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Structural Characterization of Novel 2,2'-(4-(3,4-Dimethoxyphenyl) Pyridine-2,6-diyl) Dithiazole, its Copper Complex and Antioxidant, Antimicrobial and Blood Coagulation Cascade

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Abstract: ***A novel 2,2'-(4-(3,4-dimethoxyphenyl)pyridine-2,6-diyl)dithiazole (L) and its Copper metal complex [Cu(L)Cl₂]. The ligand was prepared by facile one-pot procedure from 2-acetylthiazole and characterized by ^{1}HNMR, ^{13}CNMR, UV-Visible, Mass and Single Crystal XRD. The synthesized compounds were screened for anticoagulant, antiplatelet, antioxidant and antimicrobial studies. Study reveals the anticoagulant nature of synthetic compounds without interfering in the platelet aggregation process. The L and its [Cu(L)Cl₂] increased the clotting time of human citrated plasma in both platelet rich plasma and platelet poor plasma. Ligand increases from 180sec to 260 sec and 220sec to 300sec respectively at the concentration of 30µg and its complex is enhanced from the control 180sec to 300sec and 220sec to 340sec respectively at the concentration of 30µg. Fascinatingly, the ligand and its complex did not alter the platelet aggregation process. It is also found that the ligand and its complex exhibit promising antioxidant properties and mild antimicrobial activities. Moreover, the ligand and its complex exhibit non-toxic properties as it cannot rupture the RBC membrane.***

Keywords: anticoagulant activity, copper complex, platelet aggregation, antioxidant properties, RBC membrane safety

1. Introduction

Since the discovery of 2,2':6',2''-terpyridines (tpy) ligand in 1937, it has became one of the most extensively studied ligand because of its unique coordinating capacity with the various transition metals and lanthanides [1]. The synthesis of ligands is well reported in the literature by Kroenke pyridine ligand synthesis by using acetyl pyridine or acetyl pyrazine or acetyl thiazole as starting materials [2-3]. Many number of 4-substituted terpyridines ligands and metal complexes were reported but very few ligands and complexes of 4-(aryl)-modified-2,6-di(1,3-thiazol-2-yl) pyridine were known even though 2,6-di(thiazol-2-yl)pyridine ligand have comparable coordinating ability as that of terpyridine [4-5]. To date, only a few 2,6-di(thiazol-2-yl)pyridine derivatives and their transition-metal complexes have been reported[6]. These ligands and their various transition metal complexes were also extensively studied for their photophysical and various biological and pharmacological activities like DNA cleaving agents, DNA binding, cytotoxicity, DFT calculations, DNA interaction, anticancer activity, photoluminescence and catalytic activity, antitumor, antimicrobial, or anti-HIV agents [7-10].

Thrombosis is the primary factor associated with cardiovascular disorders which ultimately leads to death [11]. Arterial thrombosis, deep vein thrombosis and pulmonary embolism were the different types of thrombosis [12]. Currently, several synthetic anticoagulant agents such as heparin and warfarin are used to treat thrombotic disorders [13]. But, due to some side effects of synthetic molecules like vomiting, nausea and headache, their clinical use was restricted [14]. To overcome this significant problem, identifying novel anticoagulants from natural sources and synthetic drugs with the most negligible side effects could be a better solution for treating thrombotic disorders [15]. Therefore, the present study explores the beneficial efficacy of synthetic ligand with copper complex on thrombotic disorders.

A deleterious-free radical generation is an important event in the progression of many pathological conditions. During oxidative stress, these free radicals contribute a significant role in inducing cell death. Therefore, ligand(**L**) and Copper complex [Cu(**L**)Cl_2] were evaluated for their free radial scavenging potentiality.

2. Experimental Section

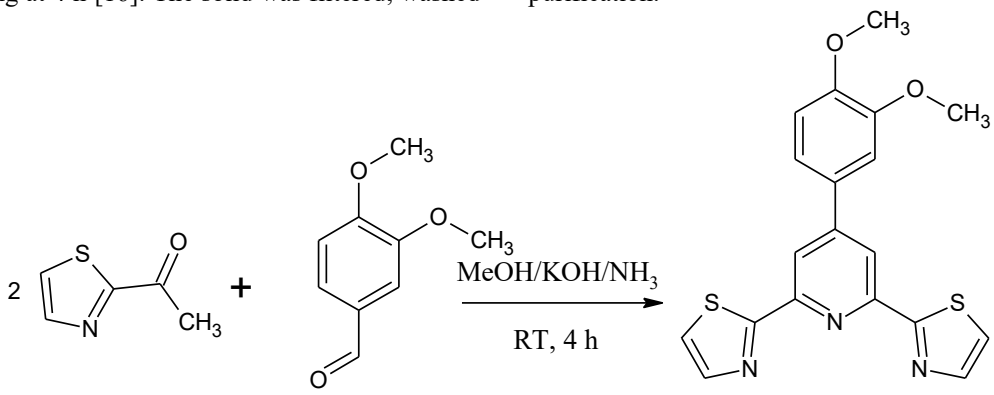
2.1 Materials and Methods

All the solvents and reagents were purchased from Sigma–Aldrich. All reactions were performed under ambient conditions. Absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer, and FT-IR spectra were measured on a PerkinElmer instrument with solid samples using a Golden Gate ATR accessory, and ^{1}H and ^{13}C NMR spectra were obtained at 400 MHz and 100 MHz.All other chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for the PlateletRich Plasma (PRP)& Platelet poor Plasma (PPP). ADP and Epinephrinewere purchased from Sigma Chemicals Company (St. Louis, USA). All the experimentation were conducted following the ethical guidelines and were approved by the Institutional Human Ethical Committee (IHEC-UOM No. 47Res/2014– 15), University of Mysore, Mysore.

2.2 General procedure for the synthesis of 2,2'-(4-(3,4-dimethoxyphenyl)pyridine-2,6-diyl)dithiazole(L):

2-acetylthiazole (2 mmol) taken in a 100 mL round-bottom flask, was added MeOH (30 mL), KOH pellets (0.560 g, 4 mmol), and 2 mL of water. The mixture was stirred for 10

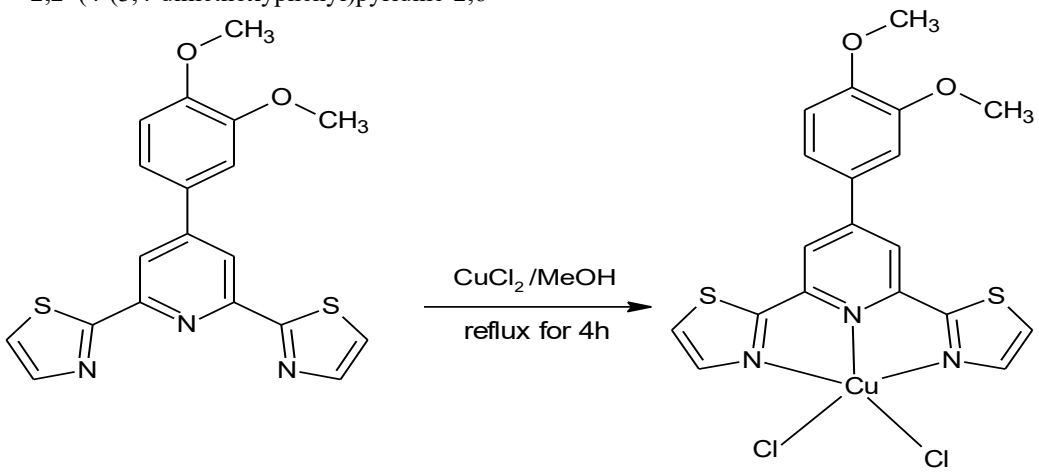
min and then added the corresponding 3,4-dimethoxybenzaldehydes (1 mmol) at room temperature continued stirring at 4 h [16]. The solid was filtered, washed with methanol, and then diethylether. The ligand obtained compound was used for complexation without further purification.



Scheme 1: Synthesis of ligand (L)

2.3 General procedure for the synthesis of copper complex [Cu(L)Cl₂]:

A solution of $CuCl_2 \cdot 2H_2O$ (1 mmol) dissolved in methanol (5 mL) was added to a methanol solution (10 mL) containing the 2,2'-(4-(3,4-dimethoxyphenyl)pyridine-2,6-diyl)dithiazole(1 mmol). The mixture was stirred at room temperature overnight. The green precipitate was collected and dried with diethyl ether recrystallized in methanol. The obtained product was identified by IR spectra and single crystal XRD.



Scheme 2: Synthesis of copper complex $[Cu(L)Cl_2]$

2.4 Preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP)

The method of Ardlie and Han [17] was employed for the preparation of human Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP). The platelet concentration of PRP was adjusted to 3.1×10^8 platelets/ml with PPP. The PRP maintained at 37°C was used within 2hr for the aggregation process. All the above preparations were carried out using plastic wares or siliconized glass wares.

2.5 Plasma re-calcification time

The plasma re-calcification time was determined according to the method of Quick et al., [18]. Briefly, the ligands and its complexes (0-30µg) were pre-incubated with 0.2ml of citrated human plasma in the presence of 10mM TrisHCl (20µl) buffer pH 7.4 for 1min at 37°C, 20µl of 0.25M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.

2.6 Platelet aggregation

The turbid metric method of Born was followed using a Chronology dual channel whole blood/optical lumi aggregation system (Model-700). Aliquots of PRP were pre-incubated with various concentrations of ligands and its complexes (0–10µg) in 0.25ml reaction volume. The aggregation was initiated independently by adding agonists, such as ADP and epinephrine, followed for 6min.

2.7 Antioxidant activity

The effect of (**L**) and $[Cu(\mathbf{L})Cl_2]$on DPPH radical scavenging activity was determined according to the method of Yamaguchi et al. [19], with slight modification, and vitamin C was used as a reference standard. Briefly, 0.1 Mmsolution of DPPH was incubated with 0–100µM of (**L**)and $[Cu(\mathbf{L})Cl_2]$for 20 min at ambient temperature in the dark, and the resulting absorbance was measured using UV-Visible spectrophotometer at 517 nm against a blank (BioMate 3S, Thermo Scientific). The percentage of free radical scavenging was calculated using this formula.

% DPPH inhibition = [(OD of control –OD of test) /(OD of control)]x100

2.8 Antibacterial and antifungal assay:

The synthesized ligand and metal complexes are screened for their antibacterial activity by using the agar well diffusion method [20] against pathogenic bacterial strains Staphylococcus aureus (MTCC 96), Escherichia coli and antifungal activity by using Aspergillusniger (MTCC 1344), Candida albicans (MTCC 854). For bacteria, the samples were incubated at 36 °C for 24 hours. For fungi, the samples were incubated at 25 °C for seven days for A. Niger and C. albicans, and the samples were incubated at 37 °C for two days. The diameters of the zone of inhibition at MIC of 100µg/mL were measured in mm concerning the ciprofloxacin, fluconazole as standard drugs for antibacterial, antifungal. MIC was determined by the lowest concentration of sample that prevents the development of turbidity. Antimicrobial activities are shown in **Table 1**. Compounds showed moderate antimicrobial activity against different strains.

Table 1: Antimicrobial activities of synthesized compounds

Compound	Bacterial strains		Fungal strains	
	S. aureus	E. coli	C. albicans	A. niger
L	9.01	8.81	9.49	9.21
$[Cu(L)Cl_2]$	11.54	12.54	12.85	12.24
Ciprofloxacin	23.0	23.0	-	-
Fluconazole	-	-	25.0	25.0

2.9 Direct hemolytic activity

Direct hemolytic activity was determined as described previously [21]. Briefly, packed human erythrocytes and phosphate buffered saline (PBS) (1:9v/v) were mixed; 1ml of this suspension was incubated independently with the various concentration of ligand and its complexes (0-10 0µg) for 1hr at 37°C. The reaction was stopped by adding 9ml of ice cold PBS and centrifuged at 1000g for 10min at 37°C. The amount of hemoglobin released in the supernatant was measured at 540nm. The activity was expressed as a percent of hemolysis against 100% lysis of cells due to the addition of water that served as positive control, and phosphate buffered saline served as a negative control.

2.10 In *silico* molecular docking:

Ligand and complex molecules were designed and synthesized. The structures were drawn in Chemdraw 11.0 (saved as mol files), and by using ADS, the energies were minimized. The minimized compounds and proteins were saved in structure data(.sd) and protein data bank (PDB) format, respectively, for further studies [22].

The docking study was performed using Accelrys Discovery Studio client version 3.5 software (AccelyrsInc., http://www.accelrys.com). The X-ray crystallographic structures of all proteins (PDB ID 2XCT bound with ciprofloxacin) were acquired from the protein data bank (PDB). A grid-based molecular docking method, the C-DOCKER algorithm, was used to dock the small molecules (ligand and complexes) into the protein active site. The designed structures were submitted to CHARMm (Chemistry at Harvard Macromolecular Mechanics) force field for structure refinement. All water molecules, bound inhibitor, and other heteroatoms were removed from the macromolecule, and polar hydrogen atoms were added. Energy minimization was carried out for all compounds using CHARMm force field to make stable conformation of protein with an energy gradient of 0.01 kcal/mol/A°. A final minimization of the compounds in the rigid receptor using non-softened potential was performed. For each final pose, the CHARMm energy (interaction energy plus ligand strain) and the interaction energy alone were calculated. The poses were sorted by CHARMm energy and the top scoring (most negative, thus favorable to binding) poses. The binding energy of the compounds was given in Table 2. Binding interaction was given in Table 2.1 Compounds showed good binding energy.

Table 2: Binding energy of the compounds

Compound	C Docker Energy	No of interaction	Interaction residues		
			Pi	Pi-Pi	H bonding
L	-87.8	11	PRO-770,PHE-771,LEU-694,GLY-695,SER-696		
$[Cu(\mathbf{L})Cl_2]$	-82.1	12	LEU-694,GLY-695,SER-696,VAL-702,LYS-704		

3. Results and Discussion

The ligand (**L**) and copper complex$[Cu(\mathbf{L})Cl_2]$were synthesized in good yield by modified Kroenke Pyridine synthesis.

3.1 ^{1}H NMR and ^{13}C NMR Spectrum:

In the ^{1}H NMR spectrum of ligand, methoxy protons appeared at δ 3.799-3.812 as singlets. Four doublets observed at 7.931-7.939 (d, 2H, J = 3.2 Hz), 7.464-7.471 (d, 2H, J =2.8 Hz) are due to protons present in the thiazole ring. A singlet observed at δ 8.380(s, 2H) due to proton present in pyridine ring. The aromatic protons observed at δ 6.941 (s, 2H, ArH) and another singlet observed at 7.035 (s, 1H, J = 7 Hz) as shown in Fig 1. In the ^{13}C NMR spectrum,methoxy (OCH_3) carbons observed at56.21 ppm. Aromatic carbons appeared at 110.10, 111.65, 117.51, 120.36, 122.09, 130.13 ppm, whereas pyridine ring carbon deshielded which observed at 144.23-151.60 ppm. The carbon of thaizole ring highly deshielded which appeared at 169.17 ppm as shown in **Fig 2**.

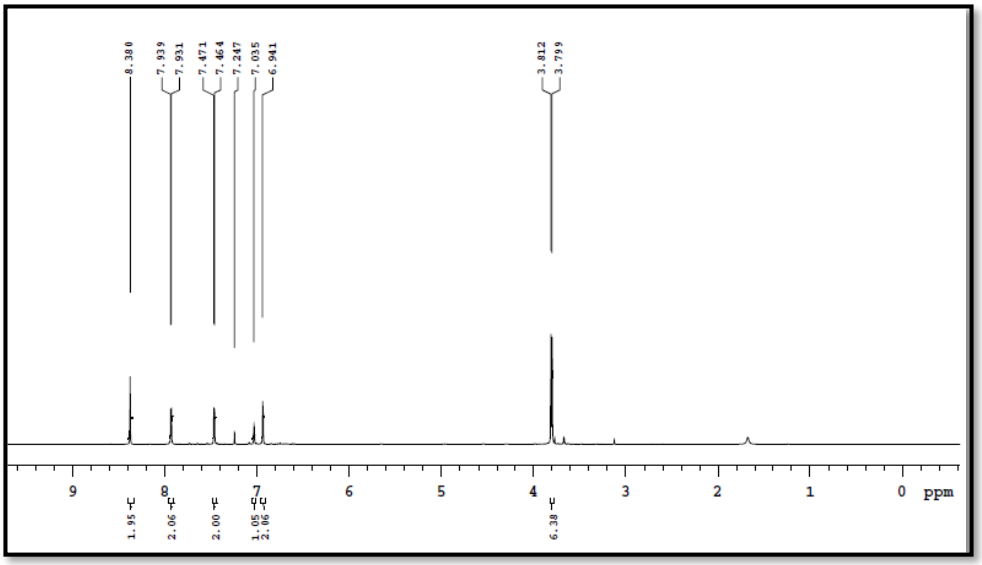


Figure 1: ^{1}H NMR spectrum of L

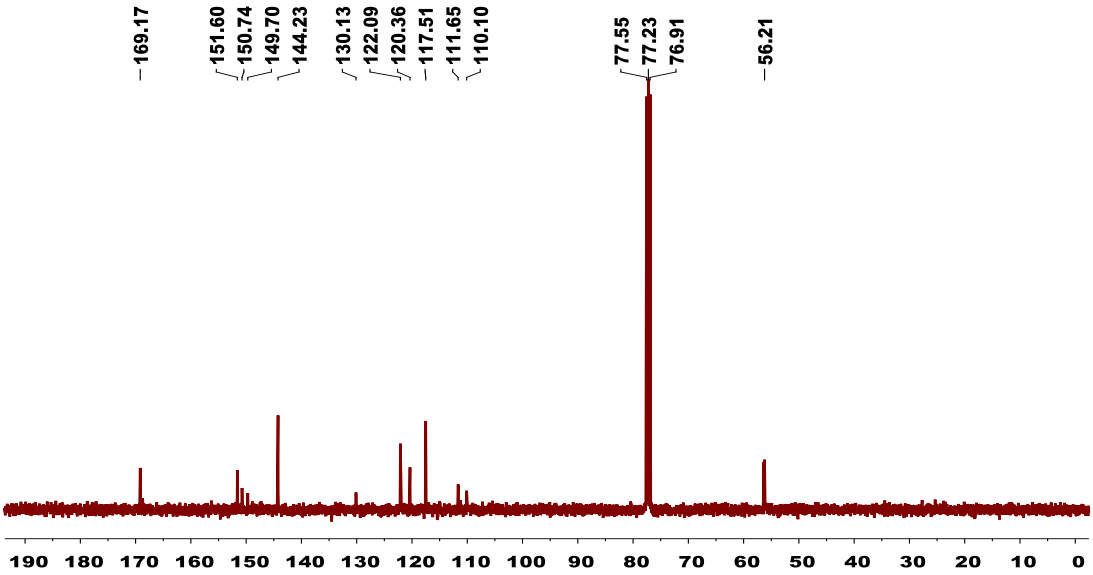


Figure 2: ^{13}C NMR spectrum of L

3.2 Mass Spectrum

The Mass Spectrum M+1 peak observed at 382 (Molecular weight 381) as shown in **Fig 3**.

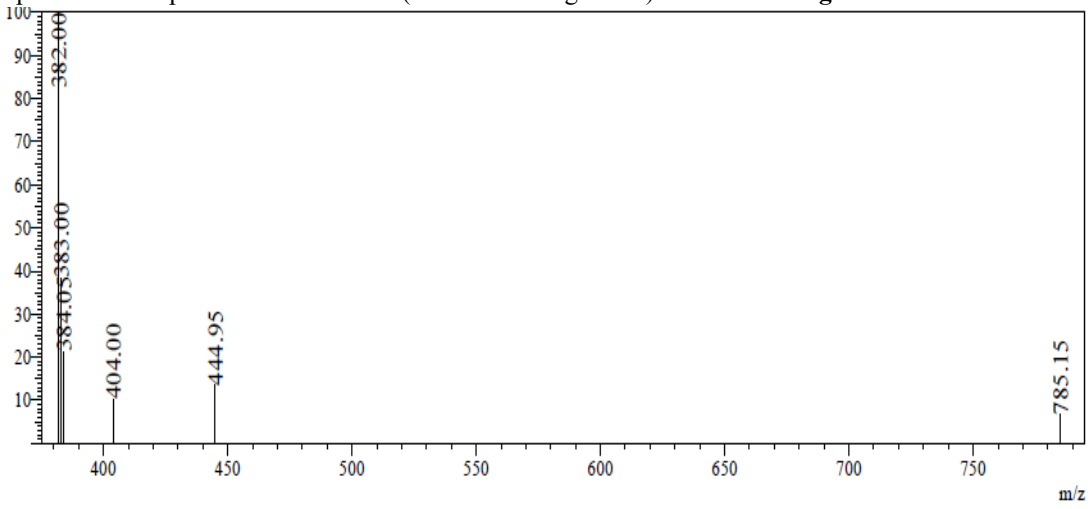


Figure 3: Mass Spectrum of L

3.3 FT-IR Spectrum:

The IR spectrum exhibits the absorption band at 1741(w) cm^{-1} due to terpyridine stretching, the absorption band at 1189(M) cm^{-1} due to -C-N stretching and 3267cm^{-1}, 3073 cm^{-1} due to –C-H stretching and 1409 cm^{-1}(s) due to (C-O) as shown in Fig 4.For copper complex, a weak peak observed at 3020 cm^{-1} for aromatic –C-H, 1625 (C-O), 1540 (C=C), 1469 (C-N), 2874(Ar C-H), 1765(C=N),1580(C=C), 1320(C-N), 764 The Cu-N band is observed in the region of 562and Cu-Cl at 798 cm^{-1} shows the formation of copper complex as shown in **Fig 5**.

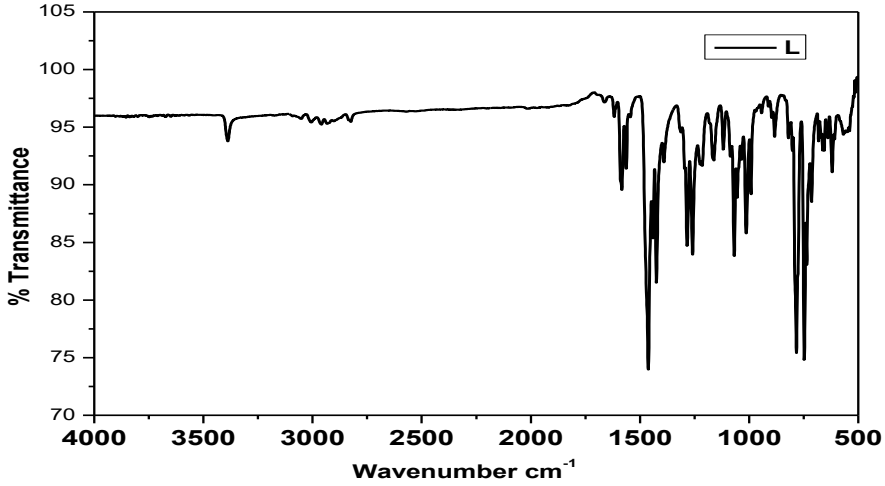


Figure 4: FTIR Spectrum of L

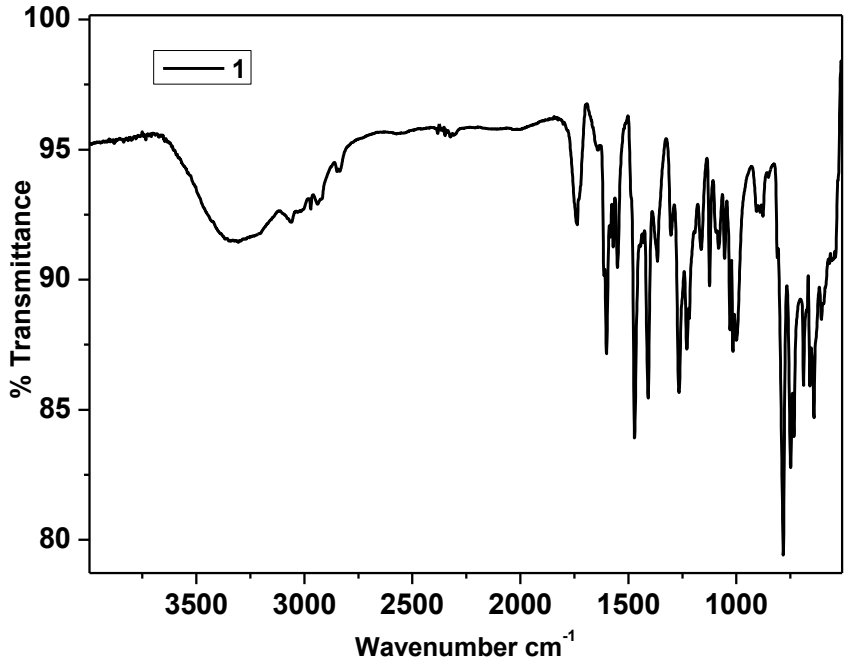


Figure 5: FT-IR Spectrum of $[Cu(L)Cl_2]$

3.4 UV-Visible Spectrum:

The UV-visible absorbance spectra of the synthesized2,2'-(4-(3,4-dimethoxyphenyl)pyridine-2,6-diyl)dithiazole were studied in $CHCl_3$ and are shown in **Fig 6**. The absorption spectra of **L** exhibit broad absorption bands between 300 and 331 nm. These absorption bands were attributed due to strong π-π* transitions of delocalized π electrons present in the **L**. The absorption spectra of **L** exhibit broad absorption bands between 227.26, 303.36 and 346.57 nm appeared due to π-π* transitions in the complex.

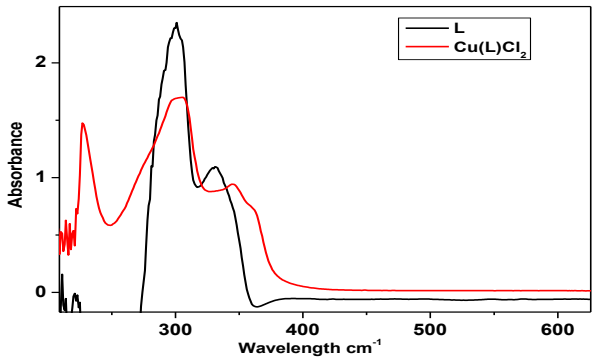


Figure 6: UV-Visible Spectrum of **L** and $[Cu(\mathbf{L})Cl_2]$

Compoud	Absorption (nm)	Band assignment	Melting point (^{0}C)
L	300.65 331.05	$\pi \rightarrow \pi*$	148-150
$Cu(L)Cl_2$	227.26 303.36 346.57	$\pi \rightarrow \pi*$	282-284

3.5 EPR Spectrum of copper complex

In the ESR Spectrum of copper complex g=2.12692 indicates the presence of free electron and the complex is paramagnetic with distorted square planar geometry as shown in **Fig7**.

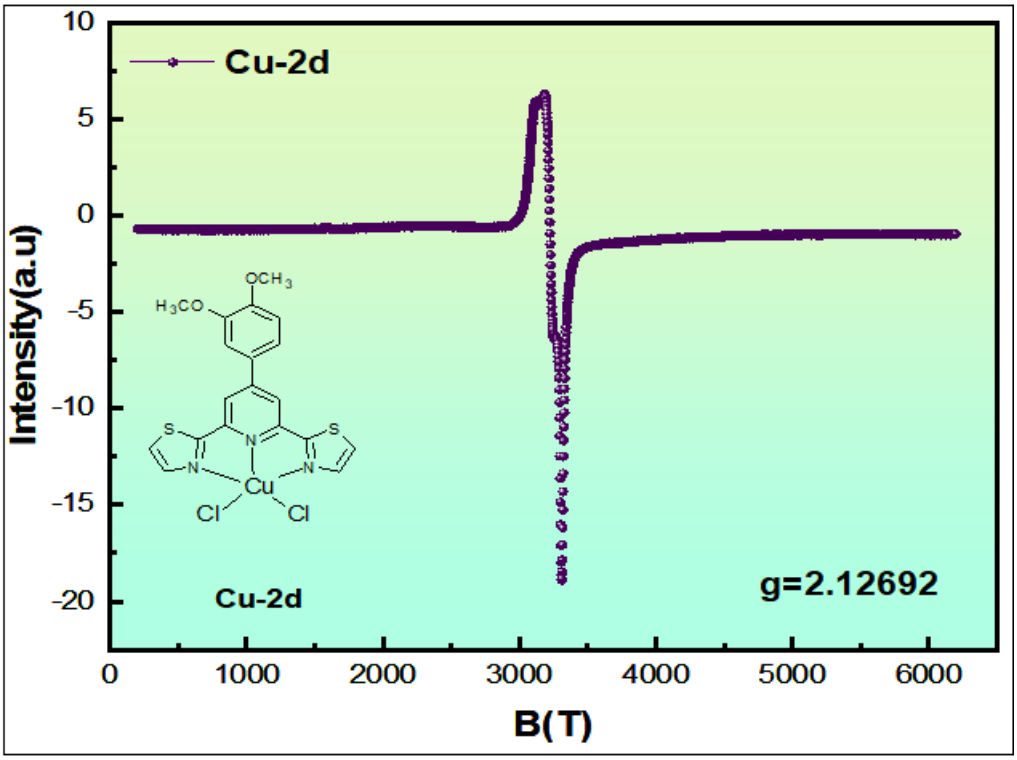


Figure 7: EPR Spectrum of $[Cu(L)Cl_2]$

3.6 Single Crystal X-ray Diffraction:

The single crystal of copper complex was determined by single crystal X-ray diffraction. The crystal data and structure refinement parameters and selected bond lengths, bond angles are given in **Table 3** and **Table 4** respectively. Refinement carried was full matrix least-squares on F^2. The bond lengths and bond angles were in good agreement with reported 2,6-(bis-thiazolyl)pyridine.

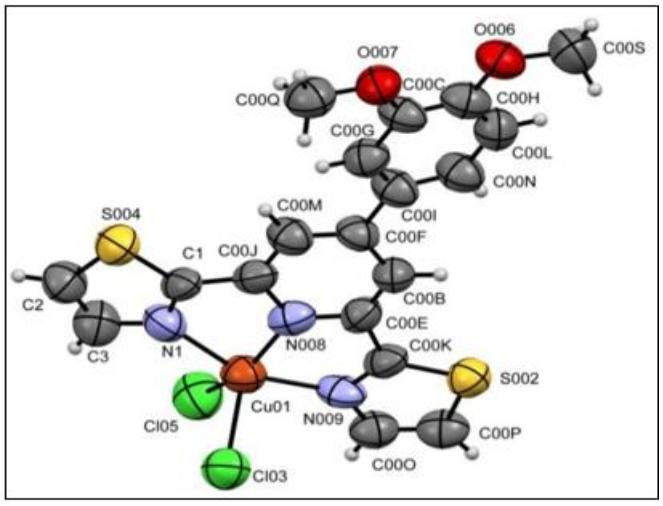


Figure 8: ORTEP diagram of $[Cu(L)Cl_2]$

Table 3: Crystallographic data and structure refinement for the Copper Complex

Parameters	
Empirical formula	$C_{19}H_{15}Cl_2CuN_2O_2S_2$
Formula weight	665.34
Temperature/K	273(2)
Crystal system	triclinic
Space group	P-1
a/Å	7.613(6)
b/Å	12.884(10)
c/Å	13.326(10)
α/°	73.610(8)
β/°	85.149(9)
γ/°	83.188(9)
Volume/Å^3	0.24 × 0.22 × 0.18 mm
Z	8
ρ_{calc}g/cm^3	1.860
μ/mm^{-1}	4.153
F(000)	672.0
2Θ range for data collection/°	3.312 to 57.374
Index ranges	-10≤h≤10, -17≤k≤ 16, -17 ≤ l ≤ 17
Reflections collected	12896
Independent reflections	5592 [R_{int} = 0.0666, R_{sigma} = 0.1020]
Data/restraints/parameters	5592/0/282
Goodness-of-fit on F^2	1.803
CCDC	**2014084**

Table 4: Selected Bond length [Å] and angles [°] in compound $[Cu(L)Cl_2]$

Cu01—N008	1.982 (11)	C00E—C00K	1.486 (16)
Cu01—N009	2.037 (12)	C00F—C00M	1.352 (17)
Cu01—N1	2.073 (11)	C00F—C00I	1.471 (16)
Cu01—Cl05	2.273 (5)	C00G—C00I	1.408 (17)
Cu01—Cl03	2.493 (4)	C00G—H00G	0.9300
S002—C00P	1.711 (13)	C00H—C00L	1.399 (18)
S002—C00K	1.720 (13)	C00I—C00N	1.387 (18)
S004—C2	1.679 (14)	C00J—C00M	1.394 (17)
S004—C1	1.686 (12)	C00L—C00N	1.405 (17)
O006—C00H	1.398 (14)	C00L—H00L	0.9300
O006—C00S	1.466 (16)	C00M—H00M	0.9300
O007—C00C	1.356 (14)	C00N—H00N	0.9300
O007—C00Q	1.438 (16)	C00O—C00P	1.376 (18)
N008—C00E	1.385 (15)	C00O—H00O	0.9300
N008—C00J	1.400 (14)	C00P—H00P	0.9300
N009—C00K	1.350 (17)	C00Q—H00A	0.9600
N009—C00O	1.356 (15)	C00Q—H00C	0.9600
N1—C1	1.317 (16)	C00Q—H00D	0.9600
N1—C3	1.369 (16)	C3—C2	1.376 (18)
C00B—C00E	1.342 (16)	C3—H3	0.9300
C00B—C00F	1.417 (16)	C00S—H00E	0.9600
C00B—H00B	0.9300	C00S—H00F	0.9600
C00C—C00H	1.351 (18)	C00S—H00H	0.9600
C00C—C00G	1.423 (17)	C2—H2	0.9300
C1—C00J	1.510 (18)		
N008—Cu01—N009	78.3 (4)	O006—C00H—C00L	122.3 (14)
N008—Cu01—N1	78.8 (5)	C00N—C00I—C00G	116.9 (12)
N009—Cu01—N1	155.3 (4)	C00N—C00I—C00F	120.5 (12)
N008—Cu01—Cl05	150.8 (3)	C00G—C00I—C00F	122.6 (13)
N009—Cu01—Cl05	97.6 (3)	C00M—C00J—N008	119.2 (13)
N1—Cu01—Cl05	97.8 (4)	C00M—C00J—C1	131.5 (12)
N008—Cu01—Cl03	105.3 (3)	N008—C00J—C1	109.2 (11)
N009—Cu01—Cl03	95.9 (3)	N009—C00K—C00E	119.4 (12)
N1—Cu01—Cl03	99.0 (3)	N009—C00K—S002	115.5 (9)
Cl05—Cu01—Cl03	103.90 (16)	C00E—C00K—S002	125.1 (11)
C00P—S002—C00K	88.2 (7)	C00H—C00L—C00N	120.3 (13)
C2—S004—C1	88.5 (7)	C00H—C00L—H00L	119.9
C00H—O006—C00S	117.5 (11)	C00N—C00L—H00L	119.9
C00C—O007—C00Q	117.1 (11)	C00F—C00M—C00J	122.3 (13)
C00E—N008—C00J	118.0 (11)	C00F—C00M—H00M	118.9
C00E—N008—Cu01	122.1 (8)	C00J—C00M—H00M	118.9
C00J—N008—Cu01	119.6 (9)	C00I—C00N—C00L	120.8 (13)
C00K—N009—C00O	109.8 (12)	C00I—C00N—H00N	119.6
C00K—N009—Cu01	113.8 (8)	C00L—C00N—H00N	119.6
C00O—N009—Cu01	136.3 (10)	N009—C00O—C00P	114.7 (13)
C1—N1—C3	111.1 (12)	N009—C00O—H00O	122.7
C1—N1—Cu01	115.1 (9)	C00P—C00O—H00O	122.7
C3—N1—Cu01	133.7 (10)	C00O—C00P—S002	111.9 (11)
C00E—C00B—C00F	121.0 (12)	C00O—C00P—H00P	124.1
C00E—C00B—H00B	119.5	S002—C00P—H00P	124.1
C00F—C00B—H00B	119.5	O007—C00Q—H00A	109.5
C00H—C00C—O007	116.5 (12)	O007—C00Q—H00C	109.5
C00H—C00C—C00G	117.9 (13)	H00A—C00Q—H00C	109.5
O007—C00C—C00G	125.6 (13)	O007—C00Q—H00D	109.5
N1—C1—C00J	117.0 (11)	H00A—C00Q—H00D	109.5
N1—C1—S004	115.8 (11)	H00C—C00Q—H00D	109.5
C00J—C1—S004	127.1 (10)	N1—C3—C2	111.5 (13)
C00B—C00E—N008	121.8 (11)	N1—C3—H3	124.3
C00B—C00E—C00K	132.1 (13)	C2—C3—H3	124.3
N008—C00E—C00K	106.0 (12)	O006—C00S—H00E	109.5
C00M—C00F—C00B	117.5 (11)	O006—C00S—H00F	109.5
C00M—C00F—C00I	123.0 (12)	H00E—C00S—H00F	109.5
C00B—C00F—C00I	119.4 (12)	O006—C00S—H00H	109.5
C00I—C00G—C00C	122.9 (13)	H00E—C00S—H00H	109.5

C00I—C00G—H00G	118.6	H00F—C00S—H00H	109.5
C00C—C00G—H00G	118.6	C3—C2—S004	113.2 (12)
C00C—C00H—O006	116.3 (13)	C3—C2—H2	123.4
C00C—C00H—C00L	121.2 (13)	S004—C2—H2	123.4

3.7 Plasma re-calcification time

To detect the role of synthesised ligand and its complex on blood coagulation cascade, plasma re-calcification time was analysed using fresh human plasma. Interestingly, newly synthesised ligand and its complex exhibit anticoagulant property as it enhanced the clotting time of citrated human plasma in both PRP and PPP from the 180sec to 260 sec and 220sec to 300sec respectively at the concentration of 30µg and its complex enhanced from the control 180sec to 300sec and 220sec to 340sec respectively at the concentration of 30µg this commends its anticoagulant property.

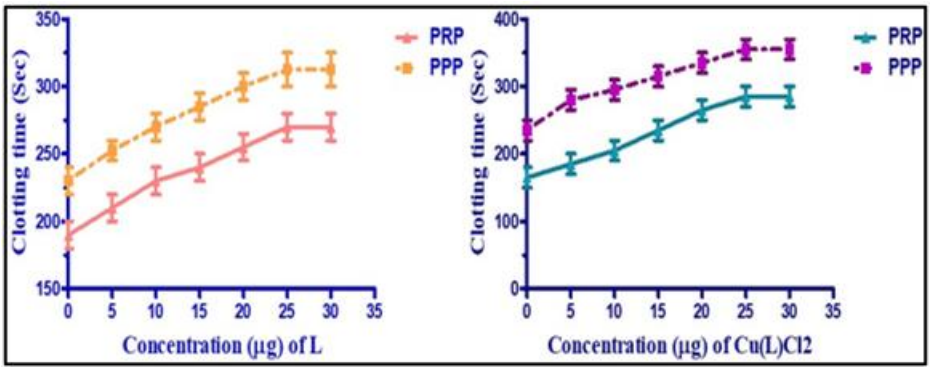


Figure 9: Plasma re-calcification time of **L** and [Cu**(L)**Cl_2]

3.8 Antiplatelet Activity

Also, to classify the probable role of ligand and its complexes on platelets, platelet aggregation assay was performed using ADP and Epinephrine as agonists. Unluckily, synthesised ligand and its complexes did not show any effect on platelets.

3.9 Free radical scavenging potentials:

Deleterious-free radical generation is the key event inprogression of many pathological conditions. Duringoxidative stress, these free radicals contribute the majorrole in inducing cell death. Therefore, **L** and[Cu**(L)**Cl_2] were evaluated for their free radial scavenging potentiality. DPPH scavenging property of **L** and[Cu**(L)**Cl_2] were evaluated, where compound **L** and[Cu**(L)**Cl_2] showedpotent radical scavenging activity when compared with positive control vitamin C Based on theseresults, **L** and[Cu**(L)**Cl_2] was considered as potent candidate as shown in the **Fig 10**.

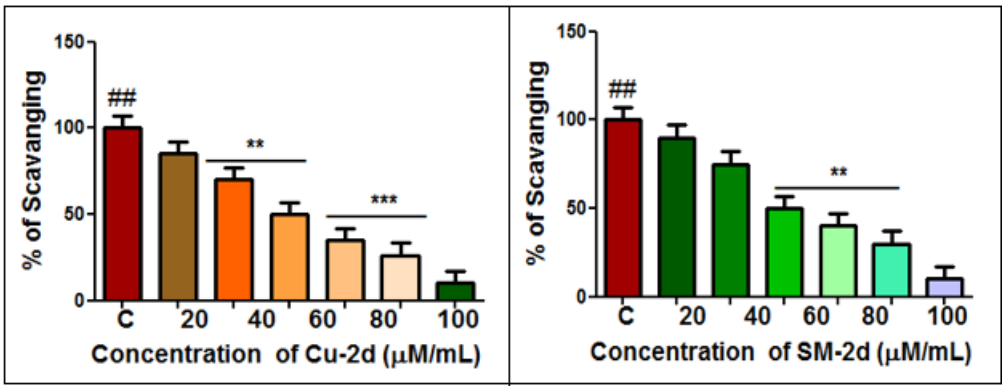


Figure 10: DPPH Scavenging of L and [Cu(L)Cl_2]

3.10Hemolytic activity

Interestingly, ligand and its complexes were non-toxic to RBC as it did not hydrolyse RBC up to the concentration of 100µg.

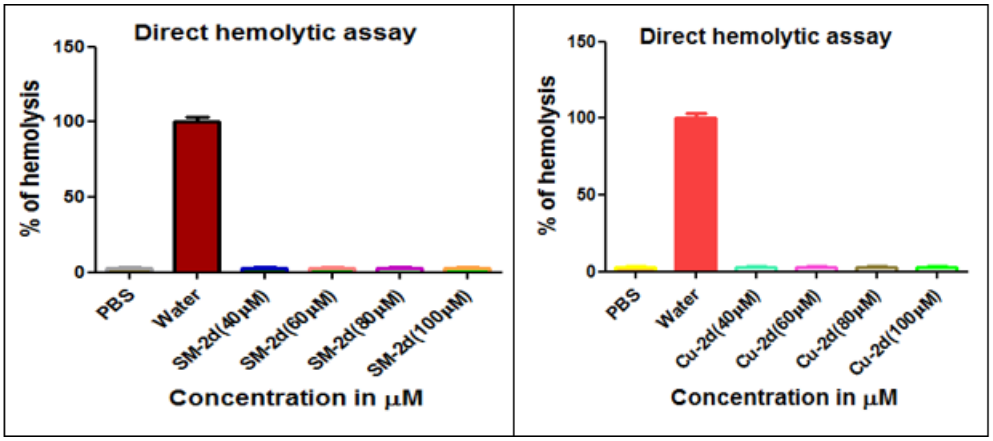


Figure 11: Hemolytic assay of L and $[Cu(L)Cl_2]$

3.11In *silico* molecular docking:

The synthesized compounds showed good binding energy and interactions with the selected proteins.

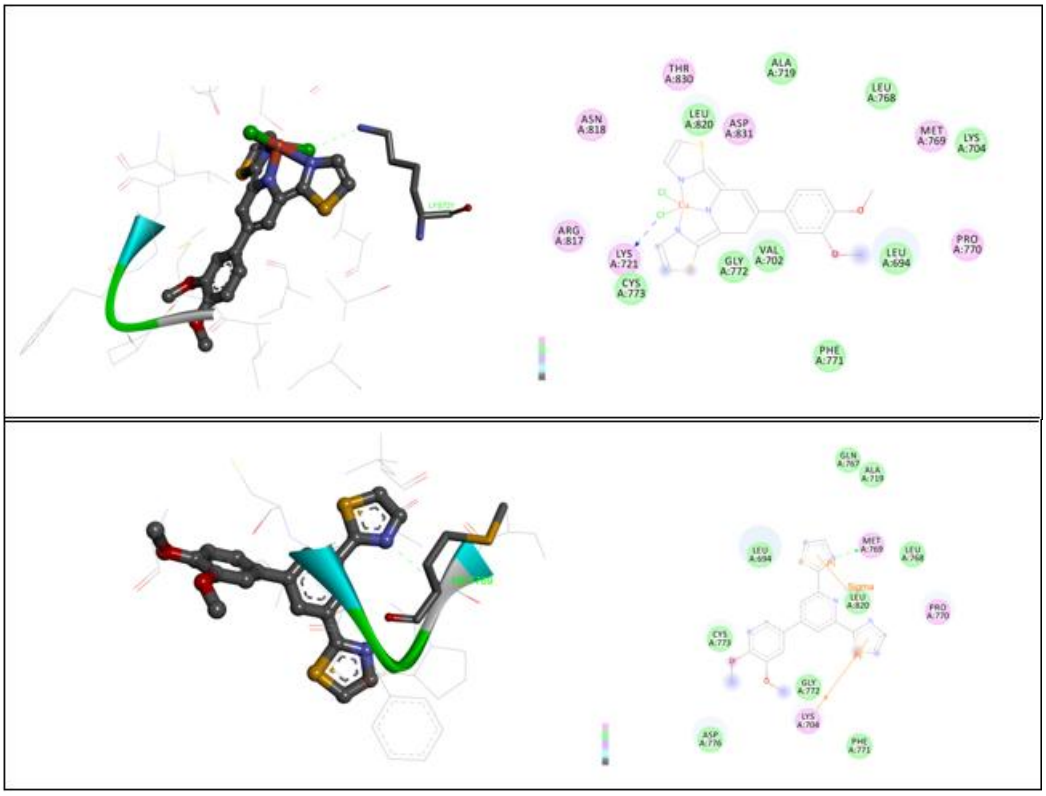


Figure 12: Binding pattern of L and $[Cu(L)Cl_2]$with target protein 2XCT

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Declaration of Conflict of Interest
The authors declared no potential conflict of interest with respect to the authorship and publication.

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