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**EXPERIMENTAL BIOLOGICAL STUDIES OF NOVEL TETRADENTATE**

**HYDRAZONE Cu(II) COMPLEXES FOR POTENTIAL APPLICATIONS**

**IN MEDICINAL CHEMISTRY**

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A series of mononuclear Cu(II) complexes were synthesized using a potential tetradentate hydrazone ligand obtained from the reaction between phenylhydrazine groups and 2,5-thiophenedicarboxaldehyde. The structures of ligands and complexes were elucidated through various spectroscopic techniques, confirming their composition. All complexes were found to adopt four-coordinated geometries, indicating the formation of stable structures. Spectroscopic analysis revealed that the hydrazone ligand coordinated with the Cu(II) metal ions as a dibasic tetradentate ligand by utilizing the phenolic oxygen and azomethine nitrogen atoms. The binding affinity of the complexes with calf thymus DNA (CT-DNA) was investigated using absorption and viscosity measurements, demonstrating their interaction through the intercalation mode. The binding studies showed that the Cu(II) complexes exhibited varying degrees of binding affinity, with Cu(L4) demonstrating the highest affinity, followed by Cu(L1), Cu(L2), and Cu(L3). Moreover, the DNA fragmentation properties of the Cu(II) complexes were evaluated, suggesting their potential utilization in pharmaceutical applications. The obtained results highlight the significance of these novel complexes in the field of medicinal chemistry.

**Keywords:** heterocyclic hydrazones; Cu(II) complex; DNA interactions; topoisomerase I inhibition

**ЕКСПЕРИМЕНТАЛНИ БИОЛОШКИ СТУДИИ НА НОВИ ТЕТРАДЕНТАТНИ ХИДРАЗОНСКИ**

**КОМПЛЕКСИ НА Cu(II) ЗА ПОТЕНЦИЈАЛНИ АПЛИКАЦИИ ВО МЕДИЦИНСКАТА ХЕМИЈА**

Серија мононуклеарни комплекси на Cu(II) беа синтетизирани со користење на потенцијален тетрадентатен хидразонски лиганд добиен од реакцијата помеѓу фенилхидразинските групи и 2,5-тиофендикарбоксалдехид. Структурите на лигандите и на комплексите беа разјаснети преку различни спектроскопски техники, потврдувајќи го нивниот состав. Утврдено е дека сите комплекси имаат четири координирани геометрии, што укажува на формирање стабилни структури. Спектроскопската анализа откри дека лигандот хидразон е координиран со јоните на металот Cu(II) како двобазен тетрадентатен лиганд преку кислородот на фенол и азотот на азометин. Сврзувачкиот афинитет на комплексите со DNA беше испитуван на тимусот на теле (CT-DNA) со помош на мерења на апсорпцијата и вискозноста, демонстрирајќи ја нивната интеракција преку режимот на интеркалација. Студиите за сврзување покажаа дека комплексите на Cu(II) покажуваат различен степен на сврзувачки афинитет, при што Cu(L4) покажа највисок афинитет, следат Cu(L1), Cu(L2) и Cu(L3). Покрај тоа беа оценети својствата на фрагментацијата на DNA на комплексите на Cu(II) што укажа на нивно потенцијално користење во фармацевтски апликации. Добиените резултати го истакнуваат значењето на овие нови комплекси во областа на медицинската хемија.

**Клучни зборови**: хетероциклични хидразони; комплекс на Cu(II); интеракции на DNA; инхибиција на топоизомераза I

1. INTRODUCTION

Heterocyclic compounds hold significant importance in the fields of medicinal chemistry and bioinorganic chemistry due to their profound biological effects. Particularly, heterocyclic compounds containing atoms such as sulfur, nitrogen, and oxygen have garnered the attention of researchers, primarily due to their pharmacological profiles.1–3 Ongoing studies in this area aim to develop novel therapeutic agents for challenging diseases, including cancer. Among the various heterocyclic structures, thiophene, characterized by its sulfur group, has emerged as a promising candidate with potential anticancer properties. Literature reports have highlighted the ability of thiophene derivatives to target and bind to proteins specific to different types of cancer, with binding properties contingent upon the positioning within the molecule.4 Furthermore, transition metal complexes of these heterocyclic structures have received considerable attention due to their coordination chemistry and beneficial pharmacological attributes.

The presence of heterocyclic compounds in fundamental biomolecules such as DNA, RNA, and proteins underscores the significance of synthetic analogues derived from these compounds in biological systems. This observation emphasizes their role in the design and development of therapeutic agents.5 This has led to the synthesis of novel thiophene derivatives and their corresponding metal complexes, each tailored with unique structural properties to target specific molecular entities in the pursuit of potential anticancer agents.3

In light of the aforementioned considerations, this study focuses on the synthesis of new hydrazone ligands capable of providing a potential tetradentate (N2O2) chelation system, as well as the corresponding Cu(II) coordination compounds based on these ligands. Comprehensive characterizations of the synthesized ligands and complexes were conducted utilizing various spectroscopic techniques. In addition, the biological activities of these newly synthesized compounds, including DNA-binding, DNA-cleavage, and inhibition of the topoisomerase I enzyme, were investigated to elucidate the structure-activity relationships.

2. MATERIALS AND METHODS

Solvents and chemicals used in the study were obtained commercially from Merck and Sigma-Aldrich. Solvents used in synthesis and measurements were freshly distilled and dried. pBR322 plasmid DNA was obtained from Fermentas. TopoGen Inc. provided DNA topoisomerase I (topo I). On a LECO 932 CHNS analyzer, microanalysis (C, N, and H) was done, and copper content was determined using atomic absorption spectroscopy on a DV 2000 Perkin Elmer ICP-AES. Attenuated total reflectance (ATR) method coupled with a Fourier transform infrared (FTIR) spectrometer (Thermo-Scientific Nicolet iS10) was used and spectra were acquired on pure solid materials from 4000 to 400 cm–1. A Sherwood Scientific MK1 Model Gouy Magnetic Susceptibility Balance was used to assess magnetic susceptibility on powdered materials at room temperature. A PG Instruments T80+ UV/Vis Spectrophotometer was used to record the electronic absorbtion spectra in DNA interaction experiments. Thermogravimetric analysis was performed using a Perkin Elmer Pyris-1 TGA thermal analyzer. 4-hydroxybenzohydrazide (Ia),6 4-aminobenzohydrazide (Ib),6 salicyloylhydrazine (Ic),7 and 2-aminobenzohydrazide (Id)7 were prepared according to the reported methods.

2.1. *Synthesis*

2.1.1*. Synthesis of hydrazine ligands*

These compounds were synthesized as follows by changing the method given in the literature.8 A solution of 0.02 mol of 4-hydroxybenzohydrazide, 4-aminobenzohydrazide, salicyloylhydrazine or 2-aminobenzohydrazide in 15 ml of ethanol was added to a solution of 0.01 mol of 2,5-thiophenecarboxyaldehyde in 10 ml of ethanol. After the addition was complete, a few drops of acetic acid was added to the reaction as a catalyst and stirred under reflux for about 3 hours. The products, which became clear at first then began to precipitate as a yellow solid. The yellow precipitates formed were filtered off. The purity of the substance was checked with thin layer chromatography (TLC) and the product was purified by crystallization from an ethanol-water (1:1) mixture.



**Scheme 1.** Synthesis and proposed structure of hydrazone ligands (4-hydroxybenzohydrazide (H2L1),

4-aminobenzohydrazide (H2L2), salicyloylhydrazine (H2L3), and salicyloylhydrazine (H2L4))

***(N',N'''E,N',N'''E)-N',N'''-(thiophene-2,5-diylbis (methanylylidene))bis(4-hydroxybenzohydrazide) (H2L1):*** Yield 72%. m.p.: 300 °C. UV-Vis. (DMF, nm) 273; 365 (sh); 383; 403(sh). FTIR (ATR, cm*−*1): 3075 br (OH), 3301 w (NH), 1636 s (C=O), 1603 s (C=N), 1246 m (C–O). 1H-NMR (DMSO\_d6, ppm): *δ* 6.67 (d, 4H), 7.44 (s, 2H), 7.81 (d, 4H)**(–Ar*H*),** *δ* 8.66, (s, 2H) **(*H*C=N)**, *δ* 10.57 (s, 2H) **(O*H*),** *δ* 11.72 (s, 2H) **(N*H*)**. Analysis: (% calculated/found) forC20H16N4O4S: C, 58.81/58.92; H, 3.95/3.95; N, 13.72/13.25.

***(N',N'''E,N',N'''E)-N',N'''-(thiophene-2,5-diylbis (methanylylidene))bis(4-aminobenzohydrazide) (H2L2):*** Yield 70%. m.p.: 295 °C. UV-Vis. (DMF, nm) 270; 392.5; 410.5 (sh). FTIR (ATR, cm*−*1): 3216 w (NH2), 3317 w (NH), 1652 s (C=O), 1598 s (C=N), 1259 m (C–O). 1H -NMR (DMSO\_d6, ppm): *δ* 5.80 (S, 4H) **(–N*H2*)**, *δ* 6.59(d, 4H), 7.39 (s, 2H), 7.66 (d, 4H) **(–Ar*H*),** *δ* 8.62, (s, 2H) **(*H*C=N)**, *δ* 11.51 (s, 2H) **(N*H*)**. Analysis: (% calculated/found) for C20H18N6O2S; C, 59.10/59.12; H, 4.46/4.45; N, 20.68/20.75.

***(N',N'''E,N',N'''E)-N',N'''-(thiophene-2,5-diylbis (methanylylidene))bis(2-hydroxybenzohydrazide) (H2L3):***Yield 73%. m.p.: 259 °C. UV-Vis. (DMF, nm) 270.5; 304.5. FTIR (ATR, cm*−*1): 3072 br (OH), 3202 w (NH), 1660 s (C=O), 1604 s (C=N), 1288 m (C–O). 1H -NMR (DMSO\_d6, ppm): *δ* 6.98(q, 4H), 7.45 (t, 2H), 7.52 (s, 2H), 7.85 (d, 2H) **(–Ar*H*),** *δ* 8.66, (s, 2H) **(*H*C=N)**, *δ* 11.73 (s, 2H) **(O*H*),** *δ* 11.92 (s, 2H) **(N*H*)**. Analysis: (% calculated/found) for C20H16N4O4S: C, 58.81/58.82; H, 3.95/3.90; N, 13.72/13.78.

***(N',N'''E,N',N'''E)-N',N'''-(thiophene-2,5-diylbis (methanylylidene))bis(2-aminobenzohydrazide) (H2L4):*** Yield 71%. m.p.: 274 °C. UV-Vis. (DMF, nm) 270.5; 386; 407.5. FTIR (ATR, cm*−*1): 3309 w (NH2), 3398 w (NH), 1651 s (C=O), 1610 s (C=N), 1248 m (C–O). 1H-NMR (DMSO\_d6, ppm): *δ* 6.38 (s, 4H) **(–N*H2*)**, *δ* 6.57 (t, 2H), 6.74 (d, 2H), 7.20 (t, 2H), 7.42 (s, 2H), 7.53 (d, 2H) **(–Ar*H*),** *δ* 8.56, (s, 2H) **(*H*C=N)**, *δ*11.66 (s, 2H) **(N*H*)**. Analysis: (% calculated/found) for C20H18N6O2S: C, 59.10/59.20; H, 4.46/4.55; N, 20.68/20.79.

2.1.2*. Synthesis of Cu(II) complexes*

A solution of 0.01 mol copper(II) acetate dihydrate in ethanol was added dropwise to a suspension of 0.01 mol of hydrazone ligand in ethanol. The resulting mixture was stirred under reflux for about 1 hour, then cooled and filtered. It was left to dry after washing with alcohol and water. The complexes were crystallized with the N, N-dimethylformamide (DMF) ether.

***For Cu(L1)***: C20H16CuN4O4S, Brown complex. Yield: 65 %; m.p.: > 350 °C. µeff = 1.70 B.M.; UV-Vis (DMF, nm) 291.5; 309.5 (sh); 322.5 (sh); 366.5 (sh); 386.5; 406; 429 and 455. FTIR (ATR, cm*−*1) 3162 w and br (OH), 1600 – 1590 m (C=N–N=C), 1168 w (C−O). Analysis (% calculated/found) for C20H16CuN4O4S C: 50.90/50.82, H: 3.42/3.44, N: 11.87/11.98, Cu: 13.46/13.43.

***For Cu(L2):*** C20H18CuN6O2S, Brown complex. Yield: 70 %; m.p.: > 350 °C. µeff = 1.70 B.M.; UV-Vis. (DMF, nm) 298.5; 331; 365.5; 372.5 (sh); 389.5; 413; 440 and 465.5. FTIR (ATR, cm*−*1) 3222 w and br (NH2), 1603 m (C=N–N=C), 1177 w (C−O). Analysis (% calculated/found) for C20H18CuN6O2S, C: 51.11/51.17, H: 3.86/3.90, N: 17.88/17.98, Cu: 13.52/13.50.

***For Cu(L3):*** C20H16CuN4O4S, Brown complex. Yield: 67 %; m.p.: > 350°C. µeff = 1.69 B.M.; UV-Vis. (DMF, nm) 275.5; 334.5; 387 (sh) 419.5 and 443.5. FTIR (ATR, cm*−*1) 3060 w and br (OH), 1621 – 1599 m (C=N–N=C), 1248 w (C−O). Analysis (% calculated/found) for C20H16CuN4O4S, C: 50.30/50.28, H: 3.42/3.42, N: 11.87/11.88, Cu: 13.46/13.52.

***For Cu(L4):*** C20H18CuN6O2S, Brown complex. Yield: 68 %; m.p.: > 350 °C. µeff = 1.72 B.M.; UV-Vis. (DMF, nm) 274; 367 (sh); 395.5; 413 (sh) and 465 (sh). FTIR (ATR, cm*−*1) 3307 (NH2), 1610 m (C=N–N=C), 1158 w(C−O). Analysis (% calculated/found) for C20H18CuN6O2S, C: 51.11/51.15, H: 3.86/3.80, N: 17.88/17.79, Cu: 13.52/13.40.



**Scheme 2.** Synthesis and proposed structure of Cu(II) complexes

2.2. *Biological studies*

2.2.1. *DNA binding studies*

The complexes DNA binding ability was assessed using UV-Vis and fluorescence spectroscopy with minor modifications to previously published protocols.9–11 Titrations were conducted at room temperature using a buffer containing 10 mM Tris-HCl and 50 mM NaCl (pH 7.6). The complex stock solution was prepared using 1% (V/V) DMF and 99% (V/V) Tris-HCl buffer. Lyophilized CT-DNA was dissolved in Tris-HCl buffer overnight at 4 °C and used within 2 days as a DNA stock solution. The UV absorbance ratio at 260 and 280 nm for the DNA solution was approximately 1.8–1.9 : 1, indicating minimal protein contamination. DNA concentration was determined from the absorbance at 260 nm using the molar absorption coefficient (6600 M−1 cm−1) at 260 nm. 9–11

2.2.1.1. *UV-Visible absorption studies*

Absorption titrations were performed by adding increasing concentrations of CT-DNA (0-100 µM) to a constant complex concentration (100 µM). The reaction mixture was incubated for 5 minutes before recording the absorption spectrum. The intrinsic binding constant (*K*b) of the complex was calculated using the Wolfe-Shimmer equation. 9–11

2.2.1.2. *Viscosity measurements*

Viscosity experiments were conducted at room temperature using an Ubbelodhe viscometer. Viscosity values were presented as (η/η0)1/3 versus the ratio of complex to DNA concentrations ([complex]/[DNA]). The obtained results are presented comparatively with 4',6-diamidino-2-phenylindole (DAPI) and ethidium bromide (ETB). 9–11

2.2.2.*DNA cleavage studies*

Plasmid DNA (pBR322) cleavage by the complex was analyzed using agarose gel electrophoresis. Optimal cleavage conditions were determined by incubating the reaction mixture at 37 °C for 1-6 hours with periodic intervals in the presence and absence of H2O2 (5 mM). Gel electrophoresis was performed at 80 V for 1.5 hours, and UV light was used to visualize the DNA bands.9–11

2.2.3.*Topoisomerase Inhibition assays*

Topoisomerase I (Topo I) relaxation assays were carried out according to the manufacturer's instructions (TopoGEN). The reaction mixture containing pHOT1 plasmid DNA was incubated with different concentrations of the complex, and Topo I activity was stopped with gel loading "stop" buffer. Gel electrophoresis was performed at 45 V for 3 hours, and the gel was stained with ethidium bromide for visualization.9–11

3. RESULTS AND DISCUSSION

3.1. *Chemistry*

According to the 1H NMR spectra of the ligands, the imino protons showed low field signals at δ 11.51 – 11.92 ppm, confirming the presence of the hydrazone ligands in the keto form. Another HC=N peak proving hydrazone formation in ligands was seen at δ 8.56 – 8.66 ppm. Protons in the aromatic ring, another of the common peaks in hydrazone ligands, appeared as doublets, triplets and singlets, usually in the range of δ 6.57 – 7.85 ppm, depending on the positions of –OH and –NH2 groups in the structure. Thiophene ring protons from the hydrogens in this aromatic ring exhibited a singlet signal at about δ 7.39 – 7.52 ppm. While peaks of H2L1 and H2L2 ligands with –OH group in their structure from hydrazone derivatives are seen as singlets at δ 10.57 and 11.73 ppm, peaks of H2L3 and H2L4 ligands with –NH2 group appear as broad singlets at δ 5.80 and 6.38 ppm as expected.12,13

In the FTIR spectra of the Cu(II) complexes of the compounds, the amide I band and the carbonyl band disappeared, and these peaks were replaced by new peaks, which likely belong to the >C=N–N=C< stretching vibrations and appeared at 1600 cm–1 (Cu(L1)), 1603 cm–1 (Cu(L2)), 1599 cm–1 (Cu(L3)), and 1610 cm–1 (Cu(L4)). This indicates the conversion of the carbonyl group in the ligands to the enolic state, which is explained by keto-enol tautomerism. Then, after deprotonation, coordination of enolic oxygen to copper(II) ion occurred. Another change that explains this is that the w (N–H) peaks observed around 3300 cm–1 in the FTIR spectra of the ligands are not observed in the FTIR spectra of the copper(II) complexes. In addition, the fact that the bands resulting from the vibrations of –OH and –NH2 groups in the para- and ortho- positions of the ligands remain unchanged, suggesting that these groups are not involved in the formation of complexes. Based on the FTIR spectral data obtained, it can be stated that the bis(aroylhydrazone) ligands of the complexes behave like dianionic O,N,N,O-tetradentate ligands and there are no other side groups involved of the coordination in the structure.

While an average of two electronic transitions were observed in the electronic spectra of the ligands, four or five electronic transitions were observed in Cu(II) complexes. The absorbances occurring around 270 – 272 nm are estimated to belong to π→π\* electronic transitions. The absorbances observed around 294 nm and 365 nm belong to the n→π\* electronic transitions in the compounds. In addition, the peaks at 400 nm and above in the complexes are the peaks of the d-d charge-transfer transitions of the complexes.7,14,15

Magnetic moment values for the Cu(II) complexes at room temperature is between 1.69 – 1.72 B.M., which are compatible with the spin only value of 1.73 B.M. for one d9 copper ion.

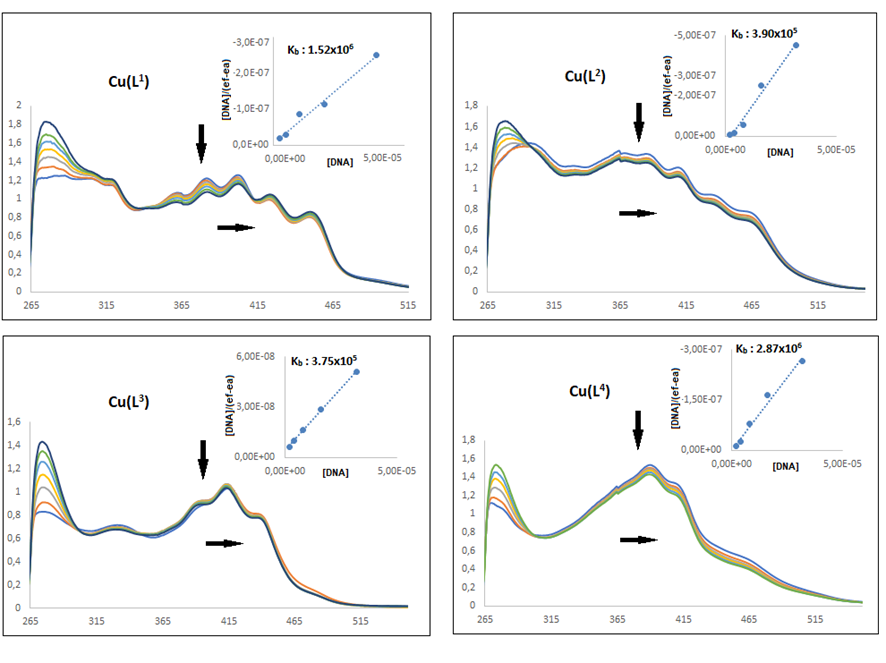
The thermogram for the all the Cu(II) complexes were recorded from 30 to 800 °C at a heating rate of 20 °C/min in a nitrogen atmosphere. The complexes decomposed completely above 900 °C, as shown in Figures S9–12. The absence of any dissociation step between 100 and 250 °C indicates the absence of any side groups inside and outside the coordination sphere.16 The decomposition step, which starts in the temperature range of about 280 – 300 °C in all complexes, may correspond to the decomposition of the part where all the complexes break down. This decomposition phase was completed around 450 °C; this corresponds to the part where the organic portion of the ligands decomposes, ultimately leaving copper oxide as residue.

3.2*. Stability*

The stability of the copper(II) complexes was examined in DMF solution for 24 and 48 hours using UV-Vis spectroscopy (Figs. S5–8). Spectra were recorded at 100 µM concentrations of the complexes. No difference was observed in the spectra of the complexes after 24 and 48 hours indicating that the complexes are stable in solution.

3.3. *DNA binding*

In order to investigate the binding interaction of new metal complexes with CT-DNA, the UV-Vis absorption spectroscopy method, which is one of the most used and effective methods, was applied. The absorption spectra of the metal complexes obtained by adding increasing concentrations of CT-DNA to the metal complexes at constant concentration are shown in Figure 1. The presence of hypochromism and bathochromism observed in the spectra of the compounds and the intercalative binding constants obtained from these graphs are related to each other. The Kb values obtained for the Cu(II) complexes were 1.52·106 M−1 (Cu(L1)), 3.90·105 M−1 (Cu(L2)), 3.75·105 M−1 (Cu(L3)), and 2.87·106 M−1 (Cu(L4)) (Table 1). As a result of the observed changes in the absorption spectrum, the Cu(II) complexes appear to be intercalated with CT-DNA.

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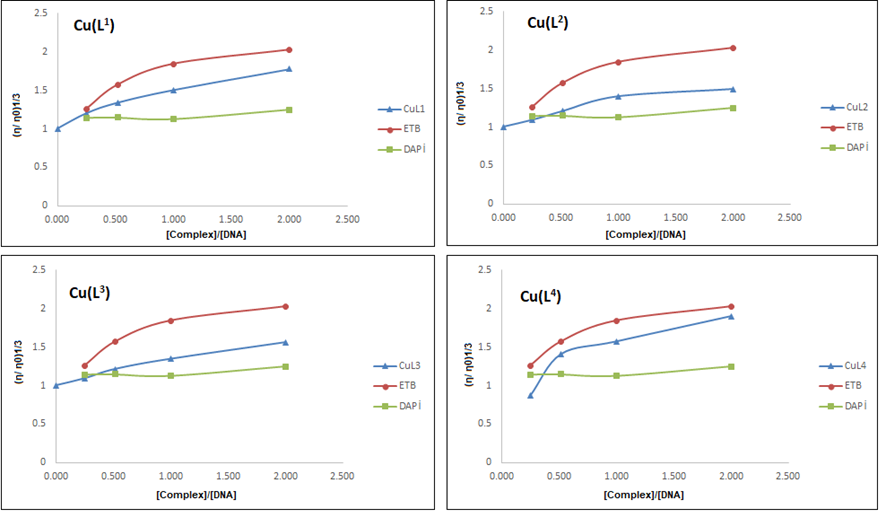
**Fig. 1.** UV-Visabsorption spectra of complexes (100 μM), upon addition of increasing amounts of CT-DNA

T a b l e 1

*The binding constant, Kb, for the intercalation of Cu(II) complexes with CT-DNA*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **λmax (nm)** | | **Δλ**  **(nm)** | **%**  **Hyp\*** | **Isobestic point**  **(nm)** | | ***K*b(M–1)** |
| **Free** | **Bound** |
| Cu(L1) | 402 | 400 | 2.0 | 7.87 | 341 | 1.52·106 | |
| Cu(L2) | 384.5 | 383 | 1.5 | 5.88 | 298 | 3.90·105 | |
| Cu(L3) | 415 | 413 | 2.0 | 5.27 | 349; 302.5 | 3.75·105 | |
| Cu(L4) | 390 | 388.5 | 1.5 | 6.80 | 305 | 2.87·106 | |
| \*Hyp, hypochromism (%Hyp = A–A0/A0) | | | | | | | |

Viscosity measurement, which is sensitive to variation in the length of DNA, is one of the most effective and simple methods of distinguishing the binding mode of DNA binding agents.17 Classical intercalation causes an increase in DNA length and thus a significant increase in the viscosity of the DNA solution. On the other hand, under the same conditions, compounds that bind only to DNA wells by partial and/or non-classical intercalation typically cause bending of the DNA helix, reducing the length of the DNA and the viscosity of the DNA solution.13,[18](https://doi.org/10.1021/bi00154a001) The viscosity of the CT-DNA was determined by measuring increasing concentrations of the complexes. The effects of all synthesized complexes on the viscosity of CT-DNA at 30 ± 0.1 °C are shown in Figure 2. Experimental data showed that with the increase in the concentration of metal complexes, an enlargement of the helix and thus an increase in the viscosity of the CT-DNA occurred. The affinity of the compounds under investigation can be determined from the increase in viscosity grade following the order Cu(L4) > Cu(L1) > Cu(L2) > Cu(L3).

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**Fig. 2.** Effect of increasing amounts of the Cu(II) complexes,

DAPI and EB on the relative viscosity of calf thymus DNA

3.4. *DNA cleavage*

DNA cleavage activities of the compounds were determined by unwinding the supercoiled form (FI) of pBR322 plasmid DNA into nicked circular (FII) and linear forms (FIII).19,20 The cleavage efficiency of complexes in pBR322 plasmid DNA was determined using the agarose gel electrophoresis method. Due to the different binding affinities of the complexes to DNA, different DNA fragmentation efficiencies of the complexes may occur.[21](https://doi.org/10.1016/j.molstruc.2011.06.006) The results obtained were analyzed in the absence of complexes by comparison with control DNA. In this case, it was observed that DNA cleavage activities of all complexes increased with increasing incubation times (1 – 6 h) at a constant concentration of 100 µM under oxidative and hydrolytic conditions (Fig. 3).



**Fig. 3.** The DNA cleavage activities of complexes obtained using agarose gel electrophoresis method

and dependent on oxidative and hydrolytic conditions (based on incubation time of 1 – 6 hours)

Similarly, DNA cleavage activity of the studied complexes increased in direct proportion to the concentration in the studies (12.5 – 200 µM), where the incubation time was kept constant (Fig. 4). It was observed that the complexes were effective on plasmid DNA even at the lowest concentration and in the first hours. In addition, it was observed that the shearing activity of the complexes was still specific at the highest concentration and highest incubation times. Besides the oxidative cutting activity being better than the hydrolytic cutting activity, it was observed that the two activities were very close to each other.



**Fig. 4.** The concentration-dependent (12.5 - 200 µM) DNA cleavage activities obtained using

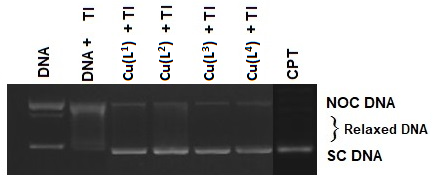
agarose gel electrophoresis method and under oxidative and hydrolytic conditions

3.5. *Topoisomerase inhibition activity*

The topoisomerase I inhibition activities of the complexes were investigated using the standard plasmid relaxation method. This method directly assesses the impact of compounds on the ability of topoisomerase I to convert supercoiled (SC) DNA into its relaxed form. Topoisomerase I enzyme converts SC DNA into relaxed DNA and nicked open circular (NOC) DNA forms. NOC DNA is a single-stranded, double-stranded DNA formed by the cleavage of the single strand of SC DNA by topoisomerase I.

In gel electrophoresis, SC DNA progresses the fastest, NOC DNA is the slowest advancing form, and Relax DNA usually progresses in layers between these two forms. When topoisomerase I shows its activity normally, the band of SC DNA, which is the fastest walking form, is expected to disappear, and bands to Relax DNA and NOC DNA are expected to appear. On the other hand, bands of Relax DNA and NOC DNA forms will not appear on the gel in cases where topoisomerase I activity is inhibited.

In this study, the inhibitory activities of the complexes against topoisomerase I enzyme were investigated by the pBR322 DNA relaxation test. At 20 µM concentration, Cu(II) complexes exhibited inhibitory activity by eliminating the relax form caused by topoisomerase I enzyme (Fig. 5). While Cu(L3) and Cu(L4) complexes showed better inhibition activity compared to Cu(L1) and Cu(L2) complexes, it was observed that the activities of the complexes were lower compared to camptothecin. The results show that Cu(II) complexes can function as potential anticancer agents due to their inhibition activities against topoisomerase I enzyme at 20 µM concentration.



**Fig. 5.** The topoisomerase I inhibition activity of Cu(II) complexes obtained using agarose gel electrophoresis method.

Camptothecin (CPT) was used as a control.

4. CONCLUSIONS

A new series of symmetrical hydrazone ligands and their mononuclear Cu(II) metal complexes were synthesized by various methods and their structures were characterized by various spectroscopic techniques. As a result of the interaction studies of the complexes with CT-DNA, it was observed that all complexes bind via intercalation with CT-DNA and the binding affinity was ordered as Cu(L4) > Cu(L1) > Cu(L2) > Cu(L3). All metal complexes were observed to cleave plasmid pBR322 DNA, in the absence of an external agent, from super-helical Form I to open circular Form II or linear Form III. Finally, in the inhibition studies of the complexes with the enzyme topoisomerase I, it was observed that the complexes inhibited the activity of the topoisomerase I enzyme at a concentration of 20 µM. Therefore, these first studies on the development of new strong metal-based complexes with practical applications are presented in the light of the information obtained from the present study, revealing the utility of these compounds as potential new therapeutic drugs.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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