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Synthesis, Spectral Characterization, DNA Cleavage, and Antioxidant Properties of Metal Complexes with a Novel Schiff Base Ligand Derived from 4-Chlorobenzoic Acid

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Abstract

A novel Schiff base ligand (DBC) synthesized from 4-chlorobenzoic acid, along with its Cu (II) and Co (II) complexes, was prepared and characterized using FT-IR, ^{1}H and ^{13}C-NMR, UV-Vis spectroscopy, as well as magnetic and conductivity measurements. Based on this, a tetrahedral structure of $[M(DBC)Cl_2]$ was proposed for the complexes. Antioxidant activity of the compounds was assessed and compared to ascorbic acid, revealing that the copper complex exhibited superior antioxidant properties compared to the cobalt complex and the ligand. Furthermore, the antibiofilm potential of the copper and cobalt complexes was assessed against five clinically relevant bacterial species (*P.aeruginosa, E.coli, K.pneumoniae, S.aureus* and *S.typhi*) using various concentrations. Biofilm inhibition was quantified through optical density measurements, revealing that the copper complex had a higher mean biofilm inhibition compared to the cobalt complex at the maximum concentration of 1000 mg/ml. Optical density measurements also indicated reduced biofilm formation with increasing metal concentrations, particularly for the copper complex. Additionally, agarose gel electrophoresis showed significant alterations in plasmid DNA profiles of bacterial isolates upon exposure to the complexes, indicating potential genetic changes and implications for bacterial adaptation.

Keywords: ligand Schiff base, 4-Chlorobenzoic Acid, DNA cleavage, Cupper complexes, Cobalt complexes, Anti-biofilm study

تحضير وتشخيص طيفي وانقسام الدنا والخصائص المضادة للأكسدة للمعقدات الفلزية مع قاعدة شف الجديدة المشتقة من 4-كلوروحامض البنزويك

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الخلاصة

تم تحضير ليكاند قاعدة شف جديد (***DBC***) من 4--كلورو حامض البنزويك, الى جانب تحضير معقداته للنحاس (***II***) والكوبلت (***II***), وتم تشخيصها باستخدام مطيافية الأشعة تحت الحمراء والرنين النووي للبروتون والكربون، والأشعة فوق البنفسجية، بالإضافة إلى القياسات المغناطيسية والتوصيلية. بناءً على ذلك، تم اقتراح

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بنية رباعية السطوح [**$M(DBC)Cl_2$**] للمعقدات. تم تقييم النشاط المضاد للأكسدة للمركبات ومقارنتها بحامض الأسكوربيك، وكشف عن أن معقد النحاس أظهر خصائص مضادة للأكسدة اعلى مقارنة بمعقد الكوبلت والليكاند.

علاوة على ذلك، تم تقييم القدرة المضادة للأغشية الحيوية لمعقدات النحاس والكوبلت ضد خمسة أنواع بكتيرية ذات صلة سريرية (زائفة زنجارية, إشريكية قولونية, الكلبسيلة الرئوية, مكورة عنقودية ذهبية, السلمونيلا) باستخدام تركيزات مختلفة. تم تحديد مقدار تثبيط الأغشية الحيوية من خلال قياسات الكثافة البصرية، والتي كشفت أن معقد النحاس كان له متوسط تثبيط أعلى للأغشية الحيوية مقارنة بمعقد الكوبلت عند أقصى تركيز 1000 مجم/مل. كما أشارت قياسات الكثافة البصرية إلى انخفاض تكوين الأغشية الحيوية مع زيادة تركيزات الفلز ، وخاصة بالنسبة لمعقد النحاس. بالإضافة إلى ذلك، أظهر التحليل الكهربائي للهلام الأجار تغييرات كبيرة في خصائص الحامض النووي البلازميدي للعزلات البكتيرية عند التعرض للمعقدات، مما يشير إلى التغيرات الجينية المحتملة والآثار المترتبة على التكيف البكتيري.

1. Introduction

Schiff Bases represent an important class of pharmaceutical organic compounds known for their biological activity and because of their biological properties, they have gained great interest from medical chemical researchers [1]. The development of new bioactive derivatives of Schiff bases relies on these properties, guiding therapeutic strategies effectively. Schiff rules find application in diverse areas such as anti-tumor, DNA-binding, anti-mycobacterial, and antibacterial treatments, as well as in biological systems like human serum albumin (HSA) and zebra fish larvae, and in analyzing water samples for sensitivity and selectivity [2, 3].

Metal complexes formed with chelating polydentate ligands containing nitrogen and sulfur atoms are significant due to their distinct pharmacological and biological activities, as well as their intriguing chemical and physical properties, as these atoms play a major role in the binding of biologically active organic molecules with metal ions [4]. Among the complexes that are more important and useful against diseases are the cobalt, copper and zinc complexes because they have a low molecular weight [5, 6].

Thiadiazoles represent a five membered aromatic ring containing three different atoms: two nitrogen atoms and one sulfur atom. The stability of the 1,3,4-thiadiazole isomer was generally controlled by the electron density in the C_2 and C_5 atoms, which was highly dependent on the substituents, making it the most thermally stable isomer. One of the most important applications of these heterocyclic compounds is that they are antibacterial and antifungal compounds, in addition to their agricultural chemical effects as anthelmintic [7, 8].
Deoxyribonucleic Acid (DNA) is a crucial molecule in living organisms and viruses, carrying genetic information essential for life processes. Biologists and chemists were interested in studying nucleic acid degradation, including investigating the ability of transition metal complexes to cleave DNA under certain physiological conditions that mimic artificial nuclease activity [9].

One of the things that helped in the design of new useful drugs and the development of cleavage DNA agents was the nature of some metal complexes in their interaction with the DNA molecule. Due to the ability of some complexes such as Cu(II) and Co(II) to specifically cleavage DNA, they serve as specific probes for DNA phosphorylation [10, 11]. Antioxidants are donors of electrons or hydrogen at the reactive site to identify free radicals, which in turn 2,2-diphenyl-1-picrylhydrazyl (DPPH) helps in evaluating the scavenging effectiveness of various organic compounds. It was observed that some of the prepared compounds, including ligands and their complexes, act as excellent antioxidants [12, 13].

This study details the synthesis and characterization of two metal complexes with ligand (DBC), derived from 4-chlorobenzoic acid, coordinated to transition metal ions Co(II) and Cu(II) with biological activity studies (antioxidants and DNA cleavage).

2. Experimental part

From commercial places of certified international companies, the solvents and chemicals used in the work were purchased as they were used without purification. Using a ultraviolet–visible spectrophotometer (Shimadzu ultraviolet-1800) at a concentration of 10^{-3}*Molar* in solvent dimethyl sulfoxide at room temperature and 1.0 cm for a quartz cell length; the ultraviolet-visible spectra of compounds were measured. Using a spectrophotometer (Shimadzu 8400s Fourier-transform infrared spectroscopy) of the cesium iodide disk in the range (4000-200) cm^{-1} and (Biotic 600 Fourier-transform infrared spectroscopy) of the potassium bromide in the range (4000 - 400) cm^{-1} the frequencies of the active groups of the prepared compounds were determined. To obtain *nuclear magnetic resonance* spectra in dimethyl sulfoxide-d^6 as a standard solvent and using tetra methyl silane as a reference compound; a Bruker Avance-*Neo Spectrometer* (400 Megahertz) was used.

2.1. Synthesis of ligand [(Z)-4-(5-((4-(dimethylamino)benzylidene)amino)-1,3,4-thiadiazol-2-yl) phenyl 4-chlorobenzoate] (DBC)

2.1.1. Preparation of 4-chlorobenzoyl chloride

A mixture of 15 mL of thionyl chloride and 5 g (0.042 mole) of 4-chlorobenzoic acid, along with 2-4 drops of dimethylformamide, was refluxed for 1.5 hours.

2.1.2. Preparation of 4-[(4-chlorophenyl)methoxy]benzoic Acid

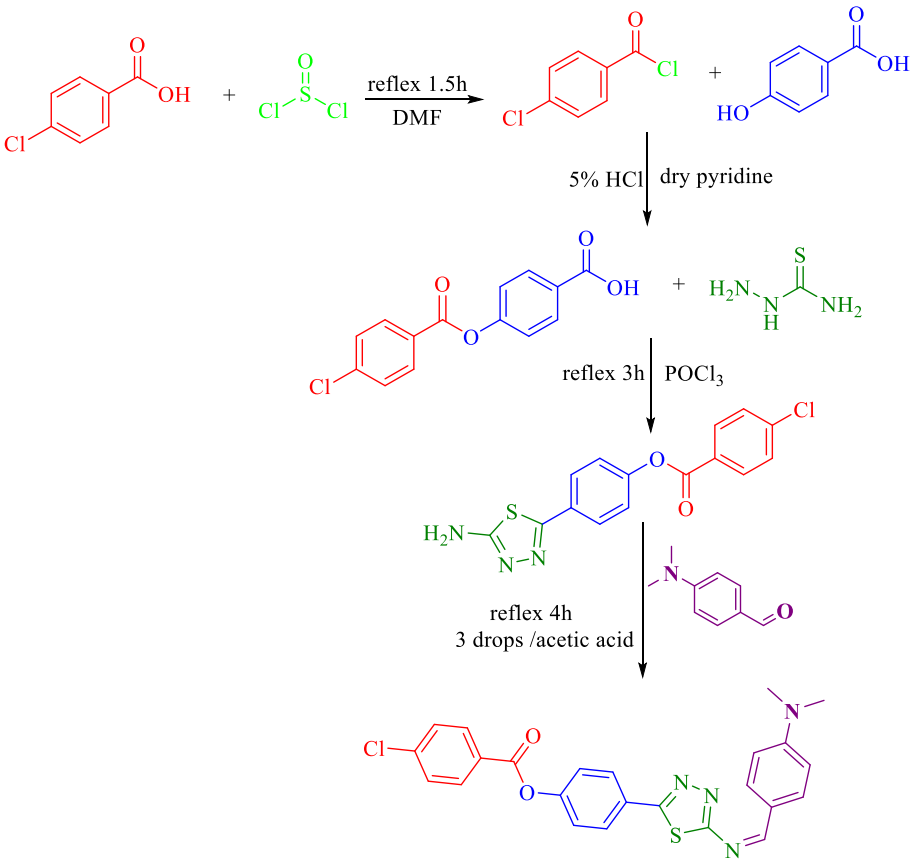
For 4 hours, 4-chloro benzoyl chloride 0.057 mole, (15 ml) was added drop by drop with continuous stirring to para hydroxy benzoic acid 7g, (0.050 mole) dissolved in dry pyridine after cooling it, and the reaction was monitored by TLC. Then the reaction mixture was poured on crushed ice, diluted with HCl, filtered to collect the precipitate, dried, and re-crystallized with ethanol.

2.1.3. Synthesis of 4- (5- amino-1, 3, 4-thiadiazol -2- yl) phenyl-4-chlorobenzoate

A mixture of 4-((4-chlorobenzoyl)oxy)benzoic acid (5g, 0.019 mole), thiosemicarbazide (6.7g, 0.074 mole) and phosphoryl chloride (20ml) was refluxed for 3 hours. The mixture was then cooled by adding drop after drop of cold water, then it was refluxed for 1 h, then cooled, filtered, and the filtrate was neutralized with sodium hydroxide, which caused the production of a deep yellow precipitate, which was filtered and dried.

2.1.4. Synthesis of Schiff's Base Ligand (DBC)

For 4 hours, the mixture of 4- (5- amino -1 ,3 ,4- thia di azol-2-yl) phenyl 4-chlorobenzoate 5g, (0.037 mole) with 4-(di-methyl amino) benzaldehyde 6.7g, (0.074 mole) in ethanol with 3-4 drops of acetic acid (glacial) was refluxed, then the mixture was cooled, filtered, and recrystallized with hot ethanol with good production 68% [14], scheme 1.



Scheme 1: Preparation course of the Ligand (DBC)

2.2. Complexes of the Ligand (DBC)

For a duration of 3 to 4 hours at a temperature of 70 ˚C under reflux, a mixture of the metal chloride solution-either 0.09 g (0.54 mmol) of $CuCl_2 \cdot 2H_2O$ or 0.13 g (0.54 mmol) of $CoCl_2 \cdot 6H_2O$ and 0.25 g (0.54 mmol) of the ligand (DBC) in ethanol resulted in the formation of a blue or green precipitate, respectively. The precipitate was filtered, washed with distilled water, and re-crystallized with EtOH. In mole ratio (M: DBC) (1:1) metal complexes were prepared [15], see Figure 1.

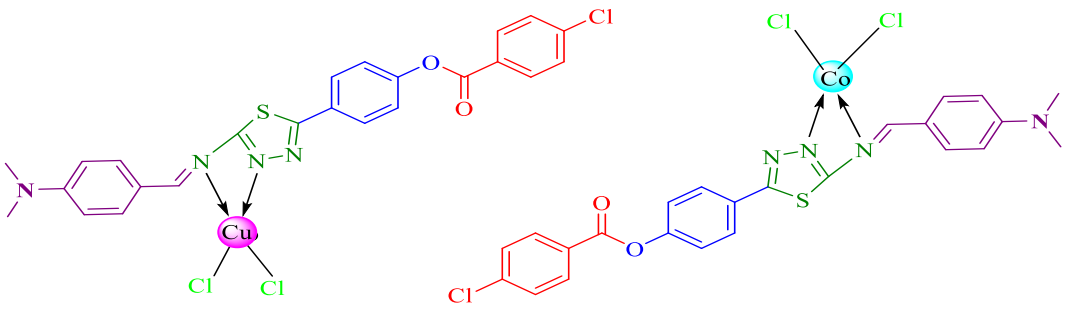


Figure 1: The synthesis of Cu(II) and Co(II) complexes

2.3. Biofilm formation Assay/ Microtitre culture plate method (quantitative assay)

All isolates were cultured at 37°C in brain-heart infusion broth media for 24 h and then 100 µl of bacterial growth culture was transferred to a tube containing 2 ml tube of normal saline with turbidity adjusted to McFarland 0.5. A volume of 180 µl of brain-heart infusion broth

media containing 1% glucose was added to sterile 96-well flat-bottom polystyrene microtiter plates. A volume of 20 µl of bacterial (*Staph.aureus*, *Salmonella*, *Pseudomonas*, *klebsiella*, *E.Coli*) suspension (from normal saline) was added to three wells of the same plates with six wells as a negative control. The covered plates were incubated aerobically at 37°C for 24 h without shaking. After incubation, all plates were gently washed three times with periphytic biofilms (PBs) and left to dry. To stabilize the biofilms, 150 µl of methanol was added to each well and allowed to sit for 15 minutes at room temperature, followed by washing and air drying. Plates were stained with 250 µl of 1% crystal violet solution for 15 min at room temperature. The wells were then washed and dried at 37°C for approximately 15 min to ensure complete dryness. The stain was re-dissolved with 200 µl of a 95% ethanol for 30 min. The optical density (OD) of each well was read at 630 nm using a microtiter plate reader. Quantitative of biofilm formation was determined by a colorimetric microtiter plate assay [16].

2.4. Antibiofilm activity of copper and cobalt complexes

The effects of copper and cobalt on biofilm activity were tested using a modified microtiter plate assay method [17]. Bacteria were cultured in 10 mL of brain heart infusion medium for 24 hours at 37°C. Subsequently, the bacterial cultures were diluted to a concentration of 1.5×10^8 colony-forming units per milliliter, as determined using a DensiCHEK device. 100 µl of each concentration of copper and cobalt plus 100 µl of the bacteria solution were added to each well of the microtiter plate. The final volume was 200 µl per well. Only sterile liquid medium was used as a control. After incubating the microtiter plates at 37 °C for 24 h, the biofilm formed was measured using crystal violet. The following equation was used to determine the biofilm inhibitory activity after measuring the optical density at 630 nm in a multiplate reader:
Biofilm reduction %= (OD of biofilm growth control – OD of treated isolate)/OD of biofilm growth control×100 ……Eq. (1) [18]
Where OD = the optical density read at an absorbance of 630 nm

2.5. Minimum inhibitory concentration.

The Both microdilution method was performed for test material determination [19]. The test material was prepared and then diluted 1:10 in sterile Hinton's broth. 100 µl of the diluted test material was transferred to the first well of a 12-well plate. An additional 100 µl of sterile Hinton's broth was added to wells 2 through 12 to create a two-fold dilution series across the plate. It was diluted by transferring 100 µl from the first well to the tenth well except for wells 11 and 12 which were used as positive and negative controls. All wells were seeded with 20 µl of a bacterial suspension similar to McFarland standard No. 5 (1.5×10^8 CFU/ml) except for the negative control. The microtiter plate was incubated at 37°C for 24 h. The MIC value was determined as the lowest concentration that inhibited bacterial growth. The growth was measured by OD_{450} determination using a microtiter plate reader.

2.6. DNA cleavage study

Protocol

First, 600 µl of the bacterial culture grown in LB medium was added to a 1.5 ml micro centrifuge tube. The Zyppy™ Plasmid Miniprep Kit can be used with the classic centrifuge-based procedure to process up to 3 ml of bacterial culture. 100 µl of 7X lysis buffer (blue) was added and mixed by inverting the tube 4-6 times. Proceed to step 3 within 2 min. Upon addition of the 7X lysis buffer, the solution's appearance transitions from opaque to transparent blue, signifying complete cell lysis. A 350 µl of cold neutralization buffer (yellow) was added and mixed well. The sample turned yellow when neutralization was completed and a yellowish precipitate was formed. The sample was inverted 2-3 additional times to ensure complete neutralization. Centrifugation at 11,000 - 16,000 x g was made for 2-4 minutes. The upper

liquid (~900 µl) was transferred to the provided Zymo-Spin™ IIN column. To avoid disturbing cell debris granules. The column was placed in a collection tube and was centrifuged for 15 seconds, the liquid waste was discarded. The column was placed back into the same collection tube. 200 µl of Endo-Wash Buffer was added to the column. Centrifuged for 30 seconds (sometimes emptying the collection tube is not necessary). 400 µl of Zyppy™ Wash Buffer was added to the column. Centrifuged for 1 minute. The column was transferred to a clean 1.5 ml microcentrifuge tube and 30 µl of Zyppy™ Elution Buffer2 was added directly to the column matrix and allowed to stand for 1 minute at room temperature. Centrifuged for 30 seconds to remove plasmid DNA [20].

2.7. Electrophoresis of DNA by Agarose Gel

Electrophoresis of DNA using agarose gel Electrophoresis was performed to identify DNA fragments after extraction or to detect the result of a polymerase chain reaction while the standard DNA was used to distinguish the size of the resulting band on the agarose gel [21].

The agarose gel was prepared by boiling the sample, usually at a concentration of 1.5%. After boiling, it was poured into a mold and left to cool. After it cools, it will solidify into a matrix. When the gel was poured into the mold, a comb was placed inside the mold at one end, so the gel contains several holes or wells. The sample was then prepared by adding a loading dye (bromophenol blue) to the DNA sample. The gel was then loaded by adding the samples to the gel. The gel was then turned on and exposed to an electric field. The gel was then stained, i.e. the DNA molecules are stained (ethidium bromide). The dye then sticks to the DNA and lines will appear on the gel wherever DNA is present. Once stained, the gel can be analyzed [22].

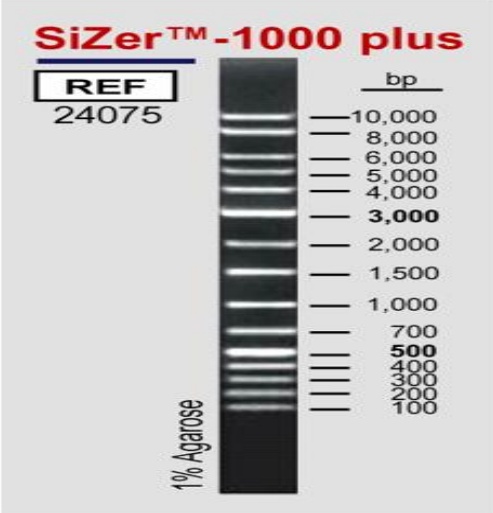


Figure 2: SiZer DNA Markers (intron)

2.8. Antioxidant activity/ preparing standard solutions

DPPH solution was prepared at a concentration of (0.1 mmol) in methanol with different concentrations of ascorbic acid limited to (25-200) mg/ml. The limiting capacity of the selected ligand and its CuII and CoII complexes to scavenge the DPPH radical was determined using the method reported by Singh and co-workers. In a brief, 30 ml of each concentration were placed in test tubes, 5 ml of DPPH were added to them, then the tubes were shaken well to homogenize the solution and placed in the dark for 30 min, then the absorbance was measured

using a UV/Vis spectrometer at a wavelength of 517 nm, ascorbic acid served as a standard [23].

2.9. Sample preparation:

Several diluted concentrations of the selected compounds were prepared and treated in the same way as ascorbic acid. The inhibitory capacity of these compounds was determined by calculating the percentage of inhibition (%I) for DPPH from the following relationship:-

$I\% = ((A_0-A_1)/A_0) \times 100$……..Eq. (2) [24]

Where %I= The percentage of inhibition of the free radical antioxidant agent DPPH; A_0= Absorbance of the free radical in the absence of the sample after 30 minutes; A_1= Absorbance of the mixture (radical and sample) after 30 minutes

2.10. Statistical Analysis

The Statistical Packages of Social Sciences-SPSS program was used to detect the effect of difference concentration in study parameters (Scavenging activity of compound). Least significant difference-LSD was used to significant compare between means (ANOVA/ One way / 0.05 and 0.01 probability) in this study [25].

3. Results and Discussion

The thermal stability of the prepared metal complexes was characterized using data obtained from melting point measurements. As for the solubility, they were good in Dimethylsulfoxide (DMSO) and Dimethylformamide (DMF) solvents. Finally, the atomic absorption data showed that the theoretical and practical results of the prepared compounds matched, as shown in Table 1.

Table 1: Results for various measurements of the prepared compounds

Compounds	Molecular weight g / mol	melting point °C	color	A.A	C.H.N.S. Measured (calculated)				Cl-content
				M%	C%	H%	N%	S%	Cl%
DBC	462.95	223-225	Orange	------	62.25 (62.27)	5.12 (4.14)	12.13 (13.00)	6.23 (6.93)	7.24 (7.66)
$[Co(DBC)Cl_2]$	592.79	250-252	Green	9.50 (9.91)	48.63 (48.65)	3.97 (3.21)	9.77 (9.46)	5.01 (5.41)	17.11 (17.95)
$[Cu(DBC)Cl_2]$	597.40	200-202	Blue	10.27 (10.64)	48.21 (48.25)	3.89 (3.21)	9.65 (9.38)	5.86 (5.37)	17.35 (17.80)

A.A=Atomic absorption, C.H.N.S.=Elemental Microanalysis

3.1. NMR Spectra of the ligand (DBC)

An integrated intensity of each signal in the NMR spectra of the ligand (DBC) was obtained, corresponding to the totals (protons or carbons) present in the prepared compounds [26], as shown in Figures. 3,4 and Table 2.

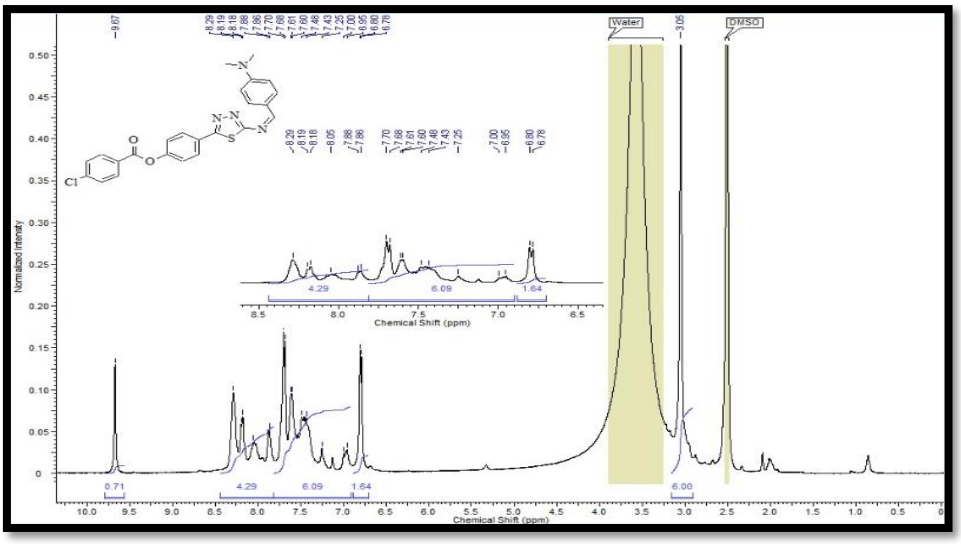


Figure 3: ^{1}H-NMR of (DBC)

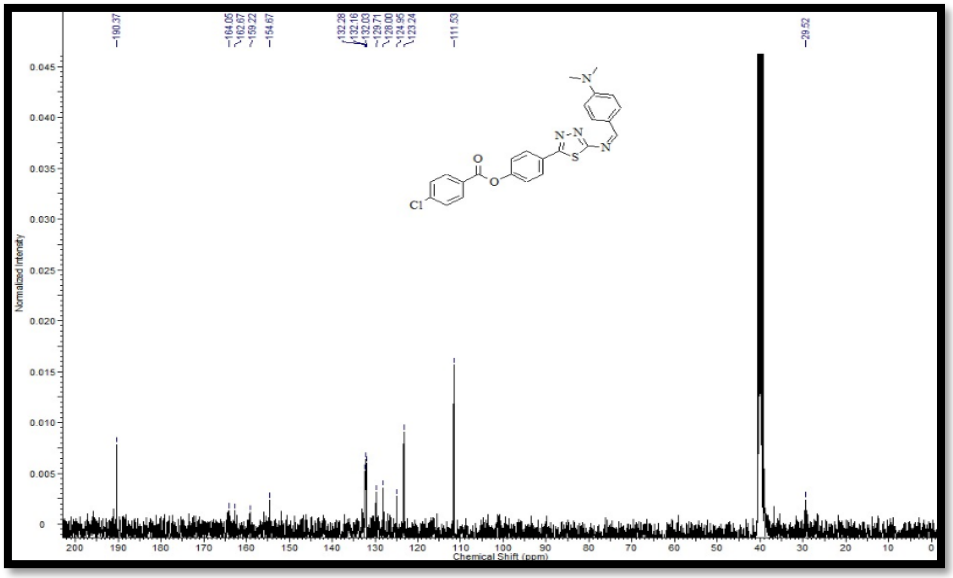
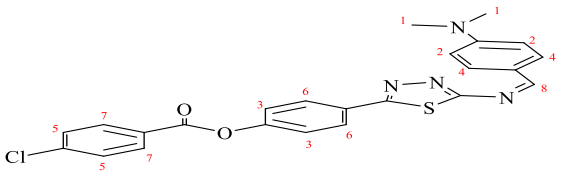
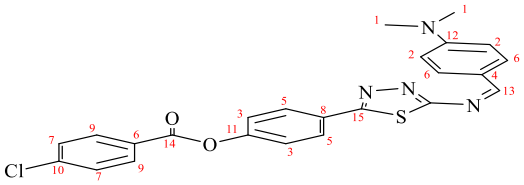


Figure 4: ^{13}C-NMR of (DBC)

Table 2: Values of chemical shifts (ppm) in the nuclear resonance spectrum of the prepared compounds



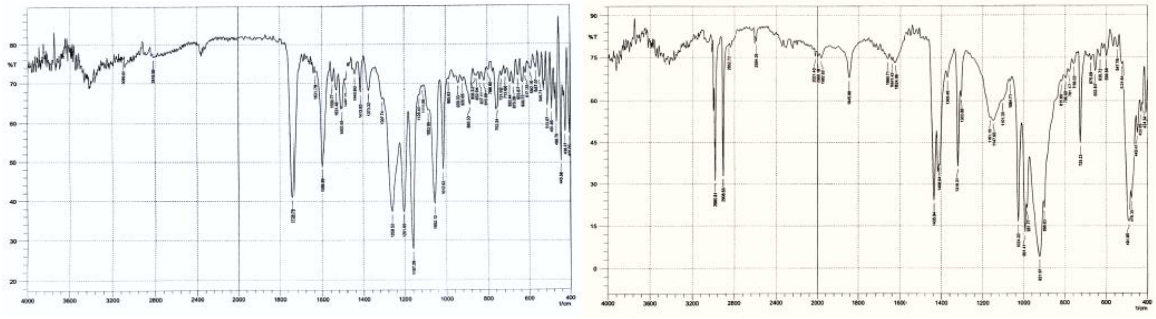


Proton groups	δ (ppm)	Carbon groups	δ (ppm)
6H (singlate), proton CH_3 group for NO.1	3.05	C1 for (CH_3), $N(CH_3)_2$ group	29.52
		C_2,C_4,C_6,C_{12} for aromatic, aldehyde group	111.53, 124.95, 129.71, 159.22
1H (douplate), proton aromatic for NO.2,4	6.78 and 6.80, 7.43 and 7.48	C_{13} for CH=N group (Schiff base)	162.67
		C_{15} for SC=N, five ring	190.37

1H (singlate), proton CH=N group for NO.8	9.67	C_3,C_5,C_8,C_{11} for aromatic, para hydroxyl benzoic acid group	123.24, 128.00, 132.03, 154.67
1H (douplate), proton aromatic for NO.3,6	6.95 and 7.00, 7.68 and 7.70	C_{14} for (CO), 4-chlorobenzoic acid group	164.05
3H (douplate), proton aromatic for NO.5,7	7.60 and 7.61, 8.18 and 8.19	C_6,C_7,C_9,C_{10} for aromatic, 4-chlorobenzoic acid group	129.71, 129.71, 132.16, 132.28

3.2. The Fourier-transform infrared spectra of compounds

When studying the infrared spectrum of the prepared compounds, a new band at 1631 cm $^{-1}$ was appeared that belongs to the frequency of the azomethine group and at 1598 cm $^{-1}$ that belongs to the frequency of the thiadiazole ring [27]. In the infrared spectrum of the prepared complexes, a band was observed at 1641 or 1643 cm^{-1}, corresponding to the υ(C=N) group. This band is shifted to a higher frequency by 10-12 cm^{-1} compared to the ligand, which proves this coordination by the emergence of a new band at M-N confirming the attachment of the nitrogen atom of the azomethine group with the metal ion. Also, the emergence of a band at 1614 or 1624 cm^{-1} dating back to the υ (C=N) group was observed, especially of the vibrations of the thiadiazole ring, and the shift from the one in the ligand to a frequency higher by 16-26, which proves this coordination by the emergence of a new band at M-N confirming the attachment of the (N) atom of the thiadiazole ring with the metal ion. The reason for the shift to higher or lower frequencies is attributed to the presence of coordination between (DBC) and the metal ion. Among the important factors that affect the frequency shift are the donor atoms and the nature of the metal ion. These bands are assigned to υ(M-N) at 524–526 cm^{-1} (in pyrrolyl compounds) and υ(M-Cl) at 318–328 cm^{-1} [28, 29]. Figure 5 and Table 3, the spectra of the (DBC) and its complexes were presented.



(1) **(2)**

Figure 5: DBC spectrum's FTIR (1) and Cu(II) Complex (2)

Table 3: Band values in the infrared spectrum of the prepared compounds

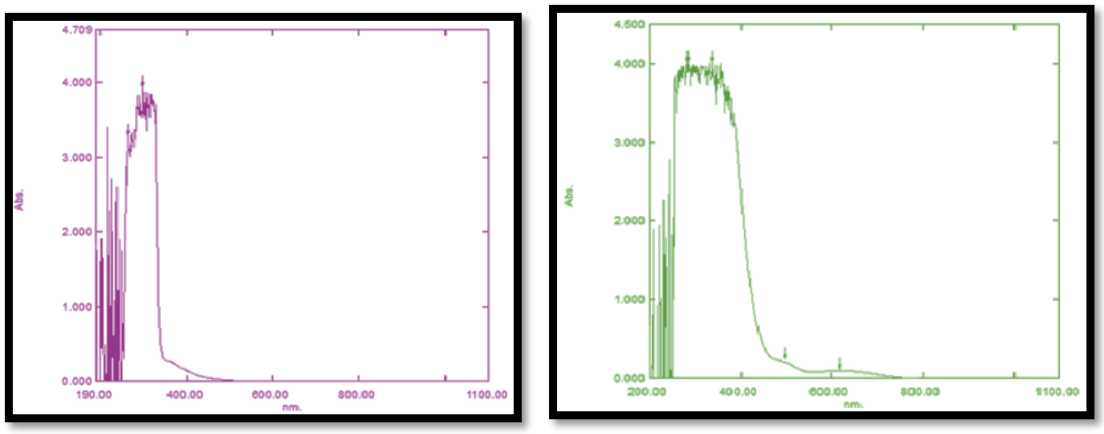
Com.	υ (HC= N) azomethine	υ (C=N) thiadiazole ring	υ(M-N)	υ(M-Cl)
DBC	1631	1598	——	——
[Co(DBC)Cl_2]	1641	1624	524	328
[Cu(DBC)Cl_2]	1643	1614	526	318

3.3. Electronic spectra of compounds

All the data obtained for the ultraviolet spectra of the prepared compounds were included in Table 4, but Figure 6 represents the spectrum of the ligand (DBC) and the complex Co(II).

Table 4: Data in the ultraviolet spectrum of the prepared compounds

Com.	Number of wave		ε maximum Molar $^{-1}$ Centi meter^{-1}	Transitions	μ_{eff} B.M.	Molar Conductivity Ohm^{-1}cm^{2}mol^{-1}
	Nano meter	Centi meter^{-1}				
DBC	275	36363	3274	π — π^*	-----	-------
	299	33444	3915	n — π^*	--	
		35714	3991	Intra-Ligand		
[Co(DBC)Cl_2]	280 336	29761	4000	C . T	4.63	18.3
	502 620	19920	211	$^4A_{2\,(F)} \rightarrow {}^4T_{1\,(P)}$		
		16129	98	$^4A_{2\,(F)} \rightarrow {}^4T_{1\,(F)}$		
[Cu(DBC)Cl_2]	300 425	33333	3861			
	1051	23529	1300	Intra-Ligand C.T	2.41	14.9
		9514	125	$^2T_2 \rightarrow {}^2E$		



(1) **(2)**

Figure 6: Electronic spectrum of (DBC) (1) and Co(II) Complex (2)

3.4. Conductivity and magnetic measurements of complexes

Table 4 presents the effective magnetic moment (μeff) values for Co(II) and Cu(II) complexes, which are 4.63 and 2.41 Bohr Magnetons (B.M.) respectively. These values are consistent with a tetrahedral geometric configuration. The molar conductivity data were also presented, which confirmed the non-electrolyte nature of the prepared compounds [30, 31].

3.5. Antioxidant activity

The metal complexes showed better activity than the free ligand (DBC). This can be attributed to the more stable tetrahedral geometric shape around the metal ions of cobalt and copper. This can be explained as after the system is complicated, the ability to stabilize the unpaired electrons and then scavenge the free radicals increases, as does the presence –N=C-S- group which makes the compound able to absorb free radicals and stabilize them through conjugated systems, thus increasing the anti-oxidant activity of the complexes relative to the free ligand [32, 33], Table 5(A-D) and Figure 7.

Table 5A: Effect of concentration in scavenging activity of compounds

Concentration µg mL^{-1}	Scavenging % (Mean ±SD)			
	Ascorbic acid	Ligand (DBC)	Complex-Co	Complex-Cu
200	75.77 ±2.60 a	40.20 ±1.91 a	54.78 ±2.40 a	80.36 ±2.80 c
100	61.95 ±1.26 b	31.28 ±5.55 b	40.70 ±3.50 b	69.48 ±3.70 b
50	48.57 ±2.80 c	20.20 ±4.8 c	21.33 ±7.00 c	54.47 ±2.40 c
25	40.04 ±5.30 d	13.27 ±0.82 d	17.39 ±4.26 c	40.43 ±7.08 d
L.S.D.	7.028 **	5.375 **	7.942 **	9.167 **
P-value	0.0001	0.0001	0.0001	0.0001

Means having with the different letters in same column differed significantly.
** (P≤0.01).

Table 5B: Comparison between ascorbic acid and ligand (DBC) in scavenging activity

Concentration µg mL^{-1}	Scavenging % (Mean ±SD)			
	Ascorbic acid	Ligand (DBC)	T-test	P-value
200	75.77 ±2.60	40.20 ±1.91	5.419 **	0.0001
100	61.95 ±1.26	31.28 ±5.55	6.075 **	0.0001
50	48.57 ±2.80	20.20 ±4.8	6.143 **	0.0001
25	40.04 ±5.30	13.27 ±0.82	6.094 **	0.0001
	** (P≤0.01).			

Table 5C: Comparison between ascorbic acid and complex-Co in scavenging activity

Concentration µg mL^{-1}	Scavenging % (Mean ±SD)			
	Ascorbic acid	Complex-Co	T-test	P-value
200	75.77 ±2.60	54.78 ±2.40	5.921 **	0.0001
100	61.95 ±1.26	40.70 ±3.50	5.882 **	0.0001
50	48.57 ±2.80	21.33 ±7.00	6.702 **	0.0001
25	40.04 ±5.30	17.39 ±4.26	6.717 **	0.0001
	** (P≤0.01).			

Table 5D: Comparison between ascorbic acid and complex-Cu in scavenging activity

Concentration µg mL^{-1}	Scavenging % (Mean ±SD)			
	Ascorbic acid	Complex-Cu	T-test	P-value
200	75.77 ±2.60	80.36 ±2.80	5.292 NS	0.251
100	61.95 ±1.26	69.48 ±3.70	5.637 *	0.0381
50	48.57 ±2.80	54.47 ±2.40	4.632 *	0.0397
25	40.04 ±5.30	40.43 ±7.08	4.180 NS	0.894
	* (P≤0.05), NS: Non-Significant.			

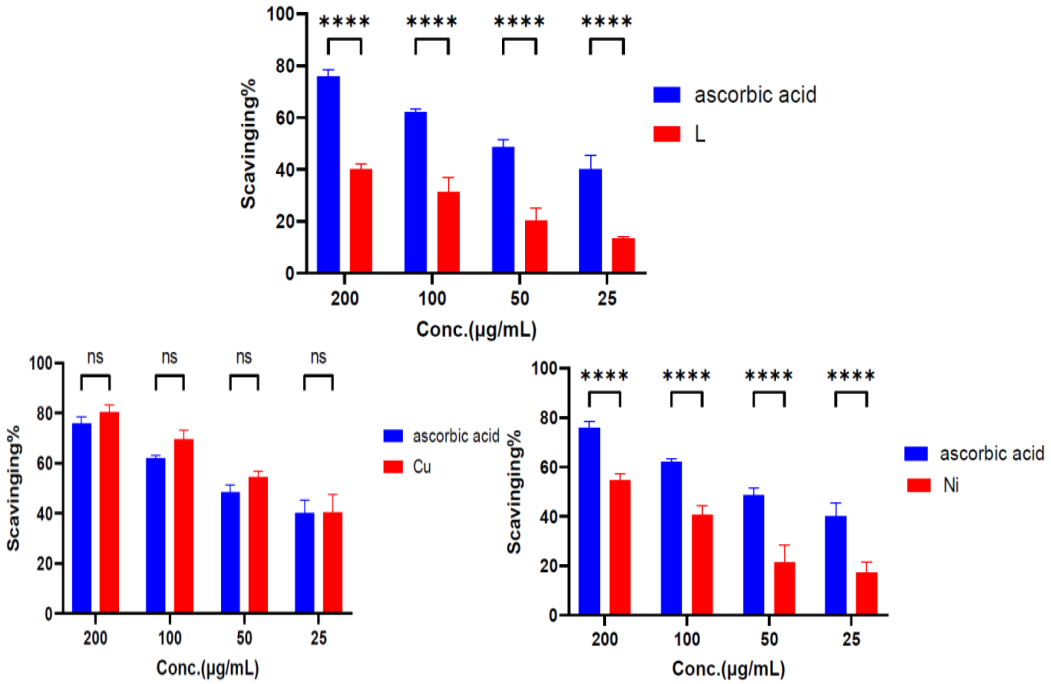


Figure 7: Scavenging activity of compounds compared with ascorbic acid as a reference

3.6. Result of Biofilm study

This study was conducted to evaluate the anti-biofilm potential of copper and cobalt complexes at varying concentrations against five bacterial species. The results present a complex landscape of interactions between metal ions, at different concentrations, and bacterial *species*, all contributing to different degrees of biofilm inhibition and optical density changes. The mean inhibition percentage of biofilm formation across all five bacterial species was notably higher for the copper complex compared to the cobalt complex at the highest concentration tested (1000 mg/ml). Specifically, the mean inhibition was 37.48% for the copper complex and 19.34% for the cobalt complex at 1000 mg/ml, as shown in Figure 8. The standard error of mean for this concentration was 5.67 for the copper complex and 7.09 for the cobalt complex, indicating somewhat higher variability in the cobalt dataset. Interestingly, for copper, a decreasing concentration from 1000 mg/ml to 250 mg/ml resulted in lower mean inhibition percentages (from 37.48% to 12.38%), demonstrating a clear dose-response relationship. The standard errors of these means were 5.67, 7.35, and 5.80 for concentrations of 1000mg/ml, 500mg/ml, and 250mg/ml, respectively. For cobalt complex, the percentages of inhibition remained relatively stable across different concentrations. In fact, the mean inhibition percentages increased slightly from 19.34% at 1000mg/ml to 21.80% at 250mg/ml. The standard error of the mean for the cobalt complex was somewhat variable but did not exhibit a clear trend across concentrations. The high p-value of 0.57 in this study proved that the observed differences in biofilm inhibition across different concentrations of copper and cobalt complexes was not statistically significant. Although the available data suggests a trend of the copper complex demonstrating greater efficacy compared to the cobalt complex at higher concentrations, the statistical analysis does not support this observation as being significant [34, 35].

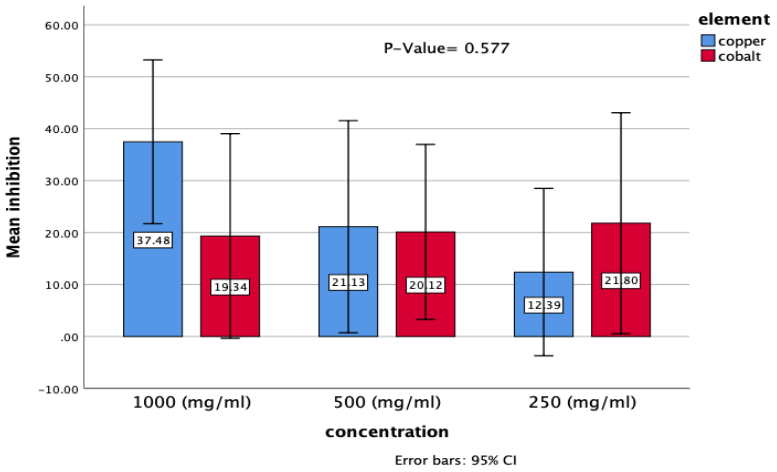


Figure 8: Mean Biofilm Inhibition Percentages and Standard Errors for Copper and Cobalt complexes at Different Concentrations

Table 6 illustrated biofilm inhibition percentages across different bacterial species (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* and *Salmonella typhi*) at varying concentrations of copper and cobalt complexes. The biofilm inhibition percentages display a broad range of values that differ significantly depending on the bacterial isolation and the metal concentration. For the copper complex, *Staphylococcus aureus* exhibits the highest biofilm inhibition at 53.3% with a 1000 mg/ml concentration, followed by *Salmonella typhi* at 44.6%. At the lowest concentration of 250 mg/ml of the copper complex, the inhibition percentages significantly decrease with *Klebsiella pneumoniae* showed the least effect at 2.6%. For the cobalt complex, the pattern is less straightforward. *Staphylococcus aureus* again appears to be the most affected at 45.3% biofilm inhibition at 1000 mg/ml, whereas *Salmonella serotype Typhi* shows the least effect at 3.2%. Interestingly, *Escherichia coli* showed an increased inhibition percentage as the concentration of cobalt complex decreases, peaking at 30.4% at 250 mg/ml [36].

Table 6: Biofilm Inhibition Percentages for Different Bacterial Strains at Various Concentrations of Copper and Cobalt complexes

Isolate	Copper complex			Cobalt complex		
	1000 mg / ml	500 mg / ml	250 mg / ml	1000 mg / ml	500 mg / ml	250 mg / ml
Escherichia coli	20	9.5	6.04	17	21.7	30.4
Klebsiella pneumoniae	31.3	24.1	2.6	20	0.15.9	18.8
Pseudomonas aeruginosa	38.2	1.06	13.3	11.2	0.12.	5
Salmonella serotype Typhi	44.6	27.7	5.5	3.2	0.08.3	8.1
Staphylococcus aureus	53.3	43.3	34.5	45.3	0.42.7	46.7

Table 7 presents the optical density (OD) measurements of biofilms for the five bacterial strains (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* and *Salmonella typhi*) those results have been obtained by the microtiter-plate method as shown in Figures 9 and 10 after treatment with various concentrations of copper and cobalt complexes. Generally, a decrease in OD values signifies a reduction in biofilm formation. For copper complex treatments, *Staphylococcus aureus* exhibited the highest OD reduction from 1.274 at 0 mg/ml to 0.594 at 1000 mg/ml. In contrast, *Pseudomonas aeruginosa* showed a slight decrease in OD from 0.749 to 0.741 when treated with 500 mg/ml of copper complex,

suggesting ineffective biofilm disruption at this concentration. For cobalt complex treatments, the OD values generally decreased with increasing concentration, although there were a few exceptions. For example, *Pseudomonas aeruginosa* displayed a slight OD increase when treated with 250 mg/ml of cobalt complex compared to 500 mg/ml [37].

Table 7: Optical Density Measurements of Biofilms for Different Bacterial Strains Treated with Various Concentrations of Copper and Cobalt complexes

Isolate		*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Salmonella serotype Typhi*	*Staphylococcus aureus*
Copper complex	0 mg/ml	0.689	0.713	0.749	0.905	1.274
	1000 mg/ml	0.551	0.489	0.462	0.5	0.594
	500 mg/ml	0.623	0.541	0.741	0.654	0.722
	250 mg/ml	0.647	0.694	0.649	0.855	0.834
Cobalt complex	0 mg/ml	0.423	0.414	0.419	0.406	0.626
	1000 mg/ml	0.351	0.331	0.372	0.392667	0.342
	500 mg/ml	0.331	0.348	0.368	0.372	0.358
	250 mg/ml	0.294	0.336	0.398	0.373	0.333

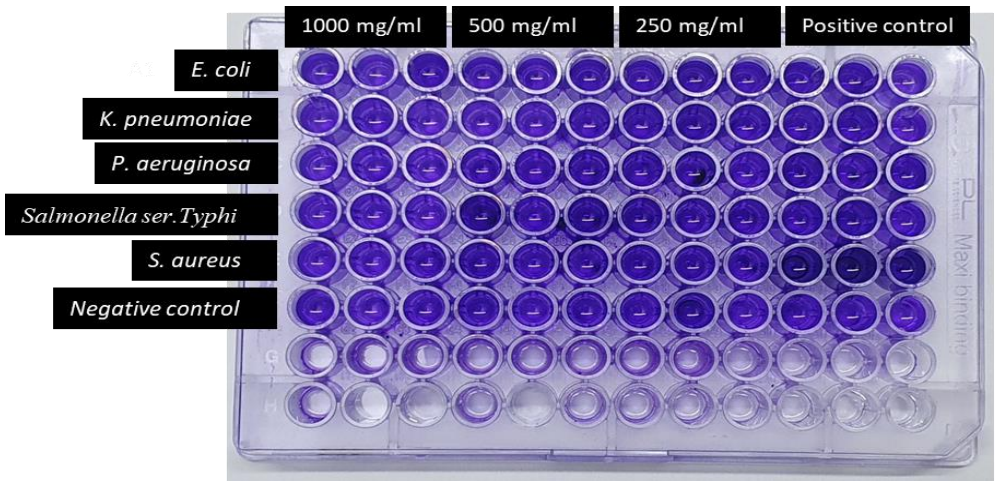


Figure 9: Biofilm intensity before and after treatment with copper complex

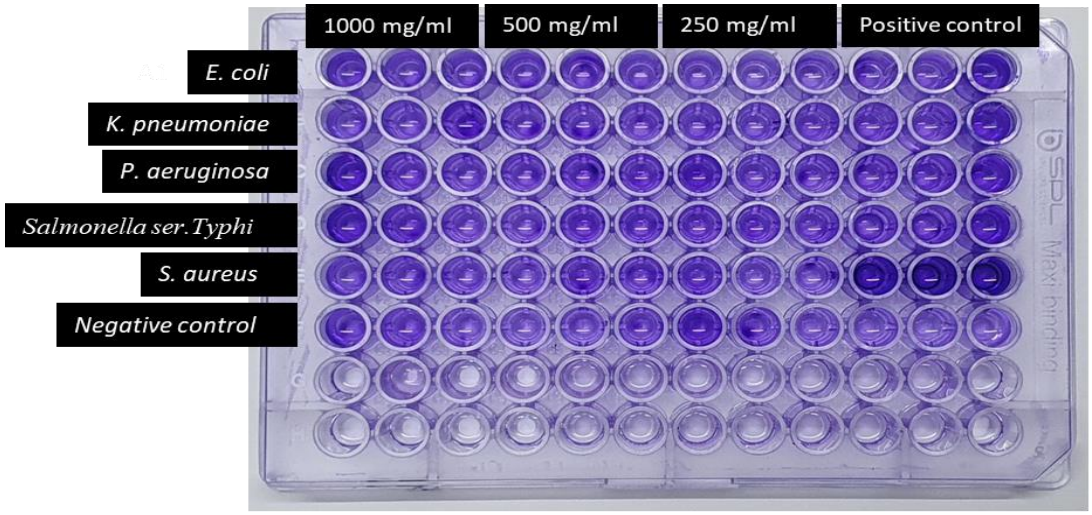


Figure 10: Biofilm intensity before and after treatment with cobalt complex

The Minimum inhibitory concentration (MIC) results indicate that a concentration of 250 mg/ml of copper complex was the lowest required for inhibiting bacterial growth across all tested strains: (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* and *Salmonella typhi*). This result suggests that the copper complex exhibits a relatively uniform bactericidal or bacteriostatic effect against a different bacterial species, at least as measured by this particular assay. On the other hand, cobalt complex also showed an MIC of 250 mg/ml for all bacterial strains except for *Staphylococcus aureus*, which exhibited an MIC of 125 mg/ml. The lower MIC value for *Staphylococcus aureus* indicates that this bacterial species is more susceptible to cobalt complexes compared to the other species tested, Table 8 [38]

Table 8: Results of Minimum inhibitory concentration of Copper and Cobalt complexes against the bacterial isolates

Isolates	Copper complex MIC (mg/ml)	Cobalt complex MIC (mg/ml)
Staphylococcus aureus	250	250
Pseudomonas aeruginosa	250	250
Escherichia coli	250	250
Klebsiella pneumoniae	250	250
Salmonella serotype Typhi	250	125

The present study set out to investigate the antibiofilm potential of copper and cobalt complexes against five clinically relevant bacterial species. To offer a full picture of the metal complexes' antimicrobial capabilities, a mix of biofilm inhibition experiments, optical density measurements, and minimum inhibitory concentration (MIC) tests were used. In addition, agarose gel electrophoresis was used as a supplemental analytical method to evaluate the influence of metal complexes on bacterial plasmid DNA profiles. Prior studies have elucidated the antibacterial properties of copper complexes and their lethal effects, which stem from their capacity to damage genetic material, thereby eliminating antibiotic-resistant genes [39].

One of the indicators that provide insight into biofilm formation and antimicrobial agent efficiency is OD. The study found that using copper and cobalt complexes reduced OD values; however the effectiveness was strain and concentration dependent. When treated with copper complex, *Staphylococcus aureus* showed a considerable reduction in OD, whereas *Pseudomonas aeruginosa* showed just a marginal decrease. This shows that, whereas the copper complex may be uniformly effective across various bacterial strains, the extent to which their efficacious can vary. The Cobalt complex, on the other hand, produced mixed outcomes, making it less predictable as an anti-biofilm agent based only on OD values. These results disagreed with the previous study that showed that complexes (Cu and Co) exhibited no difference in activity between Gram(+) and Gram(-) [40].

The inhibition rates revealed a distinct pattern. The Copper complex exhibited a dose-response relationship, where higher concentrations correlated with greater biofilm inhibition. Cobalt complex did not display such a clear trend, as its inhibition rates remained relatively stable across varying concentrations. *Staphylococcus aureus* was notably susceptible to both metal complexes, particularly at higher concentrations, while other strains like *Escherichia coli* and *Salmonella ser.Typhi* exhibited varied sensitivities. However, it's important to note that the high p-value of 0.57 suggests that these observed trends are not statistically significant, warranting further investigation to draw conclusive insights [40].

When it comes to Minimum Inhibitory Concentrations (MIC), copper and cobalt complexes both exhibited similar efficacy against most bacterial strains at a concentration of 250 mg/ml, except for *Staphylococcus aureus*, which showed a lower MIC for cobalt complex. This implies that for a broader spectrum of bacteria, copper and cobalt complexes may require similar concentration levels to achieve minimum inhibitory effects, although *Staphylococcus aureus* appears to be more susceptible to cobalt complexes [41].

3.7. DNA cleavage study

In order to explore the impact of metal complex exposure on bacterial isolates, this study employed agarose gel electrophoresis as a tool for analyzing changes in plasmid DNA profiles. Plasmid DNA was extracted from all bacterial samples-both control (untreated) and metal complex-treated. The extracted DNA samples were then subjected to agarose gel electrophoresis, in addition to a DNA sizer marker for accurate molecular weight calculation. The electrophoresis data were meticulously documented and analyzed using Gellanalyzer software, with particular attention paid to the number of bands, relative migration values, and related molecular weights. Figure 11 depicts the software results for the copper complex, whereas Figure 12 depicts the results for the cobalt complex [42].

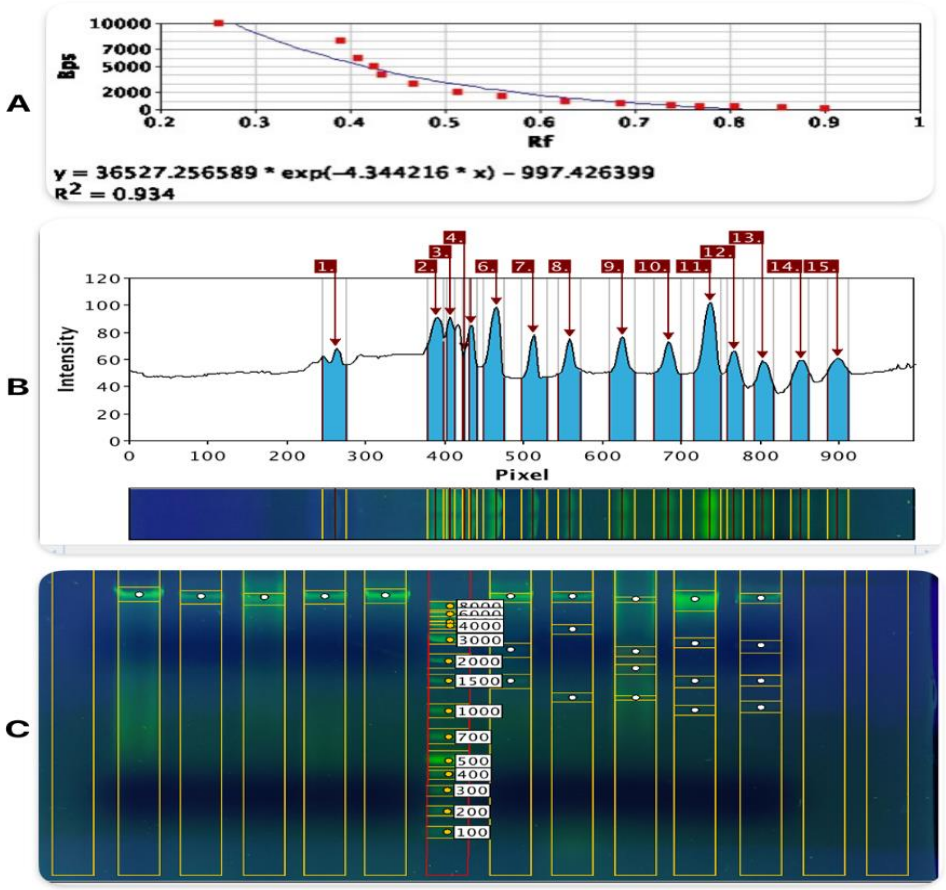


Figure 11: Gel electrophoresis results analysis of the impact of copper complex on DNA cleavage: (A) calibration curve; (B) bands influencing the DNA sizer; (C) DNA bands within the gel

This strategy was used to clarify the molecular responses of the bacterial isolates to the exposure of copper and cobalt complexes, specifically with regard to plasmid DNA migration and molecular weight changes. Agarose gel electrophoresis was used in this work to evaluate

the effect of cobalt and copper complexes on the migration of plasmid DNA in different bacterial isolates. *Staphylococcus aureus* in the absence of treatment showed a single band with a molecular weight of 6,50 Da and a relative migration of 0.31. As opposed to this, Salmonella that had not been treated showed three bands with molecular weights of 10,85, 9,44, and 6,40 Da, which matched relative migrations of 0.25, 0.28, and 0.36, in that order. *Pseudomonas* and *Klebsiella* in the absence of treatment showed two and three bands, respectively, with different molecular weights and relative migrations. Specifically, untreated *Pseudomonas* had bands at 10,75 and 6,30 Da, while untreated *Klebsiella* displayed bands at 10,60, 9,31, and 6,40 Da. *E. Coli*, another untreated isolate, exhibited bands at 10,70, 9,31, and 6,43 Da. Upon treatment with the copper complex, significant alterations were noted in band patterns. For instance, *E. Coli* treated with copper complex exhibited five bands, including new bands at 3,38 and 2,22 Da. *Klebsiella, Pseudomonas, Salmonella,* and *Staphylococcus aureus* showed six bands each upon copper complex treatment, revealing new molecular weights that were not observed in the untreated controls. For the cobalt complex treatment, untreated isolates generally displayed single bands-*Staphylococcus aureus* at 14,151 Da, *Salmonella* at 13,81 Da, *Pseudomonas* at 12,86 Da, *Klebsiella* at 13,49 Da, and *E. Coli* at 13,49 Da. Post-treatment results were diverse: *E. Coli* had three bands, *Klebsiella* showed two, and *Pseudomonas, Salmonella,* and *Staphylococcus aureus* exhibited three to five bands with distinct molecular weights and relative migrations. These findings underscore the significant impact of copper and cobalt complex exposure on the plasmid DNA profiles of different bacterial isolates, suggesting possible implications for bacterial adaptation and metal complex resistance, (Tables 9 and 10) [40, 41]

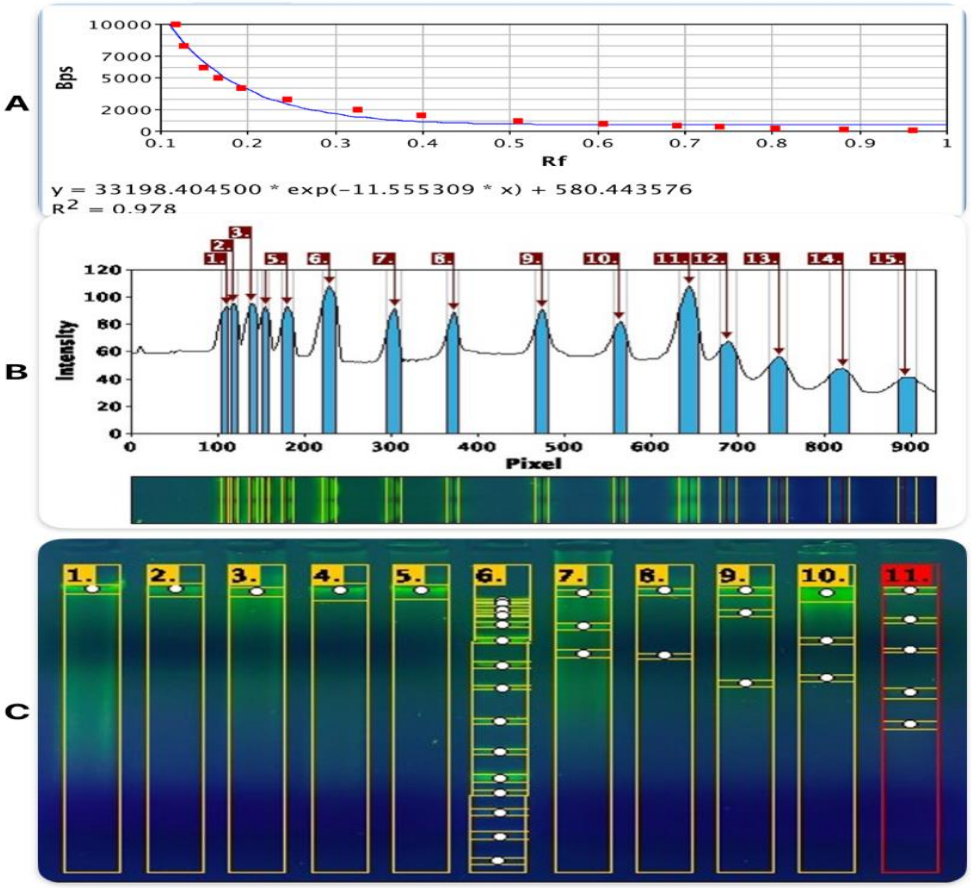


Figure 12: Gel electrophoresis results illustrating the analysis of the cobalt complex's effect on DNA cleavage (A) calibration curve (B) bands identification of the DNA sizer (C) DNA bands within the gel.

Table 9: Changes in Plasmid DNA Profiles: Relative Migration and Molecular Weights Following Copper complex Exposure.

Isolate	Number of bands	relative migrations	Molecular weight
untreated *Staph.aureus*	1	0.077	14151
untreated *Salmonella typhi*	1	0.08	13818
untreated *Pseudomonas aeruginosa*	1	0.086	12867
untreated *klebsiella pneumoniae*t	1	0.082	13493
untreated *E.Coli*	1	0.082	13493
DNA sizer marker	15	0.117	10000
		0.126	8000
		0.148	6000
		0.166	5000
		0.192	4000
		0.245	3000
		0.326	2000
		0.399	1500
		0.509	1000
		0.605	700
		0.691	500
		0.739	400
		0.803	300
		0.882	200
		0.96	100
E.*Coli* treated with cobalt complex	3	0.091	12127
		0.198	3955
		0.287	1784
Klebsiella *pneumoniae* treated with cobalt complex	2	0.08	13334
		0.295	1683
Pseudomonas *earuginusa* treated with cobalt complex	3	0.082	13493
		0.155	6128
		0.383	979
Salmonella *typhi* treated with cobalt complex	3	0.091	12127
		0.246	2510
		0.367	1060
Staph.*aureus* treated with cobalt complex	5	0.082	13493
		0.178	4801
		0.277	1926
		0.413	862
		0.515	667

Table 10: Changes in Plasmid DNA Profiles: Relative Migration and Molecular Weights Following Cobalt Exposure

bacterial isolate	bands number	relative migration	molecular weight
untreated *Staph.aureus*	1	0.313	6502
untreated *Salmonella typhi*	3	0.259	10858
		0.288	9449
		0.367	6404
untreated *Pseudomonas earuginusa*	2	0.261	10755
		0.37	6308
untreated *klebsiella pneumoniae*	3	0.264	10602
		0.291	9313
		0.367	6404
untreated *E.Coli*	3	0.262	10703
		0.291	9313
		0.366	6436
DNA sizer marker	15	0.262	10000
		0.39	8000
		0.408	6000
		0.426	5000
		0.434	4000
		0.466	3000
		0.514	2000
		0.559	1500
		0.627	1000
		0.685	700
		0.739	500
		0.769	400
		0.805	300
		0.854	200
		0.901	100
E.Coli treated with copper complex	5	0.262	10703
		0.291	9313
		0.367	6404
		0.488	3388
		0.559	2220
klebsiella *pneumoniae* treated with cobalt complex	6	0.246	11549
		0.265	10551
		0.291	9313
		0.368	6372
		0.443	4339
		0.596	1741
Pseudomonas *earuginusa* treated with cobalt complex	6	0.264	10602
		0.294	9179
		0.373	6213

Salmonella *typhi* treated with cobalt complex	6	0.492	3312
		0.531	2638
		0.596	1741
		0.241	11826
		0.292	9268
		0.373	6213
		0.475	3644
		0.558	2234
		0.624	1426
Staph.*aureus* treated with cobalt complex	6	0.243	11714
		0.267	10451
		0.371	6276
		0.479	3564
		0.559	2220
		0.618	1490

The study also explored the previously unexamined impact of metal complex exposure on bacterial plasmid DNA. Agarose gel electrophoresis revealed changes in plasmid DNA profiles post metal complex treatment, although the precise implications of these changes are not yet clear and may warrant a study of their own [41]. In the investigation of DNA cleavage through agarose gel electrophoresis, both similarities and divergences are observed when the results are juxtaposed with prior studies. The efficacious role of Cu(II) and Co(II) metal complexes in converting supercoiled DNA into other forms such as open circular or linear structures is reaffirmed, aligning with findings by [43]. These results coincide with the general understanding of DNA cleavage mechanisms, most notably oxidative and hydrolytic processes, as indicated in the work.

However, nuanced perspectives are introduced. Contrary to Sangamesh's observations where DNA cleavage was noted for all tested metal complexes, ineffectiveness in achieving the same outcome is found in certain metal complexes in this study, prompting a reevaluation of their universal applicability [44]. Additionally, the findings concerning antibacterial properties, a secondary point discussed in Nagula's research, diverge from the prevailing literature, warranting further. Therefore, the study serves as both a corroborative and challenging addition to the existing body of knowledge concerning DNA cleavage by metal complexes.

Conclusion

In the present study, the synthesis of Cu(II) and Co(II) complexes of ligand (DBC) derived from 4-Chloro benzoic Acid was conducted, and the synthesized complexes were characterized using different spectroscopic techniques. The data showed the ligand bi-dentate and the geometry complexes tetrahedral. The radical scavenging efficiency of ligand (DBC) and Cu(II) and Co(II) complexes was scrutinized using DPPH screening. A study of the compounds prepared as antioxidants showed that the functional group -N=C-S-, in addition to the presence of the donor electron, clearly affects the radical scavenging efficiency of these compounds. The study provides compelling evidence for the distinct anti-biofilm properties of copper and cobalt complexes against multiple bacterial species. While copper complex consistently demonstrated superior biofilm inhibition relative to cobalt complex, the effects varied across bacterial strains. Moreover, the significant changes detected in plasmid DNA patterns following exposure to the complexes highlight the potential genetic and adaptive consequences for the bacterial organisms. These findings advocate for the careful consideration of copper and cobalt

complexes as therapeutic agents, emphasizing the need for more in-depth exploration of their mechanisms of action and long-term implications for bacterial resistance and adaptation.

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