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IN VITRO FREE RADICLE SCAVENGING, ANTIDIABETIC AND ANTIBACTERIAL POTENTIAL OF SCHIFF BASE COMPLEXES OF COPPER(II) AND NICKEL(II) DERIVED FROM β-DIKETONE WITH AROMATIC DIAMINE

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ABSTRACT

Synthesis of a novel tetradentate β-diketone based ligand, two copper(II) and nickel(II) complexes $[CuL^2(Bipy)]$ and $[NiL^2(Bipy)H_2O]$ is describe here. The complexes were synthesized by simple reaction of copper(II) and nickel(II) metal salts with tetradentate β-diketone based ligand under the solvothermal conditions and characterized by several techniques such as elemental analyses, molar conductivity measurements, magnetic moments, spectral (UV-Vis, FT-IR, ^{1}H NMR,^{13}C NMR) techniques. The catecholase activity of the copper(II) and nickel(II) complexes were carried out using pyrocatechol as the substrate. Among the two complexes the copper(II) catalyst exhibited remarkable activity. The synthesized ligand and complexes were also screened for their anti oxidant, *in vitro* antidiabatic and *in vitro* anitbacterical activity studies.

KEYWORDS: β-diketone, aromatic diamine, catecholase activity, anti oxidant, *in vitro* antidiabatic activity, *in vitro* anitbacterical activity.

INTRODUCTION

The design of new coordination supramolecules and polymers based on transition metal compounds and multidentate organic ligands has attracted much interest in recent years.[1]

Transition metal complexes of tetradentate Schiff base ligands find applications as models of certain metal enzymes and in catalysis and materials chemistry.[2]

Metal catalysts with Schiff base ligands were mostly used in the field of catalytic hydrogenation, addition polymerisation, epoxidation reaction, bionic catalytic oxidation, etc.[3]

1,3–Diphenyl-1,3-propanedione a class of chelating compound called β–diketone. β–diketones have played and continue to play a key role in coordination compounds that have found wide application in several fields, from new materials[4] to catalysts,[5] as precursors for CVD in the microelectronic industry[6] and as potential antitumourals.[7] Due to variety of applications of this class of ligand, the studies on metal complexes of these derivatives were the subject of many researchers including ours. We herein report the novel metal complexes of this class of new ligands with copper(II) and nickel(II) metal ion.

EXPERIMENTAL

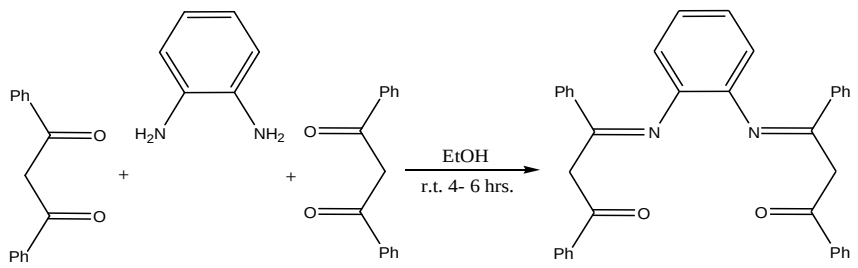
MATERIALS AND METHODS

All chemicals employed in the present study were of analytical grade and were used without purification.

The purity of compounds were checked routinely by TLC (0.5 mm thickness) using silica gel-G coated aluminium plates (Merck) and spots were visualized by exposing the dry plates to iodine vapours or by exposing UV light.

Synthesis of the Ligand (L)

The ligand was prepared (Scheme 1) by the reaction between 1,3–Diphenyl-1,3-propanedione and o-Phenylenediamine in 2:1 ratio in ethanol (30 mL). After refluxing for 4-5 h, the resulting mixture was concentrated.[8] The residue was purified by washing with cold ethanol which afforded the pure compound**.**



Scheme. 1: Synthesis of the ligand (L).

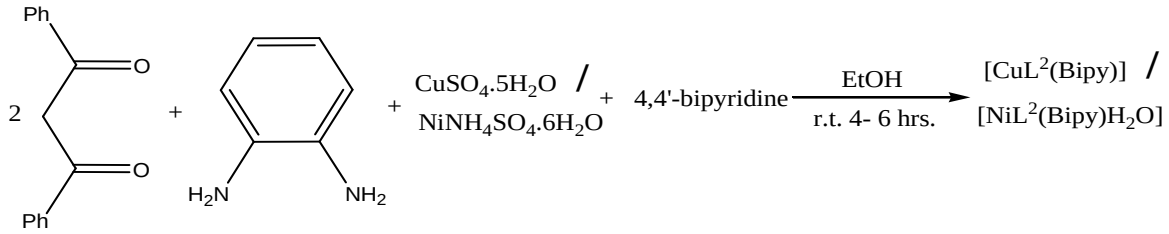
Synthesis of copper (II) and nickel (II) complexes

The synthesis of complexes is frequently performed by solvothermal methods: heating a mixture of organic linker and metal salt in a solvent system. These methods often yield crystals suitable for single crystal X-ray diffraction analysis.

An *in situ* reaction[8] of complexes in the present investigation as exemplified below (Scheme 2).

1,3–Diphenyl-1,3-propanedione and o-Phenylenediamine were taken together in ethanol (30 mL) and refluxed for 0.5 h after which copper sulphate pentahydrate / Nickel Ammonium Sulphate. $6H_2O$ (1 mmol) and 4,4'-bipyridine (1 mmol) in ethanol was added and refluxing was continued for another 3-4 h. Upon cooling, copper(II) / nickel(II) complex precipitated out. It was filtered, washed thoroughly with ethanol and dried.

The synthesised complexes were coloured solids and found to be highly stable under laboratory conditions to be stored for a long time.



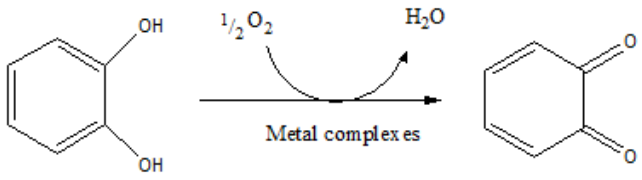
Scheme. 2: Synthesis of copper (II) and nickel (II) complexes.

Catecholase activity measurements

Kinetic measurements were carried out spectrophotometrically, following the appearance of o-quinone over time at 25°C (390nm absorbance maximum, ε=1600M^{-1} cm^{-1} in DMSO). The metal complexes (prepared *in situ*: 0.3mL of a 10^{-3}M DMSO solution) and a solution of catechol (2 mL of a 10^{-1}M solution in DMSO) were mixed in the spectrophotometric cell.[9]

Catecholase Studies

The progress of the catechol oxidation reaction was conveniently and closely monitored, by following the strong absorbance peak of quinone in the UV/Vis spectrophotometer. In all cases, catecholase activity was noted for 45 min at time intervals of 5 minutes by monitoring the increase in absorbance. A plot of log (A_∞ /$A_{\infty-t}$) versus time was obtained for each complex and the rate constants for the catalytic oxidations (Scheme 3) were calculated.



Scheme. 3: Catalytic oxidation of Catechol to o-quinone.

Antioxidant activity

DPPH scavenging activity

DPPH radical scavenging activity is a standard assay to study the antioxidant activities. It is a rapid technique for screening the radical scavenging activity of specific compounds.[10] The free radical scavenging effects of all the complexes and the ligand with DPPH radical were evaluated using concentrations 2 mg/mL of the synthesized complexes in DMF with 2 mL of 0.05 M methanolic solution of DPPH. The reaction mixture was incubated in the dark for 30 min at room temperature. The control contained all reagents without the sample while methanol was used as blank. The antiradical scavenging ability of synthesized metal complexes was determined by measuring the decrease in the absorbance of DPPH at 517 nm. The absorbance decreased when the DPPH is scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. This lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percent of inhibition (I %) of free radical production from DPPH was calculated by using the following equation.

$$\textbf{Percentage inhibition} = \frac{Ac - As}{Ao} X100$$

where, A_c - absorbance of the control: A_s - absorbance in the presence of sample solution.

Antidiabatic activity

Antidiabatic assay was done by α amylase inhibition assay as per the standard protocols.

Sample was taken in two different concentrations 10mg/ml and 25mg/ml in DMSO. It was incubated at 25°C that is at room temperature for 10 minutes. Then 1ml alpha amylase (0.5mg/ml) is added to all the samples and pre-incubated at room temperature for 10-15 minutes. After incubation 0.5 ml of 1%(w/v) starch solution was added again incubated for 5 minutes. All the solutions were prepared in 0.02M sodium phosphate buffer at pH 6.9 at 25°C. Then DNSA was added to stop the reaction followed by transferring the test tubes into boiling water bath for 5 minutes. After heating the test tubes were diluted with water and the

total volume was made up to 10 ml to dilute the solutions and then the optical density of absorbance values was taken at 540nm in a Shimazdu UV-Vis spectrophotometer.[11,12]

Total of three controls were used in the assay one is the total blank having nothing only buffer and DNSA, the other was enzyme blank where leaving enzyme (α amylase) everything was present and lastly the substrate blank having everything except substrate (starch). Total blank was used for calibrating the instrument and the other two blanks were used as controls to nullify the effect of both enzyme and substrate.

Calculations

Actual O.D was used to calculate the results.

Actual O.D. = O.D of sample - (Enzyme blank + substrate blank)

%inhibition= ((Actual O.D.)*100) / (Enzyme blank+ Substrate blank)

***In vitro* antibacterial activity**

Materials and Method

To test the *in-vitro* anti-bacterial activity of the given complexes and ligands, well diffusion method was followed as stated in Kirby Bauer Protocol. *Bacillus subtilis* (MTCC1168) and *Escherichia coli* (MTCC1886) were the pathogens used for testing based on their clinical importance in accordance with reference.[13] DMSO was used due to its high solvency. The stock cultures were stored at 4°C.

Antibacterial Susceptibility Test

The *in vitro* analysis of the culture was done by the agar well diffusion method using Muller Hinton Agar, as used *in reference*.[14] The bacterial strains *E.coli* (gram negative bacilli) and *B. subtilis* (gram positive bacilli) have been used. The anti-bacterial activity was carried out by inoculating the strains by spread plate method using sterile cotton swabs. Standard wells were created by using sterilized borers in each plate. A concentration of 1mg in 1ml was prepared from the complexes and ligands and Ciprofloxacin was used as the positive control, as used *in reference*.[15] 100µl was pipetted into each of wells. Duplicates were performed for both complexes and ligands to achieve standardized results. The zone of inhibition was observed and measured after 18-24 hours of incubation at 37°C. The effectiveness of the complexes and ligand were measured through the diameters (zone of inhibition). This procedure was performed in three replicate plates for each organism.

RESULTS AND DISCUSSION

The synthesised ligand, copper(II) and nickel(II) complexes were characterised by Elemental analyses, UV-Visible, FT-IR, ^{1}H NMR, ^{13}C NMR and Molar conductance, Magnetic susceptibility measurements and the results are given below.

Elemental analyses

The elemental analyses data for the ligand, copper(II) and nickel(II) complexes are given in the Table 1. They were in good agreement with molecular formulae.

Table. 1: Analytical data of the compounds.

Ligand/ Complexes	Empirical Formula	Formula Weight	Colour	Elemental Analysis Found (Calcd.)(%)			
				C	H	N	M
L	$C_{36}H_{28}N_2O_2$	520	Pale yellow	83.12	5.45	5.45	-
				(83.05)	(5.42)	(5.38)	
$[CuL^2(Bipy)]$	$C_{46}H_{36}CuN_4O_2$	740	Pale Green	74.87	4.93	7.60	8.75
				(74.63)	(4.90)	(7.57)	(8.58)
$[NiL^2(Bipy)H_2O]$	$C_{46}H_{38}NiN_4O_3$	752	Light Brown	74.07	5.15	7.60	7.95
				(73.32)	(5.08)	(7.44)	(7.79)

5.2 Molar conductivity and magnetic susceptibility measurements

The molar conductance values of the copper (II) and nicke(II) complexes fall in the range of 0.035 and 0.048 $\Omega^{-1}cm^2mol^{-1}$ respectively (Table 2) in ethanol suggesting that complexes are non-electrolytes.[16] The room temperature magnetic moment (μ_{eff}) value of the copper(II) complex is observed in 1.86 B.M., which corresponds to a single unpaired electron and may be concluded that the copper(II) complex have square pyramidal geometry similarly the room temperature magnetic moment (μ_{eff}) value of nickel(II) complexes is found to be 2.78 B.M. indicative of monomeric compound with octahedral geometry (Table 2).[17]

5.3 UV-Vis. Spectra

The electronic absorption spectra of ligand and complexes (10^{-3} M) were recorded in DMSO at room temperature and the data are represented in the Table 3. The band appeared around 309 nm and 359 nm corresponds to $\pi\rightarrow\pi^*$ transition of aromatic chromophore and to $n\rightarrow\pi^*$ transitions of imine moiety respectively.[18] The copper(II) and nickel(II) complexes exhibited a broad band between 596 to 620 nm due to d-d transition.[19]

Table. 2: Electronic data and some physical properties of the compounds.

Ligand/ Complexes	Charge transfer band	d-d transition	Molar conductance ($\Omega^{-1}cm^2mol^{-1}$)	μ_{eff} (BM)
L	309, 349	-	-	-
$[CuL^2(Bipy)]$	317, 355	596	0.035	1.86
$[NiL^2(Bipy)H_2O]$	312, 351	620	0.048	2.78

5.4 FT-IR spectra

The characteristic FT-IR bands of the synthesized ligand and complexes are given in Table 3. The FT-IR spectra of the ligand show a broad band in a region 3415cm^{-1}, which may be duo to υ(OH). Free υ(OH) is generally observed between 3500 and 3600 cm^{-1} . The low value of this band is due to intermolecular H-bonding,[20] which suggest the presence of keto-enol tautomeric form, at least in the solid state. The phenyl group shows υ(CH) at 3024 cm^{-1} and υ(C=N) at 1625cm^{-1}. The band at 1477cm^{-1} may be assigned to υ(C-O).[21]

On coordination the υ(C-O) band is shifted towards higher frequency suggesting the oxygen of the –OH group of the ligand and has taken part in the coordination. The strong band at 1625$cm^{-1}$for υ(C=N) azomethine of the ligand is shifted to lower frequency, suggesting coordination of the azomethine nitrogen to the metal ion.[22] Nickel(II) complex show a band in the region 3360cm^{-1}, which may be due to the presence of water molecule.[23] In all the complexes two new bands observed at 493 – 497 cm^{-1} and 453 – 457 cm^{-1} can be due to υ(M-O) and υ(M-N), respectively.[24]

All of these data confirm the fact that the ligand behaves as a tetradentate ligand forming a conjugated chelate ring, and the ligand exists in the complexes in the enolized form.

Table. 3: FT-IR data of the compounds and their assignments.

Ligand/ Complexes	υ(cm^{-1})						
	υ(H_2O)	υ(OH)	υ(CH)	υ(C=N)	υ(C-O)	υ(M-N)	υ(M-O)
L	-	3414	3024	1625	1467	-	-
$[CuL^2(Bipy)]$	-	3441	3059	1598	1471	493	453
$[NiL^2(Bipy)H_2O]$	3360	3419	3057	1595	1473	497	457

5.5 ^{1}H NMR and ^{13}C NMR spectra

A ^{1}H NMR spectrum of the ligand was carried out in DMSO-d_6 at room temperature and the important assignment is summarized in Table 4. The sharp singlet at δ 6.20 ppm, observed due to ethylene proton. The phenyl multiplets observed in the range of δ 6.73 – 8.03 ppm. The enolic nature of the ligand shows singlet at δ 11.52 ppm, due to rapid exchange

interaction of keto-enol tautomerism.[24] From the NMR data it is observed that the present ligand shows keto-enol tautomerism as shown below (Figure 1).

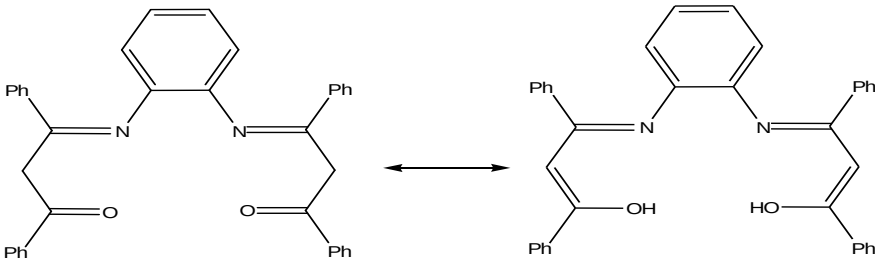


Figure. 1: Keto-enol tautomerism of ligand (L).

A ^{13}C NMR spectrum of the ligand was carried out in DMSO-d_6 at room temperature and the important assignment is summarized in Table 4. A ^{13}C NMR spectrum of the ligand showed imine carbon signal at δ 185.79 ppm and ethylene carbon at δ 77.08 ppm.

Table. 4: ^{1}H NMR and ^{13}C NMR of the lignad and their assignment.

Assignment	δ (ppm)	
	^{1}H NMR	^{13}C NMR
Ethylene	6.2	77.08
Aromatic	6.73 – 8.03	116.75 – 135.56
Enolic nature	11.52	-
Imine carbon	-	185.79

5.6 Catecholase activity measurements

The oxidation of catechol to the corresponding quinone formation is known as catecholase activity. The catecholase activity of the copper(II) and nickel(II) complexes were carried out using pyrocatechol as the substrate. 10^{-3} mol dm^{-3} solutions of copper(II) and nickel(II) complexes in dimethylsulfoxide were treated with 100 equivalents of pyrocatechol in the presence of air. The reaction was carried out spectrophotometrically at 390 nm for 45 min at time intervals of 5 min are given in Table 5. The slope was determined by the method of initial rates by monitoring the growth of the product o-quinone at the absorption band 390 nm. A graph is drawn by plotting log ($A_{\infty}/A_{\infty-t}$) *versus* time for catecholase activity (Figure 2).

The observed kinetics show a first order dependence on the complex concentration and the initial rate constant values of copper(II) and nickel(II) complexes were 4.91 x 10^{-3} min^{-1} and 0.30 x 10^{-3} min^{-1}, respectively.[25] The copper(II) complex exhibits higher activity than the nickel(II) due to redox potential of the copper(II) metal ion and the binding of the catechol with ligand molecules.

Table. 5: Catecholase activity of copper(II) and nickel(II) complexes.

Time (min)	log (A_∞ /$A_{\infty-t}$)	
	[CuL^2(Bipy)]	[NiL^2(Bipy)H_2O]
0	0	0
5	0.009	0.0005
10	0.017	0.0011
15	0.029	0.0020
20	0.046	0.0028
25	0.058	0.0035
30	0.069	0.0043
35	0.081	0.0052
40	0.092	0.0057
45	0.101	0.0063

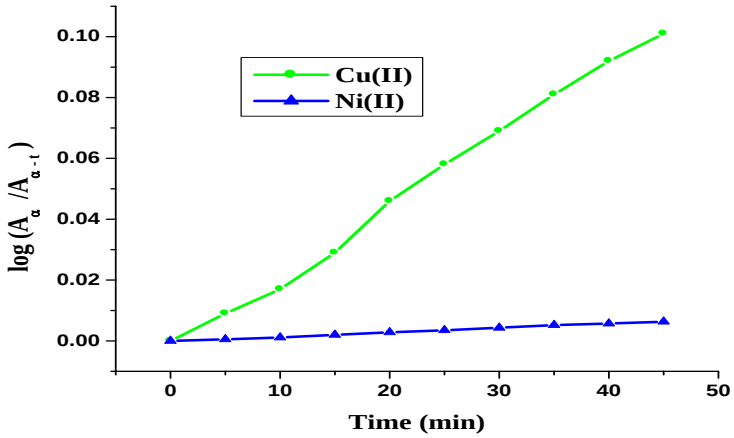


Figure. 2: Catecholase activity of complexes.

5.7 Antioxidant activity

An antioxidant can be defined as any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate. To study the *in vitro* antioxidant activity of the ligand and complexes the DPPH method was adopted. The results revealed all the synthesized compounds showed negative antioxidant activity. The DPPH scavenging activity of standard antioxidant α-tocopherol was also assayed for comparison.[26]

5.8 *In vitro* antidiabatic activity: The percentage inhibition of *α*-amylase exhibited by the ligand and complexes are depicted in Table 6 and are compared with standard drug acarbose.

Many researchers have focused on *in vivo* antidiabatic activities of copper complexes.[27] Walter *et al.*,[28] hypothesized that copper metabolism encounters the diabetic pathological

conditions. Copper can increase the tolerance of pancreatic *β*-cells against oxidative stress, which is one of the causative agents of diabetes.[29] Intramuscular injection of copper(II) acetate imidazole complex to streptozotocin (STZ) induced rats are shown to increase in glucose tolerance and consequently decrease in blood glucose level. Abdul-Ghani *et al*.,[30] proved the antidiabatic effect of bis(acetato)tetrakis(imidazole) copper(II) in STZ induced diabetic rats. Yasumatsu *et al*.,[31] stated that single intraperitonial injection of copper(II) picolinate to diabetic mice shown higher hypoglycemic effect. Moreover, Barthel *et al*.,[32] proved that, the copper chelating agent tetrathiomolybdate decreased serum copper ions and free radicals which ameliorates glucose and lipid metabolism in diabetic db/db mice model. The mechanism of action by copper has been suggested, that copper treats hyperglycemia by activating the phosphoinositide 3'kinase (PI3-K/Akt) pathway leading to GLUT 4 translocation[33] in some studies. Hence, the above studies indicate that copper(II) complexes exhibit good antidiabatic activity.

In the current study, the inhibitory effect of ligand, copper(II) and nickel(II) complexes on carbohydrate hydrolyzing enzyme *α*-amylase was investigated. This enzyme inhibitor antagonizes the activity of this enzyme and delaying the digestion of carbohydrate which prevents the sudden rise in blood glucose level, especially after meal.[34] Therefore, inhibition of this enzyme is an attractive approach for the management of diabetes. In the present study, copper(II) inhibits *α*-amylase 96.71±0.02%, nickel(II) inhibits 96.27±0.15 and ligand inhibits 97.06±0.16% (25 mg/ml); these are compared with standard drug acarbose which inhibits 88.45±0.01%. Hence, copper(II) complex is considered as moderate inhibitor when compared with ligand and nickel(II) complex. The enhanced activity of copper(II) complex may be due to the pharmacological actions of copper metal such as antiulcer, anticonvulsant, anticancer, antidiabatic and antimicrobial activity. However, further *in vivo* studies are essential to prove their antidiabatic activity and the mechanism.

Table. 6: % of Inhibition values of standard drug acarbose and the compounds.

Ligand/ Complexes	*α*-amylase % of Inhibition (mg / ml) ± SD	
	10	25
L	98.82±0.08	97.06±0.16
$[CuL^2(Bipy)]$	98.42±0.02	96.71±0.02
$[NiL^2(Bipy)H_2O]$	98.21±0.02	96.27±0.15
Acarbose	33.34±0.01	88.45±0.01

Statistical analysis

The results were expressed as mean ± S.E.M. Statistical analysis of the data were carried out using Student's t-test and the results were considered significant when $P < 0.05$.[35]

5.9 *In vitro* antibacterial activity

The zones of inhibition were compared against the standard CLSI guidelines. The diameter of the zone of inhibition is dependent on factors like rate of diffusion across the agar media, depth of the media, and the sensitivity of the pathogen towards the antibacterial sample. The zone of inhibition for Ciprofloxacin is >30mm, with reference to the standard guidelines, diameter <15mm is resistant, between 16-20mm is intermediate susceptibility and >21mm is susceptibility. The data obtained (Table 7), shows the diameter of the zone of inhibition of the samples used, in mm. Analysis of this data, reveals that each of the pathogens have intermediate susceptibility with the copper (II) complex (from reference table), as the diameter is between the range of 16-18mm. *B.subtilis* had the highest susceptibility towards copper (II), whereas it showed resistance towards nickel (II). The activity with *E.coli* was nearly the same with each complex. The results drawn from the data states that complexes have higher activity towards gram negative pathogen. In the case of ligand there was no zone of inhibition observed, stating high resistance of pathogens towards the ligand.

Copper (II) complex has the maximum zone of inhibition, with respect to both the pathogens, thus it is the best sample to be used for further analysis on antibacterial properties.

As far as the relation between structure and activity are concerned the copper (II) complex showed significantly enhanced antibacterial activity against microbial strains in comparison to the free ligand. Previous studies elsewhere suggested that chelation tended to make the ligands act as more powerful and potent bactereostatic agents,[36,37] thus inhibited the growth of bacteria more than the parent ligands did and it is similar with that of this study. It was suspected that factors such as solubility, conductivity, dipole moment and cell permeability mechanism influenced by the presence of metal ion might be the possible reason for the increase in activity.

Table. 7. The zone of inhibition for the specific complexes against *B. subtilis* and *E. coli*

Ligand/ Complexes	Zone of Inhibition (mm) ± SD		
	B.subtilis	***E.coli***	**Ciprofloxacin**
L	-	-	>30
$[CuL^2(Bipy)]$	18.0±1.41	16.5±0.71	>30
$[NiL^2(Bipy)H_2O]$	16.5±0.71	16.0±0.00	>30

6. CONCLUSION

In the present study tetradentate ligand and copper(II), nickel(II) complexes have been synthesized using β - diketones with aromatic amines, and characterized by various physico-chemical and spectral analyses. The molar conductance of all the complexes suggested their nonelectrolytic nature. The coordinating mode of the donor atoms of the ligand was confirmed by FT-IR spectra. Magnetic susceptibility measurements substantiate the paramagnetic nature of all the complexes. Based on the studies square pyramidal geometry has been proposed for the copper(II) complex and octahedral geometry has been proposed for nickel(II) complex.

The catecholase activity of the copper(II) and nickel(II) complexes were carried out using pyrocatechol as the substrate. The copper(II) complex exhibits higher activity than the nickel(II) due to redox potential of the copper(II) metal ion and the binding of the catechol with ligand molecules.

In vitro antidiabatic studies of the ligand and complexes were much higher when compared with standard drug such as acarbose. Since the ligand and complexes showed significant inhibiting activities, the current results provide a lead for the *in vivo* studies to establish the possibility of these complexes as antidiabatic agent.

In vitro antibacterial and antioxidant studies were carried out. The results revealed that among the ligand and complexes, complexes were found to exhibit very good antibacterial activity against *B.subtilis* and *E.coli*.

Among the synthesized ligand and complexes copper(II) complex was found to have good antidiabatic and antibacterial activity. The results revealed all the synthesized compounds showed negative antioxidant activity. Based on the above results, the tentative structures of the complexes can be formulated as shown in Figure 3 and 4.

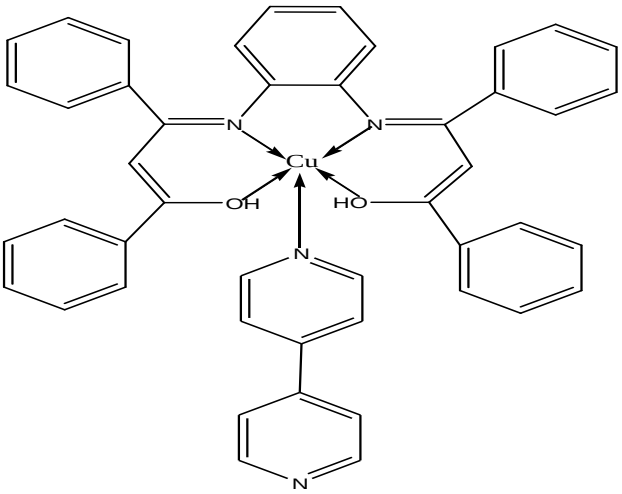


Figure. 3: The tentative structure of the copper (II) complex.

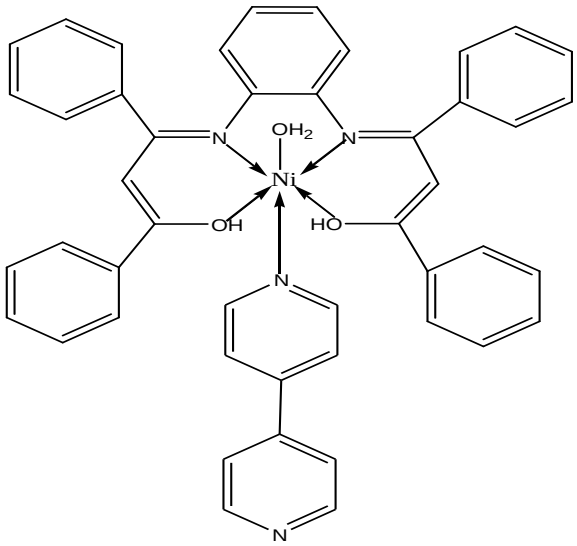


Figure. 4: The tentative structure of the nickel (II) complex.

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