized and isolated as solid products and characterized by analytical means as well as by spectral techniques such as FT-IR, ^{1}H NMR and EPR and UV spectrometry. The ligand acts as bidentate, through NO or NN, neutral in coordinating the M(II) ions. The bonding sites are the azomethine nitrogen, lactone carbonyl oxygen and respective anion counterparts. The metal complexes exhibit either octahedral or distorted octahedral structures. The complexes are found to be soluble in dimethylformamide and dimethylsulphoxide. Molar conductance values in dimethylsulphoxide indicate the non-electrolytic nature of the complexes. The compounds tested in present study have shown promising cytotoxic activity when screened using the *in vitro* method and at the same time were shown to have good activity when tested using the Ehrlich Ascites Carcinoma model. Preliminary antimicrobial screening shows the promising results against both bacterial and fungal strains.

Keywords: Thiocarbohydrazones metal complexes; Cytotoxic activity; Antitumor activity; Antimicrobial activities

1. Introduction

The compounds containing thione (>C=S) and thiol (≡C−S) groups occupy an important position among organic reagents as potential donor ligands for transition metal ions [1]. Both the organic compounds and their metal complexes display a wide range of pharmacological activity including anticancer, antibacterial and fungistatic effects. A good deal or research has been carried out on coordination chemistry of thiocarbohydrazones and thiocarbohydrazide attached to carbonyl compounds having heterocyclic moiety to give a potential ligand [2] but their metal complexes are scanty. In view of this, we planned to design a ligand of thiocarbohydrazide attached to carbonyl compounds having heterocyclic moiety, namely, 3-acetylcoumarin. The coumarins constitute an important class of compounds, with several types of pharmacological agents possessing anticancer, anti-HIV, anticoagulant, and spasmolytic and antibacterial activity among others. Of the many actions of coumarins, antioxidant and antiproliferative effects stand out. A large number of structurally novel coumarin derivatives have ultimately been reported to show substantial cytotoxic activity *in vitro* and *in vivo*. Coumarin (1,2-benzopyrone) is structurally the least complex member of a large class of compounds known as benzopyrones [3]. In addition, coumarins have been shown to inhibit *N*-methyl-*N*-nitrosourea, aflatoxin B1 and 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis in rats [4,5]. More recently, coumarin derivatives have been evaluated in the treatment of human immunodeficiency virus, due to their ability to inhibit human immunodeficiency virus integrase [6,7]. The *in vitro* effects

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of coumarins on the growth of renal cell carcinoma that derived cell lines showed that coumarin and 7-hydroxycoumarin were potent cytotoxic and cytostatic agents [8]. Several authors have reported on the use of coumarin (1,2-benzopyrone), or its metabolite 7-hydroxycoumarin, for the treatment of some human carcinomas [9–12] and to inhibit cell growth of cell lines of various types of cancer [13–17].

The coumarin derivatives have been the focus of our recent research concerning the design of new cytotoxic agents. For coumarins, generally the *in vitro* structure–activity relationship studies have shown that cytotoxicity is found with derivatives containing *ortho*-dihydroxy substituents. Also, the chemical-structure/biological activity study of the coumarins showed that the addition of a cathecolic group to the basic structure induces increased cytotoxic activity in tumor cell lines [18]. The different cytotoxic values found for the coumarins could be related to the presence and the positions of the hydroxyls in their structures. It is well known that many investigations have proved that binding of a drug to a metalloelement enhances its activity and, in some cases, the complex possesses even more healing properties than the parent drug. This has prompted us to investigate the metal binding properties of several coumarin derivatives.

Thiocarbohydrazone in the present investigation was used as the diamine with 3-acetylcoumarin to construct a tetradentate ONNO donor ligand, hoping that the toxophoric functional group (>C=S) will be away from the coordination site so that those free functional group would provide ''points of attachment'', a system which mimics certain classes of biological systems and in addition such groups could be toxic to microbes when used as drugs. Interest in complexes of these ligand systems now covers several areas ranging from general considerations of the effect of sulfur and electron delocalization in transition metal complexes to potential biological activity and practical application [19–21]. The tautomerism of these ligands and also the well-known tendency of oxygen and sulfur donors to act as bridging ligands [22,23] allow various structural possibilities for the corresponding metal complexes.

Thus, the aim of the present work is to synthesize and characterize complexes of cobalt(II), nickel(II) and copper(II) with potential biologically active Schiff base ligand, bis(3-acetylcoumarin)thiocarbohydrazone (Fig. 1) and to determine their cytotoxic activity.

2. Chemistry

Thiocarbohydrazide [24] and 3-acetylcoumarin [25] are prepared following the literature procedures. The ligand bis(3-acetylcoumarin)thiocarbohydrazone [26] is prepared by the condensation reaction of thiocarbohydrazide (0.02 mol) and 3-acetylcoumarin (0.04 mol) in presence of glacial acetic acid in ethanol medium. Complexes of the thiocarbohydrazone ligand were prepared by reaction of the thiocarbohydrazone with the transition metal salts in the appropriate molar ratios. Metal chlorides and acetates of cobalt, nickel and copper and also sulphates and nitrates in case of copper were used. The reaction sequences are outlined in Schemes 1 and 2.

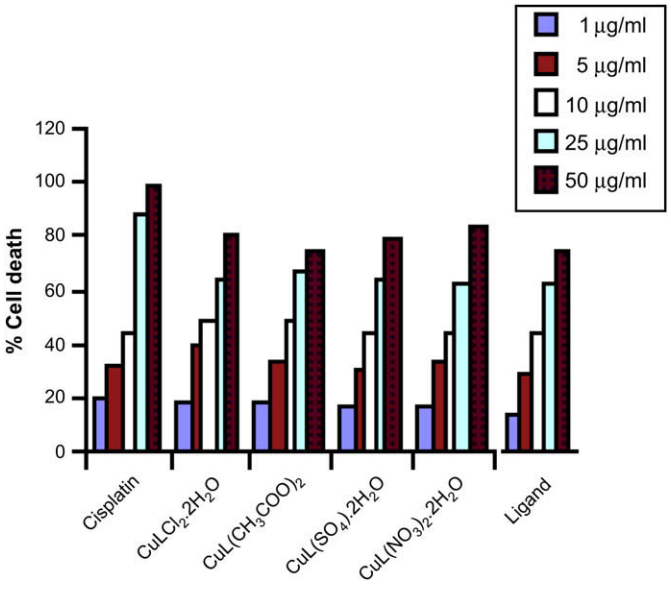
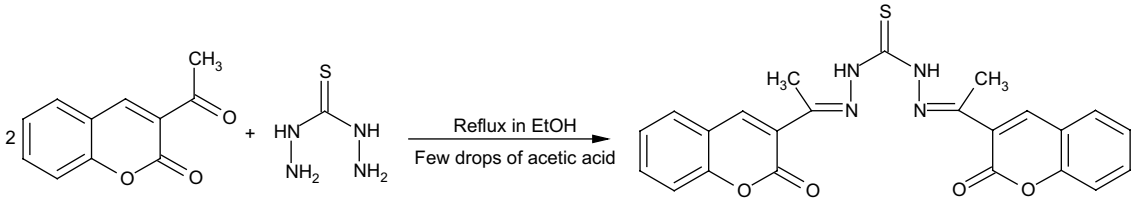


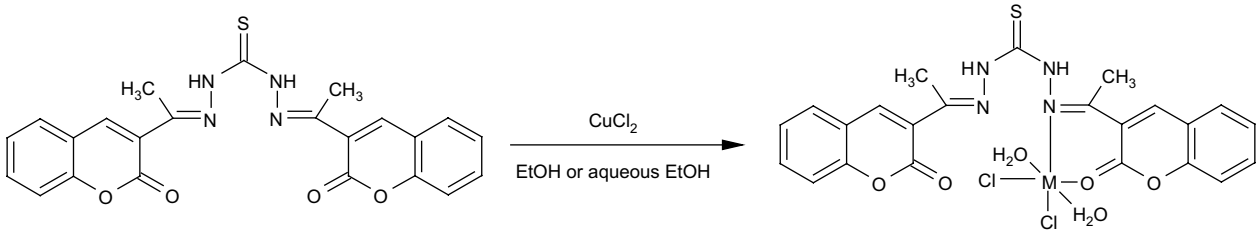
Fig. 1. Short term *in vitro* cytotoxicity of compounds towards EAC cells.

3. Results and discussion

The Schiff base ligands were readily prepared by convenient methods (Scheme 1) from a 3-acetylcoumarin and thiocarbohydrazide. The Schiff base formations were undertaken at a boiling temperature of water in non-aqueous solvent, i.e., ethanol. The ligand has been characterized by IR, ^{1}H NMR spectroscopy and elemental analysis. Reaction of metal(II) salts and the respective ligands in a 1:1 molar ratio gives rise to complexes C1–C8. The growth of single crystals of these complexes for X-ray studies is very difficult owing to their amorphous nature and we were unsuccessful in our attempts to do so. The elemental analyses of these complexes reveal 1:1 ligands to metal stoichiometry (Table 1). The complexes are found to be soluble in dimethylformamide, dimethylsulphoxide and acetonitrile but insoluble in common



Scheme 1. Synthesis of ligand.



Scheme 2. Representative synthesis of copper chloride complex.

organic solvents such as ethanol, methanol, benzene, acetone, water, etc. The composition and coordination geometry of these complexes have been established on the following experimental observations. The *molar conductance* values in dimethylsulphoxide fall in the expected range (10–32 $cm^2\,\Omega^{-1}\,mol^{-1}$) of non-electrolytes [27] indicating that chloride ions are inside the coordination sphere, and the complexes have the general formulae $[ML(X_2)]\cdot 2H_2O$. The complexes were analyzed for metal, nitrogen, sulfur and chloride. The analytical data, physical properties such as colour, conductivity and magnetic moment of the complexes are summarized in Table 1.

3.1. Infrared spectra

The infrared spectral data of the Schiff base ligand show a medium intensity absorption band at 3240 cm^{-1} due to N–H stretching and a band around 1710 cm^{-1} due to a lactone C=O stretching vibration of coumarin moiety. The bands which appear at 1550, 1240, 1100 and 760 cm^{-1} were assigned to [28–30] thioamide-I, -II, -III and -IV vibrations, respectively. Further, the ligand is expected to undergo thione ⇌ thiol tautomerism. However, the appearance of four thioamide bands in ligand indicates the existence of the ligand in the thione form. The medium intensity band at 1610 cm^{-1} is, due to $\nu_{C=N}$, a vibration of azomethine linkage [28].

The IR spectra of the complexes of cobalt(II) chloride/acetate, nickel(II) chloride/acetate and copper(II) chloride/acetate/nitrate/sulfate show a broad band at ~3450 cm^{-1} (Fig. 2). This is assigned to ν_{OH} of coordinated water. The ν_{N-H} vibration in the complexes is not changed appreciably indicating the non-involvement of N atom in coordination. The strong band appearing at 1710 cm^{-1} in the ligand, assigned to $\nu_{C=O}$, appears at the same position in the complexes, but much diminished in the intensity in the metal(II) chloride complexes. In addition, there is new band at 1645 cm^{-1} in the C1 (a representative of chloride complexes). This band may be assigned to the coordinated lactone >C=O group. This observation indicates that in the metal(II) chloride complexes 'O' atom of lactone C=O of one part coordinates to the metal atom, while other C=O remains free, whereas in metal(II) acetate complexes none of the >C=O is involved in the coordination. The $\nu_{C=N}$ observed at 1610 cm^{-1} experiences negative shift of ~5 cm^{-1} in the acetate complexes, while it remains unchanged in position in chloride complexes further a new band appears in chloride complexes (at 1590 cm^{-1}). From this observation it is obvious that, the both 'N' atoms of C=N linkage are coordinated to metal atom in the acetate complexes, while in chloride complexes only single azomethine linkage is involved in the coordination. Above observations clearly indicate that in chloride complexes one part of the ligand is coordinated to metal atom through 'O' and 'N' while other part remains uncoordinated. The $\nu_{C=S}$ observed at 760 cm^{-1} in the ligand remains at the same position in the complexes indicating non-coordination of thioamide sulfur to the metal [30].

The ν_{N-N} band assigned at 960 cm^{-1} in the ligand shifts to the higher frequency by 10–20 cm^{-1} in the complexes indicating the coordination of azomethine nitrogen to the metal [31].

The acetate complexes show band around ~1575 cm^{-1} (ν_8), ~1475 cm^{-1} (ν_3) and ~625 cm^{-1} (ν_5) (C2, a representative)

Table 1
Elemental, magnetic and molar conductance data of the complexes

Compound	Composition	Colour	M	N	S	Cl	μ_{eff}	Molar conductance
C1	$CoLCl_2\cdot 2H_2O$	Brown	10.23 (9.64)	9.72 (9.15)	5.56 (5.22)	12.33 (11.60)	5.06	8.2
C2	$CoL(CH_3COO)_2$	Brown	10.12 (9.46)	8.71 (8.99)	5.92 (5.14)	–	4.93	6.7
C3	$NiLCl_2\cdot 2H_2O$	Reddish brown	10.20 (9.60)	9.70 (9.15)	5.76 (5.23)	12.42 (11.60)	2.98	4.8
C4	$NiL(CH_3COO)_2$	Greenish yellow	9.86 (9.42)	9.42 (8.99)	4.84 (5.14)	–	2.92	4.2
C5	$CuLCl_2\cdot 2H_2O$	Brown	10.39 (10.30)	9.65 (9.08)	5.51 (5.19)	12.23 (11.52)	1.86	4.8
C6	$CuL(NO_3)_2\cdot 2H_2O$	Brown	10.12 (9.48)	12.48 (12.55)	5.03 (4.78)	–	1.89	8.2
C7	$CuL(SO_4)\cdot 2H_2O$	Brown	10.21 (9.90)	9.82 (8.73)	10.07 (9.98)	–	1.92	8.1
C8	$CuL(CH_3COO)_2$	Black	10.81 (10.12)	8.99 (8.92)	5.72 (5.10)	–	1.78	5.8

Calculated values are given in parentheses.
μ_{eff} values are given in BM.
Molar conductance values are given in λ_M mho $cm^2\,mol^{-1}$.

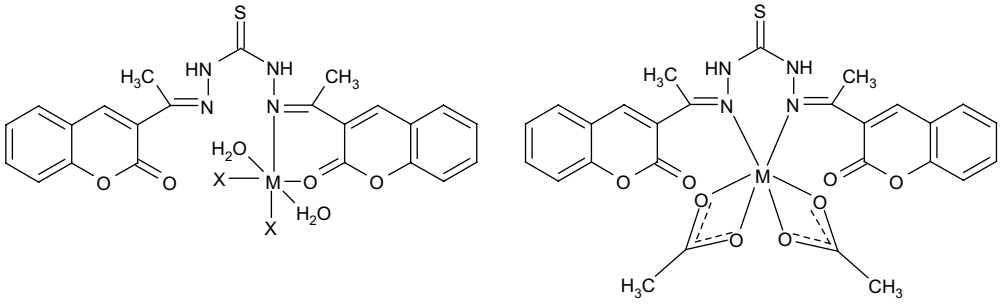


Fig. 2. Proposed structures for the complexes.

due to acetate groups. The difference $\nu_8 - \nu_3$ is of the order of 100 cm^{-1}, which is indicative of bidentate chelating behaviour of acetate group [32].

The nitrate complex of copper(II) exhibits bands at 1280, 1040, 710, 1430 and 860 cm^{-1} which can be assigned to NO_2 symmetric stretching (ν_1); N–O stretching (ν_2); N–O symmetric bending (ν_3); NO_2 symmetric stretching (ν_4); NO_2 asymmetric bending (ν_5) and out of plane rocking, respectively. These frequencies are compatible with monodentately coordinated nitrate groups [33].

In the copper(II) sulfate complex, the bands are observed at 1122, 1040, 920, 700, 620 and 450 cm^{-1} which may be assigned to coordinated sulfate groups [34]. Some bands due to free ligand also occur in this region and therefore there is difficulty in assignment of ν_{S-O} bands.

3.2. Electronic spectral studies

The distinguishable absorption bands of UV–vis spectra of the complexes in DMF solution were recorded and are listed in Table 2. The cobalt complexes (C1, C2) exhibit two bands in the range of 18,868–20,000 cm^{-1} and 8230–8928 cm^{-1} are consistent with ν_3 and ν_1 absorption bands of octahedral [35] cobalt(II) and assigned accordingly. The ν_2 band that involves a two-electron transition is not observed in spectra, but calculated from Konig equation. The ligand field parameters such as Dq, B', β, ν_2/ν_1 and LFSE have been calculated according to the procedure derived by Underhill and Billing [36]. The values are compiled in Table 2.

The nickel(II) complexes (C3, C4) exhibit three bands at 9800–10,700 cm^{-1} (ν_1), 17,200–19,050 cm^{-1} (ν_2) and 24,400–26,300 cm^{-1} (ν_3). The ligand field parameters such as 10 Dq, B', β, ν_2/ν_1 and LFSE have been calculated [37]. From the spectral data, it is concluded that these nickel(II) complexes are covalent ($\beta = 0.73$–0.95) and have distorted octahedral structures.

The electronic spectra of copper(II) complexes (C5, C6, C7 and C8) display three prominent bands in the region 16,900–17,800 cm^{-1}. This is assigned as 10 Dq band corresponding to $^2T_{2g} \leftarrow {}^2E_g$ transition [38]. In addition, there is a high intensity band in the region 23,000–27,000 cm^{-1}. This band is due to symmetry forbidden ligand → metal charge transfer transition [39]. The band above 27,000 cm^{-1} is assigned as ligand band. Hence distorted octahedral structures are proposed on the basis of electronic spectra.

3.3. Magnetic measurement data

The magnetic susceptibilities of the complexes were recorded at room temperature and are tabulated (Table 1). The magnetic moment values of cobalt(II) complexes, C1 and C2, lie in the range of 4.83–5.06 BM, and are well within the reported range 4.7–5.2 BM for octahedral complexes of cobalt(II). The magnetic moment values of nickel(II) complexes, C3 and C4 vary in the range of 2.92–3.10 BM. This is slightly higher than the spin only value (2.83 BM) corresponding to two unpaired electrons. This accounts for a slight orbital contribution to the spin only value. These magnetic

Table 2
Electronic spectral data of the complexes

Complex	λ_{max} (cm^{-1})			Dq (cm^{-1})	B'	B	ν_2/ν_1	LFSE (K cal mol^{-1})
C1	8928	19,022[a]	20,000	1009.4	815.9	0.840	2.13	23.1
C2	8230	17,557[a]	18,868	932.7	782.3	0.806	2.13	21.3
C3	10,204	19,047	26,316	1020.4	983.4	0.945	1.87	35.0
C4	10,695	19,047	24,390	1069.5	756.8	0.727	1.78	36.7
C5	17,699 (18,181)	23,529 (–)	28,169 (28,571)	1769.9 (1818.1)	–	–	–	30.3 (31.2)
C6	17,391 (18,181)	23,529 (22,222)	29,411 (30,769)	1739.1 (1818.1)	–	–	–	29.8 (31.2)
C7	16,949 (16,528)	24,390 (25,641)	30,303 (30,303)	1694.9 (1652.8)	–	–	–	29.1 (28.3)
C8	17,699 (–)	– (24,390)	27,777 (32,258)	1769.9 (–)	–	–	–	30.3 (–)

[a] Calculated value; values in nujol phase are given in parentheses.

moment values lie well within the range (2.9–3.3 BM) expected for octahedral nickel(II) complexes.

For the copper(II) complexes, C5, C6, C7 and C8, the magnetic moment values are in the range of 1.78–1.92 BM slightly higher than the spin only value (1.73 BM) corresponding to one unpaired electron. These values indicate the absence of spin–spin interaction [40] in the complexes.

3.4. EPR spectral studies

EPR spectra were recorded for powder samples for all the copper complexes at room temperature and liquid nitrogen temperature are of tetragonal characteristic. The complexes C5, C6, C7 and C8 possess a characteristic spectrum having asymmetric bands with two-g values, $g_{\|}$ and $g_{\perp}$. The data show (Table 2) considerable covalent character of metal–ligand bond in the complexes. The trend $g_{\|} > g_{\perp} > g_e$ (2.0023) observed for these complexes shows that the unpaired electron is localized in $d_{x^2} - d_{y^2}$ orbital of the Cu(II) ion and the spectra are characteristic of axial symmetry [41]. Further in all the complexes, it is expected that there is no exchange coupling between two copper centers in the solid state, as the axial symmetry parameter $G = g_{\|} - 2/g_{\perp} - 2$ is found to be more than 4 for the complexes.

4. Biological activity

4.1. Antitumor activity of complexes against Ehrlich Ascites Carcinoma in Swiss albino mice

The Brine shrimp lethality bioassay has been chosen to assess the *in vitro* cytotoxic effects of the compounds, as it is inexpensive, reliable and quick method for the purpose [42]. All the tested compounds showed considerable cytotoxic activity in the Brine shrimp lethality bioassay. LC_{50} concentrations for the compounds are tabulated in Table 3.

The compounds were tested using the short term *in vitro* cytotoxicity towards EAC (Ehrlich Ascites Carcinoma) cells as a preliminary screening technique of Tryphan Blue Exclusion Method (Cell Viability Test) for their cytotoxic potential [43]. This is one of the methods to assess cytotoxicity of anticancer compounds. This test is based on the principle that living cell membrane has ability to prevent the entry of dye. Hence, they remain unstained and can be easily distinguished from dead cells, which take the dye. The percentage of viable cells was determined. Results of the short term *in vitro* cytotoxicity of the compounds are shown in Table 4. These preliminary experiments were carried out mainly with five different concentrations of the compounds. All the compounds were found to be cytotoxic and produced 50% cell death at a concentration of 19.1 μg/ml. At 50 μg/ml concentration the standard (Cisplatin) showed 98% cell death. At 50 μg/ml concentration the $[Cu(L)Cl_2]\cdot 2H_2O$, $[Cu(L)(NO_3)_2]$ showed more than 80% cell death. All the compounds were found to have considerable cytotoxicity in the Cell Viability Test. Weight variation parameter shows that the tumor inoculated control animal gained a substantial weight by day 0. They gained a maximum weight of 19% by day 15. Cisplatin administration (on 10th post-inoculation day) significantly ($p < 0.05$) reduced weight gain as compared to control on day 15. The compounds significantly ($p < 0.05$) reduced the weight gain on day 15 as compared to control (Table 5). Comparative effects of treatments vs Cisplatin on reduction of body weights in tumor-induced mice are given in Table 6. The effect of compounds on survival of tumor bearing mice is shown in Table 7. Cisplatin significantly prolonged the median and mean survival times ($p < 0.05$) with respect to its control. It showed a significant increase in the percentage life span of animals (ILS > 50). On the other hand, all the compounds significantly prolonged the mean survival times. The influence of all the compounds on % ILS was more than 25%. By convention, a 25% increase in life span is considered as possible anticancer activity of a test compound [44]. The effect of compounds on hematological parameters is shown in Table 8. Tumor induction significantly ($p < 0.05$) increased total number of WBC almost four times. Cisplatin administration reversed this effect significantly ($p < 0.05$). All the compounds significantly ($p < 0.05$) reversed the tumor-induced rise in total counts of WBC. However, they were not as efficacious as Cisplatin in reversing the tumor-induced total counts. On differential counts, tumor induction caused a significant reduction in lymphocyte and a significant ($p < 0.05$) increase in neutrophil counts. This was significantly ($p < 0.05$) reversed towards normal by Cisplatin and the test compounds. However, the compounds were less efficacious than Cisplatin in their effects. Tumor induction caused significant decrease in RBC and Hb almost to the half of the normal animals [45]. This was significantly ($p < 0.05$) reversed towards normal by Cisplatin and the test compounds.

Table 3
Brine shrimp bioassay results of compounds

Sl. no.	Compound	Percentage deaths at 24 h			LC_{50} mg/ml
		5 mg/ml	10 mg/ml	20 mg/ml	
1	$CuLCl_2\cdot 2H_2O$	60	90	100	4.22
2	$CuL(CH_3COO)_2$	75	100	100	3.17
3	$CuL(SO_4)\cdot 2H_2O$	70	100	100	3.61
4	$CuL(NO_3)_2\cdot 2H_2O$	29	65	88	7.62
5	Ligand	20	39	49	19.22

Table 4
Short term *in vitro* cytotoxicity of compounds towards EAC cells

Compounds	Percentage cell death at different concentrations after 3 h					LC_{50} μg/ml
	1 μg/ml	5 μg/ml	10 μg/ml	25 μg/ml	50 μg/ml	
Cisplatin	20	32	44	88	98	6.6377
$CuLCl_2\cdot 2H_2O$	19	39	49	64	81	9.0698
$CuL(CH_3COO)_2$	18	33	48	66	75	10.7010
$CuL(SO_4)\cdot 2H_2O$	17	31	45	64	79	11.1884
$CuL(NO_3)_2\cdot 2H_2O$	16	33	43	62	84	10.8357
Ligand	14	30	45	63	75	12.6277

Table 5
Effect of drugs on body weight changes in tumor-induced mice

Group	Dose (mg/kg) i.p.	Percentage increase in weight as compared to day 0 (mean ± SE)				
		Day 3	Day 6	Day 9	Day 12	Day 15
Control	–	4.46 ± 1.98	7.04 ± 2.19	16.13 ± 1.75	17.15 ± 1.89	19.15 ± 2.22
Cisplatin	3.5	4.51 ± 1.15	12.04 ± 1.38	19.16 ± 1.34	9.91 ± 2.12[a]	1.32 ± 4.23[a]
$CuLCl_2 \cdot 2H_2O$	50	4.45 ± 1.01	15.34 ± 2.59	21.65 ± 5.70	14.14 ± 3.07	8.06 ± 2.33[a]
$CuL(CH_3COO)_2$	50	4.35 ± 1.03	13.29 ± 3.67	23.79 ± 5.72	15.88 ± 3.45	4.16 ± 2.33[a]
$CuL(SO_4) \cdot 2H_2O$	50	4.54 ± 1.28	15.83 ± 1.93	17.06 ± 1.82	15.52 ± 1.57	5.85 ± 2.39[a]
$CuL(NO_3)_2 \cdot 2H_2O$	50	5.34 ± 1.34	13.54 ± 1.41	18.11 ± 2.21	14.09 ± 1.23	2.66 ± 1.50[a]
Ligand	50	4.77 ± 2.05	16.43 ± 1.39	18.46 ± 1.52	14.44 ± 2.27	8.45 ± 2.39[a]

[a] $p < 0.05$ vs control.

4.2. Antimicrobial activity

In the light of interesting antimicrobial activities of the coordination complexes, the ligand and its complexes were screened for antibacterial and antifungal activity against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus aureus* and *Aspergillus niger* and *Candida albicans*, respectively by the cup plate method. On completion of incubation the activity was recorded by measuring the zone of inhibition in millimeters in case of bacterial strains and by measuring the decrease in turbidity in terms of percent transmission at 600 nm. It is evident from screening data that the ligand is active against bacteria tested in present study and the promising results were observed for the complexes $[Cu(L)Cl_2] \cdot 2H_2O$, $[Cu(L)(NO_3)_2]$ against all bacterial and fungal strains.

5. Conclusion

The present study shows that all complexes are non-electrolytes and are soluble in dimethylformamide and dimethylsulphoxide. From all possible spectral data it is found that ligand essentially coordinates through one of the azomethine nitrogen and lactone carbonyl oxygen. Their respective anion counterparts occupy the remaining coordination sites in case of M(II) chlorides, nitrates and sulphates. So in this case only one part of the ligand coordinates to metal centers. In case of M(II) acetate complexes ligand coordinates through both azomethine nitrogen and acetate group binds in bidentately chelating manner through oxygen. In conclusion, the ligand acts as neutral, bidentate, coordinating through NO or NN. The compounds showed considerable cytotoxic activity in the Tryphan Blue Exclusion Method. In the *in vivo* cancer model (Ehrlich Ascites Carcinoma model), the compounds significantly ($p < 0.05$) reversed the tumor-induced changes in the parameters monitored, *viz*., percentage increase in body weight, percentage increase in life span, tumor viable count and hematological parameters (total and DLC of WBC, total RBC and Hb). These effects were almost comparable to Cisplatin – the standard drug used in the study. The compounds however were found to have good effect in prolonging the life span (ILS) as compared to standard drug Cisplatin. These findings imply that the compounds might be having some anticancer principles. Based on the data of the present study, it is very difficult to suggest the possible mechanism for the compounds anticancer effects. The compounds tested in present study have shown promising cytotoxic activity when screened using the *in vitro* method and at the same time were shown to have good activity when tested using the Ehrlich Ascites Carcinoma model.

Though it is very difficult to conclude anything at this stage, it can be assumed that after testing against various other cancer models and at different doses these compounds may prove to be safer drugs for tomorrow.

The promising results were observed for the antimicrobial screening especially for the $[Cu(L)Cl_2] \cdot 2H_2O$, $[Cu(L)(NO_3)_2]$ complex against the fungi strains and which may be attributed to the fact that the metal complexes are potentially active against fungal cells than bacterial cells.

Table 6
Comparative effects of treatments vs Cisplatin on reduction of body weights in tumor-induced mice

Group	Dose (mg/kg) i.p.	Percentage decrease in weight as compared to respective control (mean ± SE)	
		Day 12 (mean ± SE)	Day 15 (mean ± SE)
Cisplatin	3.5	42.22 ± 11.32	93.10 ± 12.31
$CuLCl_2 \cdot 2H_2O$	50	17.55 ± 10.34	57.91 ± 9.82
$CuL(CH_3COO)_2$	50	7.41 ± 9.87	78.28 ± 8.57
$CuL(SO_4) \cdot 2H_2O$	50	9.50 ± 12.38	69.45 ± 11.49
$CuL(NO_3)_2 \cdot 2H_2O$	50	17.84 ± 8.91	86.10 ± 12.46
Ligand	50	15.80 ± 8.32	55.87 ± 8.73

6. Experimental protocols

All chemicals used were of reagent grade. Solvents were distilled prior to use in the synthetic part. The metal content of the complexes was estimated after decomposition with mixture of HCl and $HClO_4$ by gravimetric method (copper and nickel) and EDTA titration method (cobalt). Magnetic susceptibility of complexes was measured at room temperature on a Faraday balance using $Hg[Co(SCN)_4]$ as a calibrant. Electronic spectra were recorded using VARIAN CARY 50 Bio UV–visible spectrophotometer in DMSO. The IR spectra of ligand and its complexes were recorded as KBr pellets in the region 4000–400 cm^{-1} on Nicolet 170 SX FT-IR spectrometer. The EPR spectra of copper(II) complexes were recorded at both room and liquid nitrogen temperature on Varian E-4 X-band spectrometer using TCNE as *g*-marker. Conductivity

Table 7
Effect of drugs on the survival time in tumor-induced mice

Group (mg/kg)	Dose	Median survival time (days)			Mean survival time (days)		
		MST	% T/C	% ILS	(Mean ± SEM)	% T/C	% ILS
Control	–	18.00	–	–	18.33 ± 0.21	–	–
Cisplatin	3.5	34.50	191.67	91.67	34.33 ± 0.33[a]	187.28	87.28
$CuLCl_2 \cdot 2H_2O$	50	23.50	130.56	30.56	23.17 ± 0.40[a]	126.40	26.4
$CuL(CH_3COO)_2$	50	25.50	141.67	41.67	25.17 ± 0.41[a]	137.31	37.31
$CuL(SO_4) \cdot 2H_2O$	50	26.00	144.44	44.44	25.83 ± 0.31[a]	140.92	40.92
$CuL(NO_3)_2 \cdot 2H_2O$	50	29.00	161.11	61.11	28.83 ± 0.31[a]	157.28	57.28
Ligand	50	23.50	130.56	30.56	23.17 ± 0.40[a]	126.40	26.40

[a] $p < 0.05$ vs control groups, MST = median survival time.

measurements were measured on 10^{-3} M solutions of complexes in DMSO using ELICO-CM82 Conductivity Bridge provided with a cell having cell constant 0.51.

6.1. Chemistry

6.1.1. Synthesis of bis(3-acetylcoumarin) thiocarbohydrazone [26]

Thiocarbohydrazide (0.02 mol) and 3-acetylcoumarin (0.04 mol) were taken in ethyl alcohol (80 cm^3) containing acetic acid (1 cm^3). The mixture was refluxed on a water bath for 2 h. The slightly yellowish coloured crystalline mass was separated on cooling. This was filtered off, washed with ethyl alcohol, air dried and recrystallized from ethyl alcohol (m.p. 218–220 °C, yield: 90%).

6.1.2. Synthesis of metal(II) complexes

The complexes were prepared by the addition of ethanolic solution of metal(II) chloride (0.003 mol) with constant stirring to the corresponding amount of the ligand (0.003 mol) in the same solvent. The mixture was heated to reflux for 1 h. The product was filtered off, washed several times with ethanol and dried in vacuum.

Similarly, copper(II) nitrate and sulfate complexes were prepared. The syntheses were repeated using different metal–ligand ratio, but same complexes (1:1) were obtained in all the cases.

Metal(II) acetate complexes were prepared by adding an ethanolic solution of ligand (0.003 mol) to metal(II) acetate in same solvent. The resulting mixture was refluxed with constant stirring for 3 h. The complex thus formed was filtered, washed with hot water followed by ethanol and dried in vacuum.

6.2. Microbiology

6.2.1. Antitumor activity

6.2.1.1. Brine shrimp lethality bioassay [42]. The Brine shrimp lethality test was used to predict the presence of cytotoxic activity. The Brine shrimp (*Artemia salina*) eggs were procured from www.brineshrimpdirect.com. The Brine shrimp eggs were hatched in artificial sea water at room temperature and constant aeration for 48 h. After hatching 10 larve were placed in a vial containing 5 ml of artificial sea water and a drop of dry yeast suspension (3 mg in 5 ml – sea water) was added to each vial as food for shrimps. Test compounds in different concentration (10, 100 and 1000 ppm) were added to the vial before making the final volume to 5 ml with sea water. The shrimp in artificial sea water alone served as control. The vials were maintained under illumination. The experiments were set in triplicate and mean of three reading was taken as final result. After 24 h survivors were counted, by using 3× magnifying glass, and the percent deaths and LC_{50} values were calculated by using Finney Computer program.

6.2.1.2. Cell lines. Cancer cell lines, *viz*., Ehrlich's Ascites Carcinoma (EAC), to induce cancer in animal model (mice) were obtained from Amala Cancer Research Center, Amala Nagar, Thrissur, Kerala, India. The cells were maintained as

Table 8
Effect of the compounds on hematological parameters

Groups	Dose (mg/kg)	RBC (mean ± SE) (millions/mm^3)	Hb (mean ± SE) (g%)	WBC (mean ± SE) (10^3 cells/mm^3)	Differential leucocyte count %		
					Lymphocytes	Neutrophils	Monocytes
Normal	–	5.03 ± 0.28	15.72 ± 0.32	8.59 ± 0.16	86.23 ± 0.61	13.03 ± 0.46	1.07 ± 0.33
Control	–	2.14 ± 0.34 [a,c]	9.53 ± 0.32 [a,c]	32.31 ± 0.67 [a,c]	41.80 ± 0.39 [a,c]	57.35 ± 0.61 [a,c]	1.25 ± 0.43
Cisplatin	3.5	3.26 ± 0.24 [a,b]	13.08 ± 0.29 [a,b]	10.90 ± 0.19 [a,b]	77.97 ± 0.48 [a,b]	20.80 ± 0.47 [b]	1.22 ± 0.54
$CuLCl_2 \cdot 2H_2O$	50	4.56 ± 0.25 [a,b,c]	12.38 ± 0.34 [a,b,c]	24.19 ± 0.16 [a,b,c]	66.07 ± 0.43 [a,b,c]	32.80 ± 0.43 [a,b]	1.13 ± 0.49
$CuL(CH_3COO)_2$	50	4.14 ± 0.23 [a,b,c]	12.51 ± 0.28 [a,b,c]	19.24 ± 0.51 [a,b,c]	68.18 ± 0.74 [a,b,c]	30.73 ± 0.75 [a,b]	1.08 ± 0.65
$CuL(SO_4) \cdot 2H_2O$	50	4.31 ± 0.23 [a,b,c]	12.38 ± 0.22 [a,b,c]	22.31 ± 0.77 [a,b,c]	71.32 ± 0.29 [a,b,c]	27.37 ± 0.28 [b]	1.32 ± 0.48
$CuL(NO_3)_2 \cdot 2H_2O$	50	3.80 ± 0.13 [a,b,c]	12.85 ± 0.33 [a,b,c]	16.48 ± 0.11 [a,b,c]	66.57 ± 0.49 [a,b,c]	32.20 ± 0.48 [a,b]	1.23 ± 0.56
Ligand	50	4.65 ± 0.24 [a,b,c]	11.77 ± 0.17 [a,b,c]	20.39 ± 0.19 [a,b,c]	61.68 ± 0.48 [a,b,c]	34.58 ± 1.89 [a,b]	1.45 ± 0.43

[a] $p < 0.05$ vs normal.
[b] $p < 0.05$ vs control mice.
[c] $p < 0.05$ vs Cisplatin.

ascites tumor in Swiss albino mice by intraperitoneal inoculation of 1×10^6 viable cells.

6.2.1.3. Animals. Female Swiss albino mice of 6–8 weeks old (25 ± 5 g body weight) were selected from Central Animal Facility, Manipal Academy of Higher Education, Manipal, Karnataka, India. The animals were acclimatized to the experimental room having temperature 23 ± 2 °C, controlled humidity conditions, and 12:12 h light and dark cycle. The mice were housed in sterile polypropylene cages containing sterile paddy husk as bedding material with maximum of four animals in each cage. The mice were fed on autoclaved standard mice food pellets (Hindustan Lever) and water *ad libitum*. The animal experiments were performed according to the rules and regulations of the Institutional Animal Ethics Committee (IAEC).

6.2.1.4. Preparation of test solution of compounds. The solutions of the compounds were prepared by suspending them in 4% acacia and administrated intraperitoneally daily for a period of 5 days from the 10th day of tumor inoculation in the volume of 0.1 ml/10 g mouse. All the compounds were tested at the dose of 50 mg/kg body weight. The dose of Cisplatin selected was 3.5 mg/kg. This was calculated by using Body Mass Index and past experience with the drug [46].

6.2.1.5. Determination of cytotoxicity of compounds to EAC cells (in vitro studies); Tryphan Blue Exclusion Method (Cell Viability Test). In vitro short term cytotoxic activity of drug was determined using EAC cells. The EAC cells that were collected from the animal peritoneum by aspiration were washed repeatedly with phosphate buffered saline (PBS) to free it from blood. The viability of the cells was checked in a haemocytometer. The cells (1×10^6 in 0.1 ml PBS) were incubated in clean sterile tubes with the test compounds (0.01 ml, 1–50 μg/ml in dimethyl sulfoxide (DMSO)) for 3 h at 37 °C, keeping the final volume at 0.9 ml. The volume of DMSO was pegged below 0.1% of the total volume. The control tube had 10 μl of solvent. The final volume was made up to 0.9 ml with PBS. To each tube 100 μl of Tryphan blue solution was added. The live (without stain) and dead (with blue stain) cells were counted using haemocytometer and percent cell death was calculated using the formula:

$$\%\text{Cytotoxicity} = 100\,\frac{(T_{\text{dead}} - C_{\text{dead}})}{T_{\text{tot}}}$$

where T_{dead} is the number of dead cells in the treated group, C_{dead} is that in the control group and T_{tot} is the total number of dead and live cells in the test compound treated group. Cisplatin was used as the standard [43].

6.2.1.6. Induction of Ehrlich Ascites Carcinoma [47]. Antitumor activities of compounds were determined using Ehrlich Ascites Carcinoma (EAC) tumor model in mice. Female Swiss albino mice were divided into groups of 12 animals each (A: normal mice for hematological studies; B: tumor bearing mice; C: tumor bearing mice treated with one dose of Cisplatin; D: tumor bearing mice groups treated with compounds for 5 days). The ascitic carcinoma-bearing mice (donor) were used for the study, 15 days after tumor transplantation. The ascitic fluid was drawn using an 18-gauge needle into sterile syringe. A small amount was tested for microbial contamination. Tumor viability was determined by Tryphan Blue Exclusion Test and cells were counted using haemocytometer. The ascitic fluid was suitably diluted in normal saline to get a concentration of 10^6 cells/ml of tumor cell suspension. This was injected intraperitoneally to obtain ascitic tumor. The mice were weighed on the day of tumor inoculation and then once in three days thereafter. Treatment was started on the 10th day of tumor inoculation. Cisplatin (one dose) was injected on 10th day intraperitoneally. The compounds were administrated from 10th day for 5 days intraperitoneally. After the administration of last dose followed by 18 h fasting six mice from each group were sacrificed for the study of antitumor activity and hematological parameters. The remaining animals in each of the groups were kept to check the mean survival time (MST) of the tumor bearing hosts. Antitumor effects of compounds were assessed by observation of following parameters.

6.2.1.6.1. Percentage increase in weight as compare to day 0 weight. Upon weighing the animal on the day of inoculation and after once in three days in the post-inoculation period, the percentage increase in weight was calculated using the formula: % increase in weight = [(animal weight on respective day/animal weight on day 0) − 1] × 100 [48].

6.2.1.6.2. Median survival time and increase in life span (% ILS). Total number of days an animal survived from the day of tumor inoculation was counted. Subsequently, the median and mean survival time was calculated. The percentage increase in life span (% ILS) was calculated using the formula: ILS (%) = [(mean survival time of treated group/mean survival time of control group) − 1] × 100 [47].

6.2.1.6.3. Hematological parameters [49] and statistical analysis. In order to detect the influence of compounds on the hematological status of EAC bearing mice, comparison was made amongst groups of mice for each compound on the 14th day after transplantation. Blood was drawn from each mouse from retro orbital under ether anesthesia and the white blood cell total count (WBC), differential leukocyte counts, red blood cell total count and hemoglobin content parameters were evaluated. Results were analyzed by one-way ANOVA by Scheffe's *post hoc* test using SPSS computer package.

6.2.2. Antibacterial

The strains chosen for antibacterial and antifungal activity are *E. coli*, *S. aureus*, *B. aureus* and *A. niger* and *C. albicans*. The cup plate method was followed for testing. About 15 ml of base layer medium was poured into each sterilized petri dishes. Overnight grown sub cultures of bacteria and autoclaved inoculated sub cultures of fungi were mixed with agar and potato dextrose agar seed layer medium, respectively, and immediately poured into petri dishes containing base layer and allowed to attain room temperature. The cups were made

by scooping out nutrient agar with a sterile cork borer. To these cups, 0.1 ml solutions of test compounds (1 mg/ml in DMF) were added using sterile pipettes and these plates were subsequently incubated at 37 °C for 36 h. The susceptibility of these bacterial and fungal strains towards test compounds was tested by measuring the zone of inhibition (bacteriostatic and fungistatic diameter).

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