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Synthesis, Characterization, Hydrolytic Cleavage, and Biological Activity Studies of 2-[(1*e*)-*N*-{2-[(2-{(*Z*)-[1-(2-Hydroxyphenyl)Ethylidene] Amino}Ethyl)Amino]Ethyl}Ethanimidoyl]Phenol

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ABSTRACT

A Schiff base ligand 2-[(1*E*)-*N*-{2-[(2-{(*Z*)-[1-(2-hydroxyphenyl) ethylidene] amino}ethyl)amino]ethyl} ethanimidoyl]phenol L was hydrolyzed by copper cation which lead to formation of 8,8-dichloro-2H,3H,5H,6H-1,3-diaza-2-cupracyclopenta[1,3-a]1,3-diaza-2-cupracyclopentane hydrate (Complex), characterized by UV, IR, Powder XRD and by elemental analysis. In vitro antioxidant and anticoagulant, activities of L were evaluated. Antioxidant potential of L was assessed by DPPH scavenging, β-carotene bleaching test, hydroxyl radical scavenging method, ABTS radical scavenging test, and by reducing power test. In vitro anticoagulant effect of L at the 84 µg/mL; showed the maximum prolongation of plasma recalcification time which is comparable with that of the anticoagulant drug; heparin. In conclusion, results of the present investigation indicate that the ligand L can be a potential anticoagulant agent.

Keywords: Schiff base; Antioxidant; Free radicals; Anticoagulant.

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1. INTRODUCTION

Schiff bases are products that can be obtained via condensation of primary amines with carbonyl compounds and were first reported by Schiff in 1864 [1].These compounds have been extensively used as ligands in the synthesis of metal complexes [2]. In addition, Schiff bases represent an important class of organic compounds, especially in the fields of medicinal and pharmaceutical chemistry [3]. In fact, many Schiff bases display interesting pharmacological activities such as antibacterial, antifungal, antimalarial, antimicrobial, and anticancer activity [4]. Furthermore, their transition-metal complexes were found to have interesting bioactivity and play a role as potent drugs in the area of pharmacology [5]. Literature survey reveals that zinc(II) complex with 4-amino-1*H*-pyrimidin-2-one, diacetyl and glycine Schiff base showed a higher activity against *C. parapsilosis* [6].On the other hand, Schiff bases are used for colorimetric detection of Al^{3+}, these detections are very important for human health [7]. Furthermore, Schiff bases with electron donors (N, O, S…) have structural similarities with neutral biological systems and due to the presence of an imine (C=N) group, they can be used in elucidating the mechanism of transformation of rasemination reaction in biological system [8].

Several research groups have been involved in the synthesis and biological screening of Schiff bases [9]. Hranjec et al. 2011 [10] prepared a series of novel benzimidazole Schiff bases as antiproliferative agents. In addition, Ronad [11] and coworkers synthesized a series of Schiff bases from 7-amino-4-methylcoumarin and benzaldehydes, and studied their anti-inflammatory and analgesic activities. These researchers found that some of the prepared compounds were active, even more potent than a reference drug. Due to the presence of phenolic groups, phenolic Schiff bases are reported as powerful antioxidants and good free radical scavengers [12]; antioxidants have the ability to convert free radicals to stable molecules. This process safeguard cells from oxidative damage which leads to aging and diseases [13],and provide protection against cancer-causing and DNA damaging radicals [14].

We have recently prepared the pentadentate ligand 2,2'-(((azanediylbis(propane-1,3-diyl))bis(azanylylidene)) bis(methanylylidene))diphenol from salicylaldehyde and *N1*-(3-aminopropyl)propane-1,3-diamine, along with its metal

complexes [15]. Additionally, we have evaluated the antioxidant and antibacterial activities of the Schiff base and its complexes in vitro. In view of the wide interest in Schiff bases, and owing to their biological importance, we have prepared the Schiff base **1** from condensation of 2-hydroxyacetophene with *N*-(2-aminoethyl)-1,3-propanediamine. The Schiff base 2-[(1*E*)-*N*-{2-[(2-{(*Z*)-[1-(2-hydroxy phenyl)ethylidene]amino}ethyl)amino] ethyl}ethanimidoyl]phenol, was characterized by a panel of spectroscopic techniques such as UV, IR, NMR, and mass spectrometry, and by elemental analysis. In addition, the antioxidant, and anticoagulant activities of this Schiff base were examined.

2. MATERIALS AND METHODS

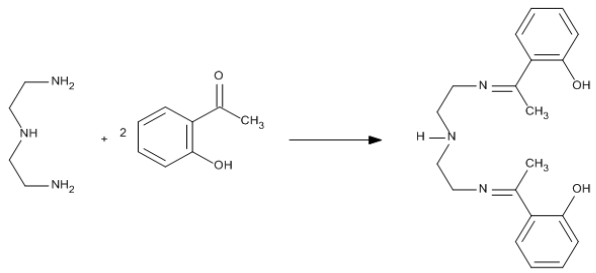
2.1. Materials

Each of the following chemicals was purchased and used without further purification: 2'-hydroxyacetophenone (Aldrich), diethylenetriamine, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, linoleic acid, butylated hydroxy toluene (BHT), and TWEEN 40 (Sigma–Aldrich). Reactions were monitored by thin layer chromatography (TLC) using silica gel plates (60 F-254, Merck); plates were visualized under UV light. Melting point was measured with a Stuart melting point apparatus. Infrared spectra (IR) were obtained, as KBr disks, on a Schimadzu FTIR-8400S spectrophotometer. The electronic absorption spectra in the 200–900 nm range were recorded on a UV-1800Schimadzu UV–visible spectrophotometer.^{1}H and ^{13}C NMR spectra were obtained with a Bruker-Avance III 500 MHz spectrometer with DMSO-$d_6$as solvents and TMS as an internal standard. Chemical shifts are expressed in δ units, and coupling constants (*J*-values) for ^{1}H-^{1}H, are given in Hertz. High resolution mass spectrum (HRMS) was acquired by electrospray ionization (ESI) technique with the aid of a Bruker APEX-2 instrument. The mass spectrum displayed the correct molecular ion peak for which the measured high resolution (HRMS) data are in good agreement with the calculated value. Elemental analysis (C, H, and N) was carried out with a EuroVector EA3000 instrument.

2.2. Methods

2.2.1. Synthesis of Schiff base ligand 2-[(1E)-N-{2-[(2-{(Z)-[1-(2-hydroxyphenyl)ethylidene]amino} ethyl)amino] ethyl}ethanimidoyl]phenol, L

The Schiff base, 2-[(1*E*)-*N*-{2-[(2-{(*Z*)-[1-(2-hydroxyphenyl)ethylidene]amino}ethyl) amino]ethyl}ethanimidoyl]phenol (**L**), (**Scheme1**) was prepared according to a procedure outlined by Charef et al. [16], which involved reaction of diethylenetriamine (1.032 g, 10.0 mmol) with 2'-hydroxyacetophenone (2.72 g, 20 mmol) in 100 mL of absolute ethanol. The mixture was refluxed for 3h at 50 °C, with stirring. After cooling, the mixture was concentrated under reduced pressure to afford the desired product as a yellow solid, which was filtered and dried in vacuum.

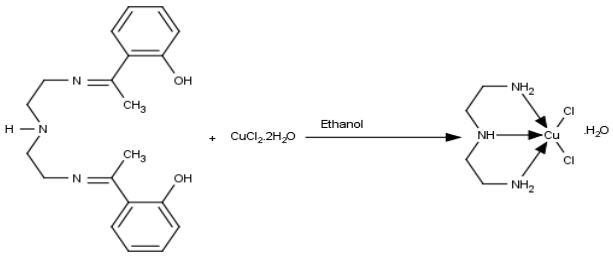


Scheme1. Synthesis of Schiff base **L**.

Yield 98%. M.p.: 93-94 °C. UV–Vis (EtOH) λ_{max} (nm) 321, 389. IR (KBr, cm^{-1}): ν_{max}: 3453 (br, OH), 1616 (C=N). ^{1}H-NMR (500 MHz, DMSO-*d6*) δ(ppm): 2.33 (s, 3H, CH_3), 2.88 (t, *J* = 13.0 Hz, 2H), 3.61 (t, *J* = 12.6 Hz, 2H), 6.69 (dd, *J* = 24.9, 16.8 Hz, 2H), 7.21 (t, *J* = 14.9 Hz, 1H), 7.57 (d, *J* = 16.2 Hz, 1H,), 16.49 (s, 1H, OH). ^{13}C-NMR (75 MHz, DMSO-*d6*) δ(ppm): 14.8(CH_3), 49.3 (CH_2), 49.9 (CH_2), 116.7 (CH), 118.8 (CH), 119.2 (C), 129.2 (CH), 132.8 (CH), 164.9 (C), 173.2 (C).HRMS (EIMS) *m/z*: calcd. for $C_{20}H_{26}N_3O_2$ [M + H]$^{+}$340.20250, found 340.20308. Anal.Calcd for $C_{20}H_{25}N_3O_2$: C, 70.77; H, 7.42; N, 12.38. Found: C, 70.69; H, 7.38; N, 12.32%.

2.2.2. Hydrolysis cleavage of 2-[(1E)-N-{2-[(2-{(Z)-[1-(2-hydroxyphenyl) ethylidene]amino} ethyl)amino] ethyl}ethanimidoyl]phenol, L

8,8-dichloro-2H,3H,5H,6H-1,3-diaza-2-cupracyclopenta[1,3-a]1,3-diaza-2-cupracyclopentane hydrate (Complex) was formed from the complete hydrolysis of the ligand L, which involved reaction of L (0.002mol, 0.68g) and $CuCl_2.2H_2O$ (0.002mol, 0.34g) in 50 mL of absolute ethanol, the reaction mixture was refluxed for 24h at 75°C. After cooling the crystalline powder was washed several times with absolute ethanol and diethyl ether and dried in vacuum **(Scheme 2)**.



Scheme 2. Hydrolysis cleavage of the ligand L

Yield 76%. UV–Vis (EtOH) λ_{max} (nm) 247. IR (KBr, cm^{-1}): ν_{max}: 3276-3243 (br, NH_2), Anal.Calcd for $C_4H_{15}Cl_2CuN_3O$: C, 18.79; H, 5.91; N, 16.44. Found: C, 19.53; H, 5.54; N, 16.82%.

2.3. In vitro *antioxidant activity*

2.3.1. DPPH radical scavenging activity

A methanolic solution of **L** or BHT at different concentrations (500 µL) was mixed and incubated in the dark for 30 min with 500 µL of DPPH solution (0.25 mM), and the absorbance was then measured at 517 nm [17]. The DPPH radical scavenging activity of **L** was calculated using the following equation:

$$\text{Scavenging activity (\%)} = 100x(A0 - A1) / A0$$

A_0 is the absorbance of the blank and A_1 the absorbance of **L** or of butylated hydroxytoluene (BHT) used a standard.

2.3.2. B-carotene/linoleic acid bleaching method

Four milligrams of β-carotene was dissolved in 2 mL of chloroform and placed in a 100-mL round-bottom flask. After chloroform evaporation, 25 µL of linoleic acid, 200 mg of Tween 40, and100 mL oxygenated water were added to the flask with vigorous shaking. Aliquots (2.5 mL) of this emulsion were added to 350 µL of tested compounds (2 mg/mL). The absorbance was measured at 470 nm, each 15 minute for 2h. Inhibition of lipid peroxidation was calculated from the equation [18];

$$\text{Inhibition (\%)} = (\text{Asample} / \text{ABHT}) \times 100$$

A_{sample} is the absorbance of the compound and A_{BHT} is the absorbance of BHT (used as a standard).

2.3.3. Hydroxyl scavenging activity

A reaction mixture (1.1mL) containing 500 µL of $FeSO_4$ (1.5 mM), 350 µL of H_2O_2 (6 mM), 150 µL of sodium salicylate (20 mM) and 100 µL of different concentrations of the tested compound was made. The mixture was incubated at 37ºC for 30 min and the absorbance was measured at 562 nm. The percentage scavenging effect was calculated from the formulae: Scavenging activity (%) = $[1 - (A1 - A2) / A0] \times 100$, where $A_0$is the absorbance of the control (without sample), $A_1$is the absorbance in the presence of the sample, and $A_2$is the absorbance without sodium salicylate. Ascorbic acid was employed as astandard[19].

2.3.4. ABTS radical scavenging activity

An aqueous solution of ABTS (7 mM) was mixed with ($K_2S_2O_8$) solution (2.45 mM), and the mixture was incubated at room temperature in the dark for 16 h. The mixture was diluted with ethanol to obtain an absorbance of 0.750 ± 0.025 at 734 nm. Then, 2 mL of this solution was added to 20 µL of **L** solution at different concentrations. After 30 min of incubation, the $ABTS^{\bullet+}$ radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = (1 - AS / AC) \times 100$$

Where A_C is the initial concentration of the $ABTS^{\bullet+}$ and A_S is absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of **L** or BHT [20].

2.3.5. Reducing power test

To 200µL of sample solution at different concentrations, 200 µL of 0.2 M phosphate buffer (pH 6.6) and 200 µL of potassium ferricyanide (1%) were added. After incubation for 20 min at 50ºC, 200 µL of trichloroacetic acid (10%) were added, and the reaction mixture was centrifuged for 10 min at 1000 rpm. An amount of 200µL of supernatant was mixed with 200µL of distilled water and 40 µL of ferric chloride (0.1%), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power [21],[22].

2.4. Anticoagulant activity by plasma recalcification time

Blood was freshly collected from normal individuals and mixed with 3.8% trisodium citrate in a volume ratio of 9:1.A volume of 200µLof plasma was added to 100µL of **L** solution of different concentrations and 300 µL of $CaCl_2$ (25 mM), and the mixture was incubated at 37ºC in a water bath. The time of clot formation was recorded with a stopwatch [23]. The anticoagulant drug; Heparin was used as standard.

3. RESULTS AND DISCUSSION

3.1. Characterization

The Schiff base **L** has been prepared according to a procedure published by Charef et al. [16] which involve condensation of diethylenetriamine with 2'-hydroxyacetophenone in a 2:1 M ratio. The reaction gave rise to the desired Schiff base 2-[(1*E*)-*N*-{2-[{2-{(*Z*)-[1-(2-hydroxyphenyl) ethylidene]amino}ethyl)amino] ethyl}ethanimidoyl]phenol (**L**) as a yellow solid in 98%. M.p. 93-94 ºC (lit.105ºC). R_f = 0.88 (ethyl acetate: ethanol, 7:3 was used as mobile phase). The prepared Schiff base has been characterized by elemental analysis and by a panel of spectroscopic techniques such as Ft-IR, ^{1}H- and ^{13}C-NMR, and mass spectrometry. The data, detailed in the experimental section are consistent with the suggested structure. Thus, the mass spectra display the correct molecular ion peak for which the measured high resolution (HRMS) data are in good agreement with the calculated values. DEPT experiments were used to distinguish secondary hydrogens from primary and tertiary ones. Furthermore, FT-IR and ^{1}H NMR data are similar to those reported in the literature [24].

3.1.1. IR and UV–Vis spectra

The IR spectra of the ligand L and the complex (**Figure 1**), revealed the difference between these compounds by the absence of the band of azomethine group (1616 cm^{-1}) in IR spectra of the complex, and the appears of tow bands at 3276 and 3243cm^{-1} of primary NH_2. Results indicate the complete hydrolysis of the Schiff base ligand L.

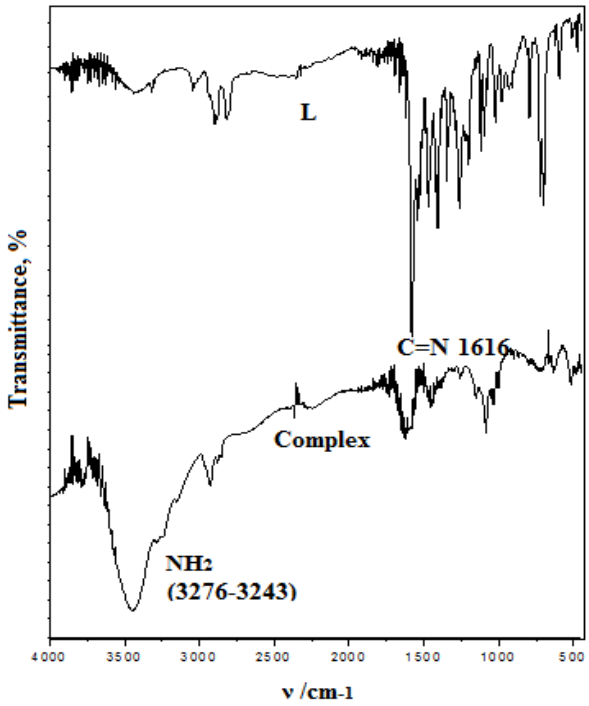


Figure 1. IR spectra of L and complex

The UV-visible spectra of **L** recorded in ethanol, showed two bands at 321 and 389 nm. The first band can be attributed to π-π^* transition within the aromatic ring, whereas the second band could be due to n-π^* transition within -C=N group. In addition, the UV-visible spectra of the complex recorded in distilled water, showed the absence of the precedents bands of aromatic ring and(-C=N) group.

3.1.2. Powder XRD

Results represented in **Figure 2** revealed XRD patterns of the complex and demonstrate the sharp crystalline peaks which confirmed crystalline phase of the complex; with 2Θ ranging from 0 to 80º.

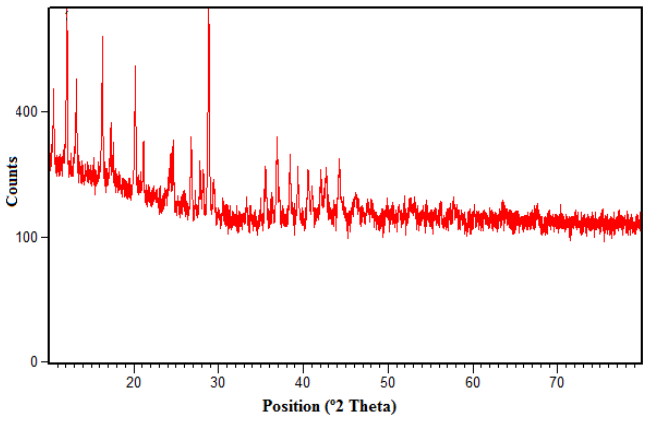


Figure 2. Powder XRD pattern of the complex

3.2. Antioxidant activity

The antioxidant activity of the synthesized Schiff base ligand was investigated in vitro by different methods and through the use of various assays. Certainly the use of various methods leads to more reliable and satisfactory results [25]. The first test was the effect of L against DPPH, which is a free radical, used as a model for measuring the antioxidant activity. The reaction between antioxidant agents and DPPH, allows conversion of latter to the stabilized form (1,1-diphenyl-2-picrylhydrazine). Stabilization of this radical indicates the hydrogen-donating capacities of antioxidants [26].Results presented in **Figure 7**; reveal that scavenging activity of **L** was concentration-dependent. The inhibition concentration of 50% (IC_{50}) of DPPH radical was 3.37 ± 0.13 $mg.mL^{-1}$ for **L** and 0.087 ± 0.001 mg/mL for BHT. In the present investigation, the presence of two hydroxyl groups at aromatic rings in the **L** molecule, leads to stabilization of the free radicals. This is in agreement with results reported in the literature which indicate that molecules with multiple OH groups attached at the aromatic ring may have higher free radical-scavenging activity [27]. In addition, the antioxidant capacity of **L** was evaluated against free radicals formed in linoleic acid oxidation. Results displayed in **Figure 8**, show that **L** can inhibit the oxidation of β-carotene after a reaction time of 2 h with inhibition percentage of 59.07%. The effect of **L** against lipid peroxidation was studied and results are in agreement those previously obtained Charef et al. [16] and by Zai-Qun [28], who showed the importance of hydroxyl-substituted Schiff bases in the protection against radical-induced peroxidation of triolein in micelles, hemolysis of human red cells, and oxidation of DNA.

The powerful reactivity of hydroxyl radicals causes higher interaction with biomolecules and can easily cross biomembranes and causes cell damage [29]. Detection of OH• is achieved by the hydroxylation of salicylate in the system of Fenton reaction (presence of $FeSO_4$ and H_2O_2). Displayed in **Figure 9** are results related to the scavenging activity of **L** against hydroxyl radicals, with an IC_{50} of 116.52µg/mL. Results agree with previous studies. [30], which proved that good electron donors may accelerate the conversion of H_2O_2 to H_2O. The presence of active hydrogen (in a phenolic group) could be responsible for the scavenging activity of **L**. On the other hand, the ABTS radical cation assay was used to confirm the free radical-scavenging activity. Results showing the ABTS radical scavenging activity of **L**, expressed as percent inhibition (I%), are displayed in **Figure 10**. These results reveal significant scavenging activity of **L** with an IC_{50}= 22.8 µg/mL, where IC_{50} of the standard (BHT) is 10.74 µg/mL.

To evaluate the reductive capacity of the Schiff base **L**, the effect toward the transformation of Fe^{3+} to Fe^{2+} was investigated. Results that express the reducing power of the prepared Schiff base are shown in **Figure 11**. Results indicate low capacity of **L** for $Fe^{3+} \rightarrow Fe^{2+}$ transformation with an IC_{50} of 3.02 mg/mL. In this assay, the reducing power of a tested substance may serve as a significant indicator for its potential antioxidant activity [31]. All of these results, confirmed the reducing character of the ligand L predicted by the theoretical calculations.

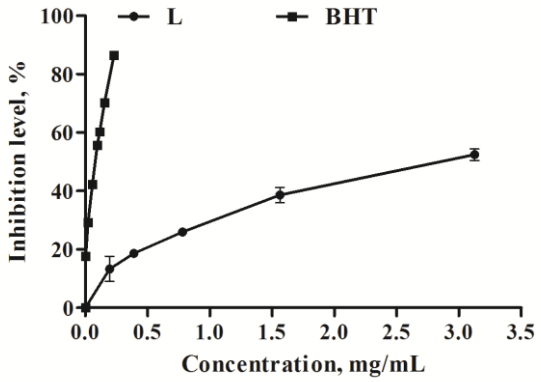


Figure 7. DPPH radical scavenging activity of **L** and BHT.

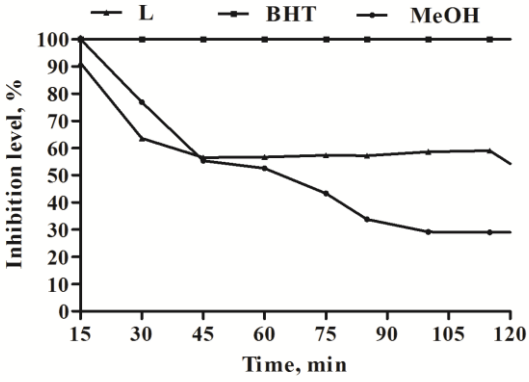


Figure 8. Kinetics of bleaching of β-carotene at 470 nm of **L**, BHT and methanol during 2 h.

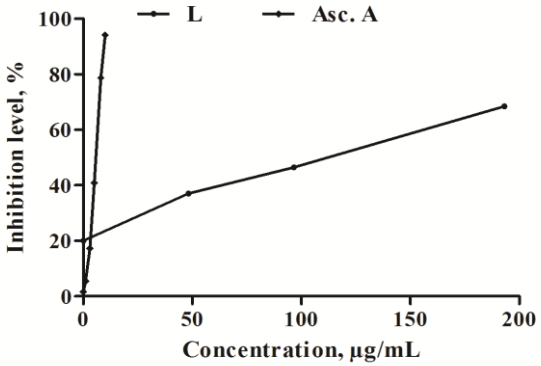


Figure 9. OH• scavenging activities of **L** and ascorbic acid.

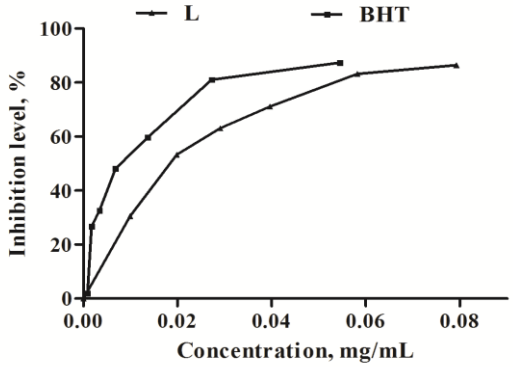


Figure 10. Radical scavenging activity **L** and BHT against ABTS.

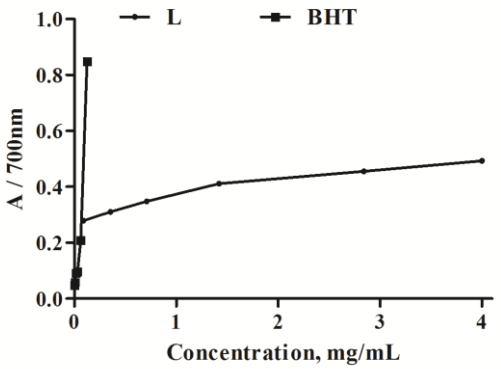


Figure 11. Reducing power assay of **L** and BHT.

3.3. Anticoagulant activity

Anticoagulant agents play an important role in the prevention and the treatment of thromboembolic disorders [32]. An investigation by Sawant et al. [33] showed that Schiff bases display anticoagulant activity. The effect of **L** on plasma recalcification time is depicted in **Figure 12**. Results show the ability of **L** to prolong the time of coagulation in plasma. The normal recalcification time was 5.53 min; the recalcification time was prolonged to 12.23 min with the increase of concentration (84 µg/mL), which is comparable with that the anticoagulant drug (heparin 15.13 min at 38.13 µg/mL). Our results, are in good agreement with those of Sawant et al. [33], who found that most synthesized Schiff bases derived from 1,3-Oxazines exhibit significant anticoagulant activity by measuring the time taken for the clot to form. Our results show that **L** increases the time of recalcification in plasma, which explains the higher anticoagulant activity of **L.**

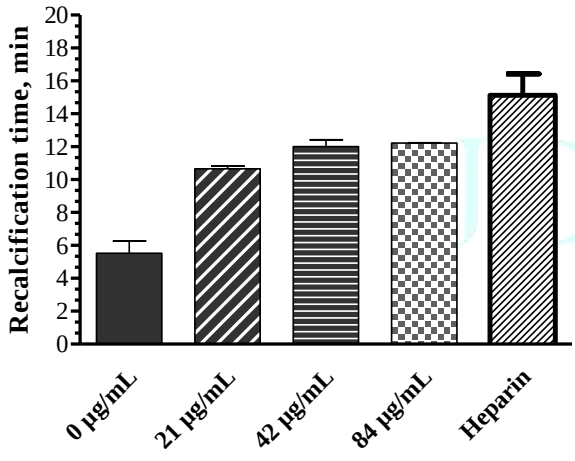


Figure 12. Effect of **L** and **Heparin** on plasma recalcification time

4. CONCLUSIONS

A Schiff base, **L**, derived from diethylenetriamine and 2'-hydroxyacetophenone was synthesized and characterized. **L** was hydrolyzed by copper cation. The antioxidant properties of **L** were evaluated by several methods such as DPPH, β-Carotene bleaching assay, hydroxyl radical scavenging, ABTS radical scavenging, and reducing power. Results revealed that **L** has important antioxidant activities. Furthermore, **L** displayed significant anti-recalcification activity. Results obtained in the present studies indicate that the ligand **L** can at least be a potential antioxidant and anticoagulant agent.

ACKNOWLEDGMENTS

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