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Anti-inflammatory and antimicrobial studies of biosensitive Knoevenagel condensate *β*-ketoanilide Schiff base and its Co(II), Ni(II), Cu(II) and Zn(II) complexes

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Abstract

A new series of transition metal complexes of Co(II), Ni(II), Cu(II) and Zn(II)**,** has been synthesized from the Knoevenagel condensate Schiff base ligand(L) derived from *β*-ketoanilide and furfural with *o*-phenylenediamine and diethylmalonate. Structural features were determined by spectral and analytical techniques. Square-planar geometry has been adopted by the complexes except cobalt complex which has an octahedral geometry. The synthesized ligand and its complexes were screened for their anti-inflammatory activity in male albino rats (Carrageenan-induced rat paw oedema model). Among these complexes, copper complex shows significant anti-inflammatory activity. It exhibits significant dose dependent activity in acute inflammation. The doses of 100 and 200 mg kg^{-1} bw produced 38.3 % and 42.8 % inhibition respectively after 3 h as compared with that of the standard drug (indomethacin) which showed 48.5 % inhibition. The *in vitro* antimicrobial activity of the ligand and its complexes was monitored by disc diffusion method. It has been found that complexes have higher antimicrobial activity than that of free ligand.

Keywords: Schiff base; Anti-inflammatory activity; Antimicrobial activity.

1. Introduction

The Schiff bases have been subject of great of interest for a number of years because of their various chemical and structural characteristics, and also their proved applications as biologically active molecules [1]. Their complexes are known to be biologically important and act as models to understand the structure of biomolecules and metalloproteins. They have also a variety of applications, including biological, clinical, analytical and industrial purposes [2].

With increasing incidence of deep mycosis in recent years [3], there were more and more studies for screening new and more effective antimicrobial broad-spectrum drugs with low toxicity. The interest in the study of Schiff bases and their complexes containing oxygen and

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nitrogen donor atoms arises from their significant antifungal activity [4-6]. Inhibiting properties toward tumour growth were recently demonstrated for some Schiff base complexes [7].

The imine bond in Schiff bases can be easily reduced to give amino derivatives. The comparison of the ligand coordinating properties of reduced Schiff bases with their Schiff base parents showed that the basicity of N atoms is enhanced and also a greater flexibility as consequence of the hydrogenation of the C=N bond, leading to more stable complexes [8-11]. For these reasons, the reduced Schiff bases have gained particular attention [12].

Bearing all these in mind, we have synthesized and characterized the novel Knoevenagel condensate β-diketanilide Schiff base derived from furfuraldehyde, β-ketoanilide, *o*-phenylenediammine and diethylmalonate and its copper(II), cobalt(II), nickel(II) and zinc(II) complexes. Usually such complexes have pharmacological applications. Hence, we decided to evaluate the anti-inflammatory, antibacterial and antifungal activities of the synthesized complexes as compared to the standard drugs.

2. Experimantal

2.1. Chemicals

All reagents and chemicals were procured from Merck (Darmstadt, Germany). Solvents used for electrochemical and spectroscopic studies were purified by standard procedures [13].

2.2. Instrumentation

Elemental analyses of the complexes were carried out on a CHN analyzer Carlo Erba 1108, Heraeus. Infrared spectra (4000–400 cm^{-1}, KBr discs) of the samples were recorded on a Perkin–Elmer 783 series FTIR spectrophotometer. Electronic absorption spectra (in DMF at room temperature) in the range of 200–1100 nm were obtained on a Shimadzu UV-1601 spectrophotometer. ^1H NMR spectra (300 MHz) of the ligand and its zinc complex were recorded on a Bruker Avance DRX 300 FT-NMR spectrometer using $CDCl_3$ and DMSO-d_6 as solvents, respectively. Fast atom bombardment mass spectra (FAB-MS) were obtained in a 3-nitrobenzyl alcohol matrix using a VGZAB-HS spectrometer. X-band EPR spectra of the complexes were recorded at room temperature and at −196 °C, respectively. Molar conductivity of 10^{-3} mol L^{-1} solution of the complexes in DMF was measured at room temperature with a Deepvision Model-601 digital direct reading deluxe conductivity meter. Magnetic susceptibility measurements were carried out by employing the Gouy method at room temperature on a powdered sample of the complex. $CuSO_4 \cdot 5H_2O$ was used as the calibrant. Metal contents of the complexes were determined according to a literature method [14]. Chloride ion was determined gravimetrically as silver chloride. Purity of the ligand and its complexes was evaluated by column chromatography and thin layer chromatography. Electrochemical studies were performed on a CHI620C electrochemical analyzer with three electrode system of a glassy carbon electrode (GCE) as the working electrode, a platinum wire as auxiliary electrode and Ag/AgCl as the reference electrode. All the electrochemical measurements were carried out in a 10 mL electrolytic cell. Solutions were deoxygenated by purging with N_2 prior to measurements.

2.3. Synthesis of Ligand

The Knoevenagel condensate Schiff base ligand and its Co(II), Ni(II), Cu(II) and Zn(II) complexes were prepared as follows: Condensation of acetoacetanilide with furfuraldehyde was performed by stirring with equimolar amounts (10 mM) in 20 mL ethanol for 2 h in the presence of 0.2 mL of piperidine as the catalyst. The solution was then cooled and the condensed product, yellow coloured solid Knoevenagel condensate β-ketoanilide, was isolated by filtration, washed and recrystallized from ethanol.

Knoevenagel condensate β-ketoanilide (10 mM) was dissolved in ethanol (30 mL) and refluxed with *o*-phenylenediamine (20 mM) and diethylmalonate (10 mM) in ethanol (30 mL) with the addition of 1 g anhydrous K_2CO_3 for about 6 h. The solvent was reduced to one-third and the pasty mass so obtained was treated with hot water and set aside in refrigerator for 10 h. The solid Schiff base ligand formed was removed by filtration and recrystallized from ethanol.

2.4. Synthesis of metal complexes

A solution of the Schiff base ligand (5 mM) in ethanol (20 mL) was added to a solution of metal chlorides (5 mM) in ethanol (10 mL) and the mixture was refluxed for 3 h and concentrated to one-third volume and kept at 0 ^{o}C for 2 h. The solid product formed was filtered, washed with ethanol and dried *in vacuo*.

2.5. Anti-inflammatory assay

The synthesized ligand and its complexes were tested for their *in vivo and in vitro a*nti-inflammatory activity against Male Wistar Albino rats. Male Wistar Albino rats weighing about 150-170 g from inbred stock were used throughout the experiments. They were given commercial diet (Hindustan Lever Ltd., Bangalore) and tap water *ad libitum*. For experiment, rats were randomly selected into groups comprising of 6 in number. The animals were given one-week time to get acclimatized with laboratory conditions. The animals were made to fast over night before the experiment.

Dose selection of the test drug, copper complex was based on the acute toxicity test carried out over a dosage range of *M. tridentata* varying from 50, 100, 200, 400, 600 and 800 mg kg^{-1} bw orally [15]. According to the results of acute toxicity test, the doses of 100 and 200 mg kg^{-1} bw were chosen for the experiment. The test drug in the form of 2% suspension in normal saline was fed orally in a volume of 10 mL kg^{-1} bw. The standard drug, Indomethacin (Ciba Geigy, India) was used as a 2% suspension in normal saline in a dose of 10 mg kg^{-1} bw.

The Carrageenan-induced oedema in rats was divided into four groups of 6 rats each. Group I received normal saline and served as control. Group II received the doses of 100 mg kg^{-1} bw and group III received the doses of 200 mg kg^{-1} bw of copper complex, respectively. Group IV received the standard drug, indomethacin 10 mg kg^{-1} bw. Oedema was induced by injecting 0.1 mL of carrageenan (1% W/V; Sigma, USA) in normal saline into the sub plantar region of the left hind paw of all the four groups of rats after 1 h of drug administration [16]. The paw volume was measured with the help of mercury replacement plethysmometer (Model 7140, UGO Basile, Italy) first at zero hour and then at 1, 2, 3 and 4 h after administration of drugs. The percentage inhibition of oedema compared with that of the control was taken as anti-inflammatory activity. The percentage inhibition of oedema was calculated by the formula:

$$\text{Percentage Inhibition of oedema} = (A - B)/ A \times 100$$

where 'A' represents the paw volume of the control group at 3 h and B represents the paw volume of the test drug treated group at 3 h.

The data were expressed as mean ± SEM. Statistical analysis was performed one-way ANOVA followed by Dunnett's multiple comparison test using sigma stat software (version 2.0, Jandel Scientific Inc. USA). The values of 'p' less than 0.001 were considered as significant.

2.6. Antimicrobial assay

The synthesized ligand and its complexes were tested for their *in vitro* antimicrobial activity against three Gram-negative (*Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella typhi*) and two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacterial strains and for *in vitro* antifungal activity against *Aspergillus niger*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans* by disc diffusion method using potato dextrose agar as medium. The stock solution (10^{-2} mol L^{-1}) was prepared by dissolving the compounds in DMF and the solutions were serially diluted in order to find the Minimum Inhibitory Concentration (MIC) values. In a typical procedure [17], a disc was made on the agar medium inoculated with microorganisms. The disc was filled with the test solution using a micropipette and the plate was incubated, 24 h for bacteria and 72 h for fungi at 35 ^{o}C. During this period, the test solution diffused and the growth of the inoculated microorganisms was affected. The inhibition zone was developed, at which the concentration was noted. Streptomysin and nystatin were used as control drugs for bacteria and fungi respectively.

3. Results and discussion

The synthesized ligand and its Co(II), Ni(II), Cu(II) and Zn(II) complexes were found to be air stable. The ligand is soluble in common organic solvents but the complexes are soluble only in DMF and DMSO. The physical properties and analytical data of the complexes are enlisted in Table 1. Analytical data of the complexes correspond well with the general formula $[ML]Cl_2$ except for the cobalt complex whose formula is $MLCl_2$. Monomeric nature of the complexes was confirmed from their magnetic susceptibility data. The observed high molar conductivity of the complexes in DMF at room temperature is consistent with the electrolytic nature of the complexes due to the counter ion (chloride ion) in the structure of all the complexes except for the cobalt complex which has low molar conductivity indicating its non-electrolytic nature. Elemental analysis results for the metal complexes agree with the calculated values showing that the complexes have the metal/ligand ratio of 1:1. Presence of the chloride ion is evident from the Volhard's test.

Table1
Physical characterization, analytical, molar conductance and magnetic susceptibility data of the complexes.

No	Compound	*wi*(calc.)/mass % *wi*(found)/mass %				Λ_m S cm^2 mol^{-1}	μ_{eff} (BM)	Molecular weight
		M	C	H	N			
1	L	-	71.9 (71.6)	5.3 (5.2)	13.5 (13.1)	-	-	518
2	$[CuL]Cl_2$	9.8 (9.5)	57.1 (56.8)	4.2 (4.2)	10.7 (10.5)	122	1.94	652
3	$[CoLCl_2]$	9.1 (8.9)	57.5 (57.2)	4.2 (4.1)	10.8 (10.6)	12.4	4.61	648
4	$[NiL]Cl_2$	9.1 (9.0)	57.5 (57.1)	4.2 (4.2)	10.8 (10.5)	131	-	648
5	$[ZnL]Cl_2$	10.0 (9.7)	56.9 (56.5)	4.1 (4.0)	10.7 (10.4)	106	-	654

3.1. Mass spectra

FAB-MS spectra of the synthesized ligand and its complexes were recorded and the obtained molecular ion peaks confirm the proposed formulae. Mass spectrum of the ligand shows a molecular ion peak (M+1) at *m/z* 518 corresponding to $[C_{31}H_{27}N_5O_3]^+$ ion. Its copper complex shows the M^+ peak at *m/z* 652 and another important peak appeared at *m/z* 518, which suggests the presence of two chloride ions. These data confirm the stoichiometry of $[CuL]Cl_2$ type. This is further supported by the mass spectra of all the complexes. The observed peaks are in good agreement with their empirical formulae as indicated by the microanalytical data. Thus, the mass spectral data support the conclusions drawn from the analytical and high molar conductivity values. The cobalt complex shows the (M+1) peak at *m/z* 648 and the absence of the peak at *m/z* 518, which confirms its stoichiometry as $[CoLCl_2]$. This is further supported by its analytical and low molar conductivity values.

3.2. Infrared spectra

The infrared spectra gave some important information regarding to the skeleton of the complexes. The IR spectrum of the ligand shows characteristic bands for ν(N-H) at 3172 cm^{-1}, ν(C=N) at 1632 cm^{-1} and ν(C=O) at 1710 cm^{-1}. In all the complexes, ν(N-H) bands were shifted by 30-45 cm^{-1} to lower frequencies, due to coordination of the NH groups. The ν(C=N) bands were also shifted by 20-25 cm^{-1} to lower frequencies, due to the participation of azomethine groups in coordination. On the other hand, the stretching vibration of ν(C=O) was not affected in all the complexes, which indicates that the carbonyl groups are not involved in coordination to the metal ion. The coordination of nitrogen to the metal atom is supported by the appearance of a new band in the region 420 – 460 cm^{-1} assigned to ν(M-N) vibration [18].

3.3. Electronic absorption spectra and magnetic moment measurements

The electronic spectra serve as a tool to distinguish between the square-planar, octahedral, and tetrahedral geometries of the complexes. The absorption region, assignment of the absorption bands and the proposed geometry of the complexes are given in Table 2. The copper complex is magnetically normal with a magnetic moment of 1.94 B.M. Electronic absorption spectrum of copper(II) complex exhibits two intraligand charge transfer bands (INCT) at *ca.* 33212 cm^{-1} and 25012 cm^{-1}, and a d-d band at *ca.* 17452 cm^{-1} which is due to $^2B_1g \rightarrow {}^2A_1g$ transition. This d-d band strongly favors a square-planar geometry around the metal ion. The absence of absorption below 10000 cm^{-1} excludes the possibility of tetrahedral geometry for the complex [19].

The observed zero magnetic moment confirms the square-planar geometry for nickel(II) complex in conformity considering the fact that all known square-planar complexes of nickel(II) are diamagnetic. The Ni(II) complex shows four bands, two intraligand charge transfer bands at *ca.* 35320 cm^{-1} and 26280 cm^{-1}, two d-d bands at *ca*. 22988 cm^{-1} and 19801 cm^{-1} which are assigned as $^1A_{1g} \rightarrow {}^1A_{2g}$ and $^1A_{1g} \rightarrow {}^1B_{1g}$ transitions respectively which are characteristic for a square-planar geometry. Electronic spectrum of the cobalt complex exhibits a broad and weak band in the regions of 11559 cm^{-1}, 13895 cm^{-1}, and 17458 cm^{-1}, suggesting an octahedral geometry. The room temperature magnetic moment (4.61 B.M.) determined for the Co(II) complex is higher than the spin-only magnetic moment (μ_{eff} = 3.87 B.M.) of three unpaired electrons, which is due to the spin-orbit coupling contribution and indicates high spin nature [20].

Table 2
Electronic absorption spectral data of the complexes.

No	Compound	Absorption (cm^{-1})	Band assignment	Geometry
1	L	35020	INCT	---
2	$[CuL]Cl_2$	33212 25012 17452	INCT INCT $^2B_{1g} \rightarrow {}^2A_{1g}$	Square- planar
3	$[NiL]Cl_2$	35320 26280 22988 19801	INCT INCT $^1A_{1g} \rightarrow {}^1A_{2g}$ $^1A_{1g} \rightarrow {}^1B_{1g}$	Square- planar
4	$[CoLCl_2]$	33350 24980 11559 13895 17548	INCT INCT $^4T_{1g}$ (F) → $^4T_{2g}$ (F) $^4T_{1g}$ (F) → $^4A_{2g}$ (F) $^4T_{1g}$ (F) → $^4T_{2g}$ (P)	Octahedral

3.4. Nuclear magnetic resonance spectra

The ^{1}H–NMR and ^{13}C–NMR spectra of the ligand and its zinc complex were recorded in $CDCl_3$ and DMSO-d_6 respectively. ^{1}H NMR spectrum of ligand in $CDCl_3$ gives the following signals: phenyl as multiplet at 6.8-7.4 δ, =C–CH_3 at 2.4 δ, –N–CH_3 at 3.6 δ, -NH-Ar at 8.1 δ and –CONH– protons at 9.3 δ. The –CONH- proton signal (9.6 δ) in the spectrum of the zinc complex is shifted downfield compared to the free ligand, suggesting deshielding of the amide nitrogen group due to the coordination with metal ion and no change in the prominent peak of –NH-Ar which indicates that –NH-Ar nitrogen is not involved in the coordination to central metal ion. Thus, ^{1}H NMR spectra reinforce the conclusion drawn from the IR spectra. The ^{13}C–NMR spectral data of the ligand and its zinc complex are assigned as given in Table 3. The spectra of free ligand and its zinc complex show some upfield and downfield shifts, but these shifts are not large. This indicates the coordination of the ligand to the metal ion.

3.5. Electron paramagnetic resonance spectra

The EPR spectra of copper(II) complex was recorded in DMSO at room temperature and -196 ^{o}C. The room temperature spectrum shows an isotropic pattern, expected for Cu^{2+} in solution, but the spectra for the frozen solutions show the usual anisotropic pattern as expected for powder sample. The absence of half field signal at 1600 G, corresponding to the ΔMs = ±2 transition, rules out any Cu-Cu interaction in the EPR spectra [21]. The frozen DMSO solution is axial with $g_{\|} > g_{\perp} > 2.0036$, indicating a $d_{x^2-y^2}$ ground state [22] which is in agreement with the electronic absorption spectroscopic assignments. The frozen solution spectrum of the complex shows four-line hyper fine splitting $A_{\|}$ with signals penetrating to ^{63}Cu and ^{65}Cu slightly resolved at the low field component (Fig.1). The most remarkable feature is that the $g_{\|}$ value (2.31) is substantially higher than the majority of known copper(II) complexes. A factor potentially contributing to increase of $g_{\|}$ is distortion from square-planar geometry [23].

The degree of geometrical distortion was ascertained by a parameter $g_{\|}/A_{\|}$ ($A_{\|}$ in cm^{-1}) with the values less than 140 associated with the square-planar structures, whereas higher values indicate distortions towards tetrahedron [24].

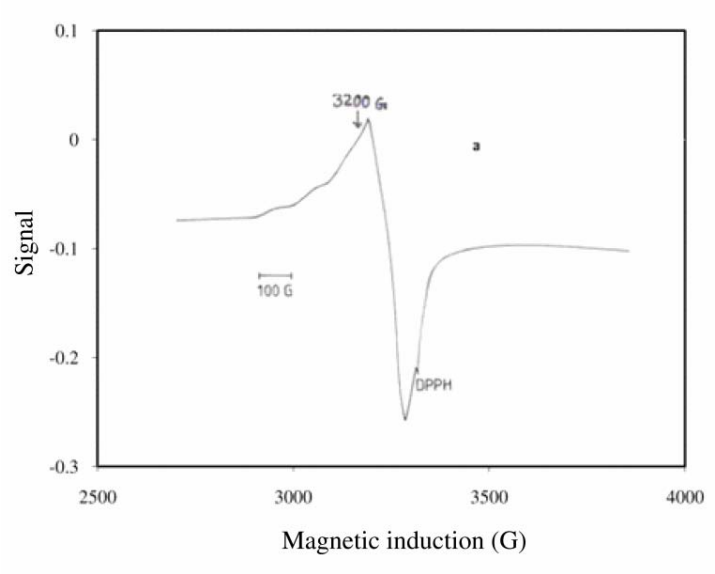
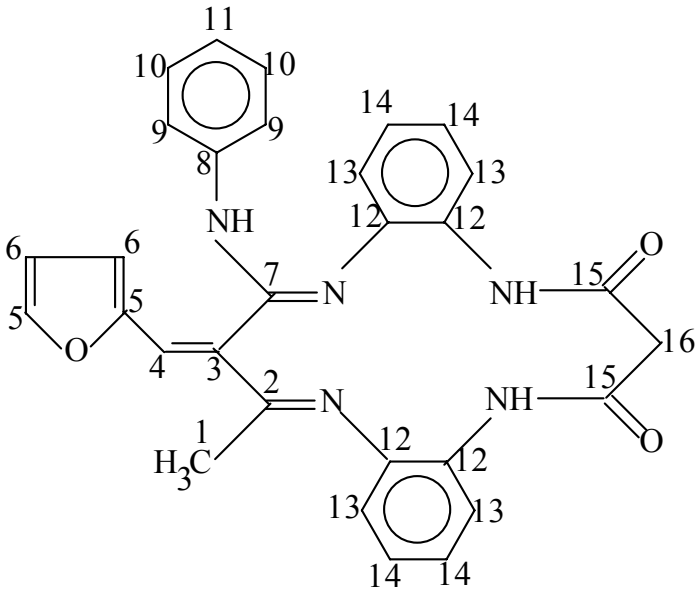


Fig. 1. EPR spectra of the copper complex at −196 °C.

Table 3
^{13}C- NMR spectral data (ppm) of ligand (L) and its Zn(II) complex.

	Ligand (L)	*[ZnL]Cl₂*
C_1	36.2	36.5
C_2	152.0	149.8
C_3	97.4	97.5
C_4	116.2	116.3
C_5	120.2	120.1
C_6	118.9	118.8
C_7	152.3	150.3
C_8	136.1	136.0
C_9	136.4	136.3
C_{10}	138.6	138.4
C_{11}	139.2	139.2
C_{12}	140.9	140.1
C_{13}	121.6	121.5
C_{14}	121.9	121.9
C_{15}	154.8	154.1
C_{16}	130.3	130.2



For the present complex, the $g_{\|}/A_{\|}$ is 145 which is in agreement with significant deviation from planarity and is further confirmed by the bonding parameter α^2 whose value is less than unity. The electron spin resonance and optical spectra have been used to determine the covalent bonding parameters for the Cu(II) ion in various ligand field environments. Since there has been wide interest in the nature of bonding parameters in the system, we adopted the simplified molecular orbital theory [25] to calculate the bonding coefficients such as in-plane π- bonding (β^2), out-plane π- bonding (γ^2) and in-plane σ-bonding (α^2). The observed α^2 (less than unity) and β^2 (greater than 0.5) values indicate that the present copper(II) complex has some covalent character. The observed value for the exchange interaction parameter for the copper complex (G

= 4.8) suggests that the ligand forming complex is regarded as a weak field, and the local tetragonal axes are aligned parallel or slightly misaligned, and the unpaired electron is present in the $d_{x^2-y^2}$ orbital [26] and the exchange coupling effects are not operative in the present complex.

3.6. Cyclic voltammogram study

Cyclic voltammogram of the complexes were recorded in DMF solution with tetrabutylammonium perchlorate as the supporting electrolyte. It showed two quasi-redox couples. In the reduction process, it showed a cathodic peak at 0.111 V for Cu(III) → Cu(II) (Epa = 0.805 V, Epc = 0.111 V, ΔEp = 0.694 V and $E_{1/2}$ = 0.458 V). It also showed a cathodic peak at -0.499 V for Cu(II) → Cu(I) (Epa = -0.141 V, Epc = -0.499 V, ΔEp = 0.358 V and $E_{1/2}$ = -0.320 V) reduction. The above results are quite close to the mononuclear complex [27].

The cyclic votammogram of the [$CoLCl_2$] showed two quasi-redox couple (Fig. 2). In the reduction process, it showed a cathodic peak at 0.294 V for Co(III) → Co(II) (Epa = 0.598 V, Epc = 0.294 V, ΔEp = 0.304 V and $E_{1/2}$ = 0.446 V). It also showed a cathodic peak at -0.398 V for Co(II) → Co(I) (Epa = -0.224 V, Epc = -0.398 V, ΔEp = 0.174 V and $E_{1/2}$ = -0.311 V) reduction.

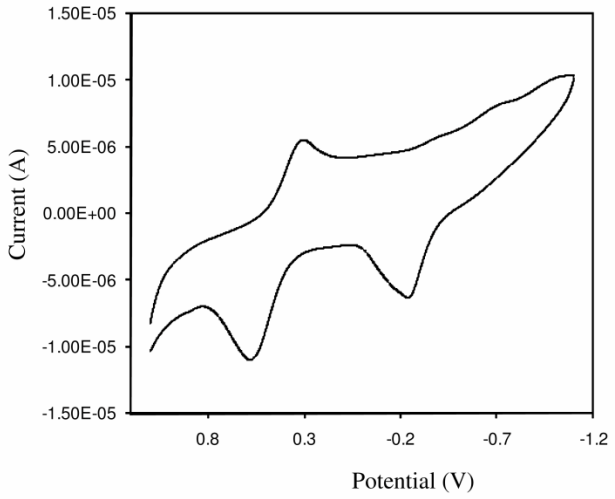


Fig. 2. The cyclic votammogram of the complex [$CoLCl_2$] in DMF solution with tetrabutylammonium perchlorate as the supporting electrolyte.

The cyclic votammogram of the [NiL]Cl_2 showed one quasi-redox couple (the potential range from +1.2 V to -1.2 V) in the negative region, characteristic of the Ni(II) → Ni(I) reduction at Epc = -1.08 V, with the associated anodic peak at Epa = -0.68 V for the Ni(I) → Ni(II) oxidation (ΔEp = 0.40 V and $E_{1/2}$ = -0.38 V). Based on the above spectral data, the structures of the ligand and its complexes are assigned as shown in Fig. 3 and Fig. 4 respectively.

3.7. Anti-inflammatory assay

Anti-inflammatory activities of the synthesized compounds are very interesting. Oedema, which develops after carrageenin inflammation, is a biphasic event. The initial phase is attributed to the release of histamine and serotonin. The oedema maintained between the first and the second phase is due to kinin like substances [28]. The second phase is said to be promoted by prostaglandin like substances. It has been reported that the second phase of oedema is sensitive to drugs like hydrocortisone, phenylbutazone and indomethacin [16]. The preliminary pharmacological screening of metal complexes on carrageena induced rat hind paw oedema (Table 4).

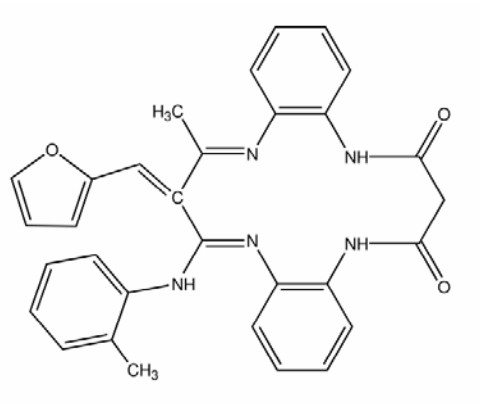


Fig. 3. Structure of the ligand.

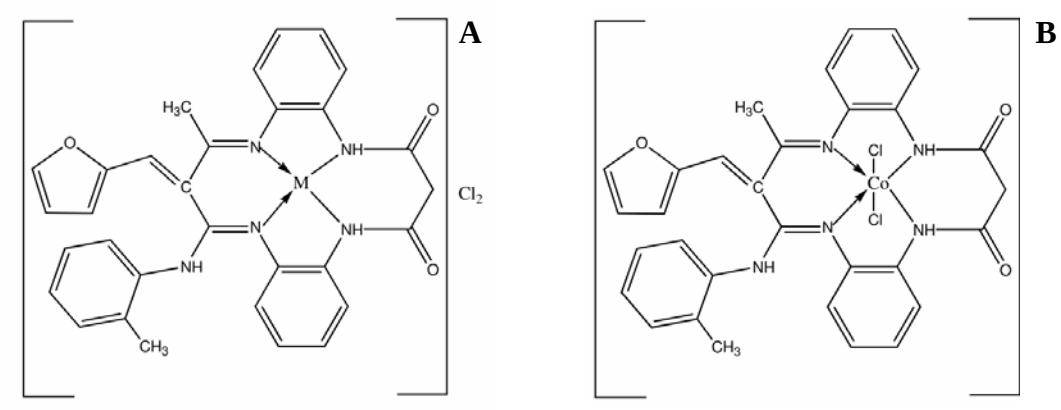


Fig. 4. Structure of complexes: $[ML]Cl_2$ general type (A); $[CoLCl_2]$ (B).

Table 4
Preliminary pharmacological screening of metal complexes on carrageena induced rat hind paw oedema.

No.	*Drug*	Dose (mg kg^{-1} bw)	Increase in paw volume after three hours	% Inhibition in paw volume
1	Control	0.5	0.34 ± 0.01	---
2	Indomethacin	10	0.12 ± 0.01	48.5 ± 2.01
3	Ligand	100	0.32± 0.01	15.6± 3.14
4	$[CoLCl_2]$	100	0.28 ± 0.01	17.7 ± 3.06
5	$[NiL]Cl_2$	100	0.24 ± 0.01	29.4 ± 1.97
6	$[CuL]Cl_2$	100	0.16 ± 0.01	38.3* ± 1.97
7	$[ZnL]Cl_2$	100	0.22 ± 0.01	35.3* ± 2.04

∗P < 0.001 as compared to Control (ANOVA followed by Dunnett's t test)
Each value is the mean ± SEM of 6 rats weighing 150-170 g

Among the metal complexes, copper complex has significant activity. The copper complex produced a dose dependent inhibition of carrageenin induced paw oedema. The inhibition started at 1 h, which continued till 3 h when compared with that of the control. Oral administration of 100 and 200 mg/kg bw of the copper complex produced 38.3% and 42.8% inhibition

respectively after 3 h as compared with that of the standard drug which showed 48.5% inhibition (Table 5).

Table 5
Anti-inflammatory activity of copper complex against carrageenan induced paw oedema.

Treatment	% Increase in paw volume					Inhibition in paw volume (%)
	Post insult time of assay in hours					
	0	1	2	3	4	
Control (0.5 ml kg^{-1} bw)	39.91 ± 1.53	69.32 ± 3.12	97.83 ± 8.13	108.59 ± 9.09	109.81 ± 8.33	-
$[CuL]Cl_2$ (100 mg kg^{-1} bw)	27.92 ± 1.68	51.24 ± 4.20	70.56 ± 5.40	67.31* ± 5.30	63.26* ± 5.12	38.3
$[CuL]Cl_2$ (200 mg kg^{-1} bw)	29.37 ± 2.57	47.68 ± 4.30	72.17 ± 5.30	62.16* ± 4.70	59.92* ± 4.90	42.8
Indomethacin (10 mg kg^{-1} bw)	27.90 ± 0.92	33.80 ± 1.83	38.80 ± 2.32	55.90* ± 3.21	58.82* ± 3.92	48.5

∗P < 0.001 as compared to Control (ANOVA followed by Dunnett's t test)
Each value is the mean ± SEM of 6 rats weighing 150-170 g.

The results of carrageenan induced rat paw oedema model indicated the dose dependent anti-inflammatory activity *i.e.* the dose effect of 200 mg/kg bw of the copper complex was more active than 100 mg/kg bw which was found to be statistically significant (Table 6). The present study revealed significant anti-inflammatory activity of copper complex. It may be concluded that the results of the present study support the use of this complex in some inflammation and painful conditions which confirm the presence of heterocyclic moieties in the ligand system.

Table 6
Anti-arthritic activity of ethanol extract of M. *tridentata* against Freund's adjuvant induced arthritis.

Treatment	% Increase in paw volume									
	Post insult time of assay in days									
	1	3	5	7	9	11	13	15	17	19
Control (Inflamed)	101.8 ± 4.18	183.6 ± 5.2	200.9 ± 7.56	206.8 ± 4.2	192.8 ± 7.3	180.2 ± 7.8	201.3 ± 6.54	219.4 ± 8.19	239.6 ± 9.7	243.9 ± 6.81
Control (Non-Inflamed)	12.5 ± 0.71	24.5 ± 1.6	29.8 ± 1.72	42.8 ± 3.67	26.2 ± 1.52*	25.6 ± 1.34*	27.8 ± 1.69*	23.5 ± 1.38*	19.8 ± 1.20*	18.2 ± 0.89*
M. tridentata extract (100 mg/kg)	78.8 ± 5.6 (22.59)	106.6 ± 7.4 (41.94)	95.4 ± 4.8 (52.51)	89.5 ± 5.3 (56.72)	75.4 ± 4.8* (60.89)	88.9 ± 6.2* (50.67)	104.3 ± 8.2* (48.24)	113.9 ± 9.4* (48.09)	127.4 ± 7.3* (47.77)	122.7 ± 8.1* (48.97)
M. tridentata extract (200 mg/kg)	78.9 ± 4.2 (22.49)	101.8 ± 8.3 (44.55)	88.9 ± 5.1 (55.75)	83.6 ± 4.9 (59.57)	72.1 ± 3.7* (62.60)	82.5 ± 5.8* (54.22)	99.4 ± 7.2* (50.62)	107.5 ± 9.1* (51.00)	117.6 ± 8.6* (50.96)	115.8 ± 9..5* (51.67)
Indomethacin (10 mg/kg)	69.8 ± 4.32 (31.43)	90.2 ± 5.15 (50.87)	73.7 ± 4.61 (63.32)	81.1 ± 4.82 (60.78)	90.2 ± 6.45* (53.22)	96.7 ± 4.98* (46.34)	97.4 ± 6.05* (51.62)	104.9 ± 6.27* (52.19)	107.1 ± 8.24* (55.30)	108.6 ± 5.72* (55.47)

∗P < 0.001 as compared to Control (ANOVA followed by Dunnett's t test)
Each value is the mean ± SEM of 6 rats weighing 150-170 g.
The number in the parentheses indicates the percentage inhibition of the inflammation.

3.8. Antimicrobial assay

The *in vitro* antimicrobial activity of the compounds was tested against the four Gram-negative (*Escherichia coli, Shigella flexeneri, Pseudomonas aeruginosa, and Salmonella typhi*) and two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacterial strains and for in vitro antifungal activity against *Trichophyton longifusus, Candida albicans, Aspergillus flavus, Microsporum canis, Fusarium solani, and Candida glaberata* by disc diffusion method. The minimum inhibitory concentration (MIC) values of the compounds are summarized in Tables 7 and 8. A comparative study of the ligand and its complexes (MIC values) indicates that complexes exhibit higher antimicrobial activity than the free ligand. From the MIC values, it was found that the $[CuL]Cl_2$ was more potent among the other investigated complexes.

Table 7
Minimum inhibitory concentration of the synthesized compounds against the growth of bacteria (μg mL^{-1}).

No	Compound	MIC (μg/mL)				
		E.coli	*P.aeruginosa*	*S.typhi*	*B.subtilis*	*S.aureus*
1	L	56	72	60	52	48
2	$[CuL]Cl_2$	16	18	24	10	20
3	$[NiL]Cl_2$	20	20	28	14	26
4	$[CoLCl_2]$	22	24	34	16	34
5	$[ZnL]Cl_2$	30	32	42	28	46
6	Streptomycin	10	12	14	6	18

Table 8
Minimum inhibitory concentration of the synthesized compounds against growth of fungi (μg mL^{-1}).

S.No	Compound	MIC (μg/mL)				
		A.niger	*C.albicans*	*A.flavus*	*R.stolonifer*	*R.bataicola*
1	L	60	58	46	54	64
2	$[CuL]Cl_2$	8	6	5	11	10
3	$[NiL]Cl_2$	11	12	10	14	14
4	$[CoLCl_2]$	9	8	7	13	11
5	$[ZnL]Cl_2$	10	10	6	12	13
6	Nystatin	12	10	8	16	12

Such increased activity of the complexes can be explained on the basis of Overtone's concept [29] and Tweedy's Chelation theory [30]. According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favours the passage of only the lipid-soluble materials due to which liposolubility is an important factor, which controls the antifungal activity. On chelation, the polarity of the metal ion will be reduced to a greater extent due to the overlap of the ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Further, it increases the delocalization of π-electrons over the whole chelate ring and enhances the lipophilicity of the complexes. This increased lipophilicity enhances the penetration of the complexes into lipid membranes and blocking of the metal binding sites in the enzymes of microorganisms. These complexes also disturb the respiration process of the cell and thus block the synthesis of the proteins that restricts further growth of the organism. Furthermore, the mode

of action of the compound may involve formation of a hydrogen bond through the azomethine group with the active centre of cell constituents, resulting in interference with the normal cell process.

4. Conclusions

Knoevenagel condensate Schiff base and its Co(II), Ni(II), Cu(II) and Zn(II) complexes have been designed and synthesized. They were characterized by analytical and spectral techniques. The anti-inflammatory activity was carried out using carrageenan-induced rat paw oedema model. The results obtained from antifungal and antibacterial tests together showed that all the complexes are more active towards fungi than bacteria. It has been found that the activities of the complexes are higher than the free ligand.

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