

Synthesis and Evaluation of the Genotoxic Properties of Coumarin Derivative and its Complexes with Copper and Cobalt

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تخليق وتقييم السمية الجينية لمشتق الكومارين ومترابطاته مع النحاس والكوبلت

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Received: July 07 2026

Accepted: September 17, 2026

Published: October 03, 2026

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Abstract:

A new 6-hydroxy-4-methyl-5-(phenylazo)coumarin derivative (L) was successfully synthesized and characterized. Copper (II) and cobalt (II) complexes of L were also prepared and characterized using elemental (CHN) analysis, (FTIR), (¹H NMR), (MS), UV–Vis spectroscopy, melting-point measurements, and molar conductivity. The spectroscopic and elemental analysis results supported the synthesized ligand and its metal complexes. The complexes exhibited low molar conductivity values, indicating their non-ionic nature. The genotoxic properties of 6-hydroxy-4-methylcoumarin, (L), and its complexes were evaluated using the alkaline comet assay by measuring the percentage of tail DNA (% Tail DNA). Hydrogen peroxide (H₂O₂) was used as a positive control (PC), while untreated cells served as the negative control (NC). The findings demonstrated that H₂O₂ induced the highest level of DNA damage, with a mean % Tail DNA of 32.04% in prostate cancer patient cells. In contrast, ligand L exhibited the lowest mean % Tail DNA (7.51%), significantly lower than both the H₂O₂-treated group (p < 0.0001) and the negative control group (p = 0.0043). The 6-hydroxy-4-methylcoumarin, cobalt (II), and copper (II) complex treatments also significantly reduced DNA damage compared with the H₂O₂ group, while their % Tail DNA values were not significantly different from those of the negative control or L. These findings indicate that L and its complexes did not increase DNA damage under the tested experimental conditions and may exhibit protective effects against oxidative DNA damage.

Keywords: Coumarin, Complexes, Genotoxic, Comet Assay.

الملخص:

تم بنجاح تحضير وتشخيص مشتق جديد من 6-هيدروكسي-4-ميثيل-5-فينيل آزو) كومارين (L). كما تم تحضير معقد النحاس (II) والكوبالت (II) للمركب (L)، وتشخيصهما باستخدام (CHN)، (FTIR)، (¹H NMR)، ومطيافية الكتلة، ومطيافية الأشعة فوق البنفسجية-المرئية (UV–Vis)، وقياس درجة الانصهار، وقياسات التوصيلية المولارية. وقد دعمت

نتائج التحاليل الطيفية والعنصرية التراكيب المقترحة للربطة المحضرة ومعداتها الفلزية. كما أظهرت المعقدات قيمًا منخفضة للتوصيلية المولارية، مما يشير إلى طبيعتها غير الأيونية. تم تقييم الخصائص السمية الجينية لكل من 6-هيدروكسي-4-ميثيل كومارين، والربطة L، ومعدني النحاس (II) والكوبالت (II) باستخدام اختبار المذنب القلوي (Alkaline Comet Assay)، وذلك من خلال قياس النسبة المئوية للحمض النووي في ذيل المذنب (% Tail DNA). واستخدم فوق أكسيد الهيدروجين (H_2O_2) كمجموعة سيطرة موجبة، بينما استُخدمت الخلايا غير المعاملة كمجموعة سيطرة سالبة. أظهرت النتائج أن مجموعة H_2O_2 أحدثت أعلى مستوى من تلف الحمض النووي، بمتوسط % Tail DNA بلغ 32.04% في خلايا مرضى سرطان البروستاتا. في المقابل، أظهرت الربطة L أقل متوسط % Tail DNA، حيث بلغ 7.51%، وكان أقل بصورة معنوية مقارنةً بمجموعة H_2O_2 ($p < 0.0001$) وكذلك مقارنةً بمجموعة السيطرة السالبة. ($p = 0.0043$) كما أدت معاملات 6-هيدروكسي-4-ميثيل كومارين، ومعد الكوبالت (II)، ومعد النحاس (II) إلى خفض معنوي في تلف الحمض النووي مقارنةً بمجموعة H_2O_2 ، في حين لم تكن قيم % Tail DNA لهذه المعاملات مختلفة معنويًا عن مجموعة السيطرة السالبة أو الربطة L. تشير هذه النتائج إلى أن L ومعدني النحاس (II) والكوبالت (II) لم يؤديوا إلى زيادة تلف الحمض النووي تحت الظروف التجريبية المستخدمة، وقد تكون لهم تأثيرات وقائية محتملة ضد تلف الحمض النووي الناجم عن الإجهاد التأكسدي.

الكلمات المفتاحية: الكومارين، المعقدات، السمية الجينية، اختبار المذنب.

Introduction:

Inorganic biochemistry is a field situated at the crossroads of inorganic chemistry and biology. The emergence of inorganic biochemistry is linked to the general development of science. This field encompasses the study of the functions, processing, storage, and applications of metal ions and compounds in biological systems [1,2]. Certain minerals are essential for biological systems, contributing to a variety of vital processes. For example, manganese acts as a redox center in many enzymes. The iron porphyrin complex in hemoglobin is utilized to transfer and store oxygen in the body. Iron and copper centers are also prominently involved in photosynthesis, contributing to the transport of electrons along or across membrane proteins [3-5].

Coumarins are abundant in nature, and their derivatives can be found in many plants and some essential oils. Coumarins are "also found in fruits and microorganisms [6]. They are widely used in biomedical applications and chemical sensing. Bilel (2016) reported that coumarins possess anticancer, antioxidant, antimicrobial, and other properties. The role of coumarins as anticancer agents is supported by research conducted by Zahag and his team (2014), who claimed that 7-hydroxycoumarin inhibits cyclin D1 release. Nasr et al. have also noted the anticancer activity of coumarin [7-9]. However, current research focuses on the use of coumarin as a cytotoxic agent.

Coumarins are highly potent biological agents, possessing anticonvulsant, antioxidant, anticoagulant, antibacterial, antiviral, and antifungal properties. Extensive data exists regarding the antitumor effects of coumarin derivatives, which act at various stages of cancer development. Some exhibit cell-growth inhibition properties, while others have cytotoxic activity [10]. Although some coumarin derivatives exhibit color and fluorescence, they are actually colorless. To enhance their optical stability and induce pathochromatic shifts, coumarin pigments typically contain perfluoroalkyl groups. Coumarin pigments are known to produce shades ranging from yellow-green to orange when applied to polyester fabrics. Furthermore, various types of coumarin have been used in research related to their ability to form complexes. Coumarin has also demonstrated its effectiveness in solar and laser applications [11,12].

According to Wang et al., coumarin derivatives are ideal as colorants and fluorescent dyes, which are widely used as indicators in chemical sensors. Azo compounds are among the most applied dyes because of their diverse applications in different dyeing fields, such as textiles, fibers, and other materials, as well as in coated plastics, biomedical studies, and advanced applications in organic synthesis [13]. Mezgebe, and his team have detailed the structure and dyeing properties of azo compounds in numerous patents and research papers [14]. Hydroxyazocoumarin compounds are mostly applied as analytical reagents, as they are known as metal indicators containing functional groups capable of forming microcomplexes and binding to major metal ions. The synthesis of coumarin pigments has long been of interest to chemists, given that many natural products contain this heterocyclic nucleus. Furthermore, these pigments are effectively used in the chemical, biochemical, physical, and pharmaceutical fields [15,16].

Transition-metal complexes of coumarin derivatives have attracted considerable attention because coordination with metal ions can modify the structural, electronic, physicochemical, and biological properties of the parent ligands. In particular, copper(II) and cobalt(II) ions have attracted considerable attention in coordination chemistry due to their ability to form stable complexes with ligands containing oxygen and nitrogen donor atoms [17,18].

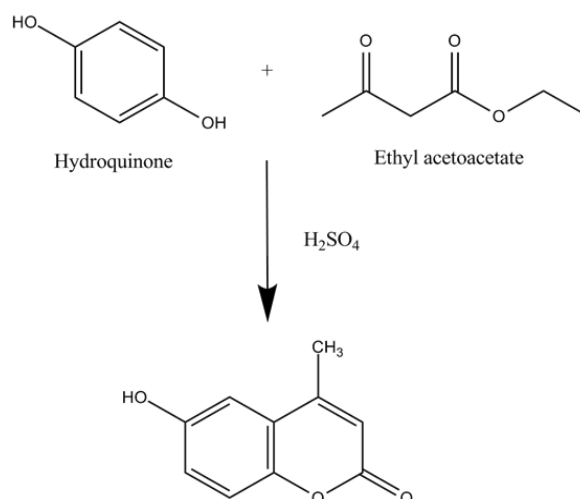
Previous studies have reported Cu (II) and Co (II) with different hydroxycoumarin derivatives. For example, cobalt (II), copper (II), and manganese (II) complexes of 6-hydroxy-4-methylcoumarin have been investigated and evaluated for their biological activities. Other studies have also described Cu(II) and Co(II) complexes containing hydroxycoumarin, coumarinyl, and related donor groups [19]. These studies demonstrate that coordination of coumarin derivatives with transition-metal ions can produce compounds with biological properties different from those of the corresponding free ligands [20, 21]. In particular, the genotoxic properties of (L) and complexes require further investigation. It is therefore important to determine whether these compounds induce DNA damage or alter DNA integrity. Accordingly, the present study was designed to synthesize and characterize (L) and complexes and to evaluate their genotoxic properties using the comet assay. The study also compares the effects of (L) and complexes with appropriate control groups in order to assess their influence on DNA damage.

Material and methods:

Synthesis of coumarin:

The synthesis of coumarin was carried out using hydroquinone and ethyl acetoacetate as starting materials, with concentrated sulfuric acid serving as the reaction medium. The procedure was adapted from a previously published method [12], with suitable modifications.

For the synthesis, 43 mL of concentrated sulfuric acid was initially transferred into a 100-mL glass beaker and cooled using an ice-salt bath. After the temperature had fallen below 10 °C, 20 g of hydroquinone was added gradually to 25 mL of ethyl acetoacetate while the mixture was continuously stirred. The reaction temperature was carefully maintained below 10 °C throughout the addition by means of the ice salt bath. The addition required approximately 5 h to complete.

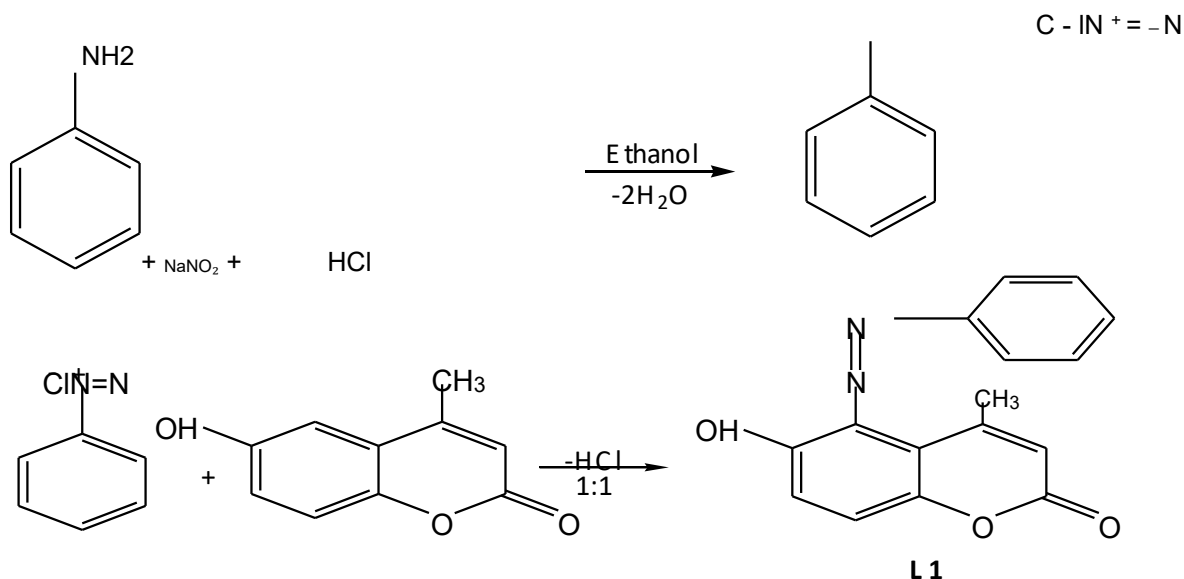


Synthesis of Ligand (L):

The 6-hydroxy-4-methyl-5-(phenylazo) coumarin ligand (L) was synthesized through a diazotization and coupling reaction involving aniline and 6-hydroxy-4-methylcoumarin. The procedure was slightly modified from a previously reported method [18].

A solution of aniline (0.01 M) in ethanol (40 mL) and 2 M hydrochloric acid (20 mL) was stirred thoroughly and cooled to 0–5 °C in an ice-cold brine bath, followed by diazotization with an aqueous sodium nitrite solution (20 mL, 0.01 M). The resulting diazonium solution was slowly added at 0–5 °C to a stirred solution of coumarin (0.01 M) in ethanol (100 mL) containing 10 g of sodium hydroxide, and the mixture was subsequently stirred at room temperature for 1 h.

Then added 100 mL of 2.5 M hydrochloric acid to formation the azo-coumarin derivative. Then recrystallization from ethanol to obtain the 6-hydroxy-4-methyl-5-(phenylazo)coumarin ligand (L)". The reaction procedure was slightly modified from the method reported by [12].



Synthesis of Complexes:

Complexation was carried out separately for Cu (II) and Co (II) using ligand L. For each preparation, 0.001 mol of the metal ion was dissolved in hot ethanol, followed by the addition of ligand L in an equimolar amount to give a 1:1 metal-to-ligand composition. The resulting solutions were brought to a pH of 6.5–7.5 by gradual addition of dilute ammonia solution (1:10) [7].

Determination of complexes:

The metal content of the synthesized solid compounds was determined following the procedure reported by Abdel Latif et al. (2007), with appropriate modifications. A 25–35 mg portion of the solid compound about a 50 mL, then 3 mL of concentrated sulfuric acid and 2 mL H₂O₂. The mixture was gently heated until vapors were evolved and was then boiled. Additional hydrogen peroxide was added when necessary, and the solution was heated until the digestion was complete.

For the determination of the transition-metal ions, the pH of the solution was adjusted to approximately 10 using an ammonia buffer solution. Erythrochrome Black T (EBT) was used as an indicator. The resulting red-colored solution was titrated with a standard 0.01 M ethylenediaminetetraacetic acid (EDTA) solution until a pure blue color was obtained. For the cobalt (II) and copper (II) ions, murexide was used as an additional indicator under the specified alkaline conditions. The cobalt (II) solution produced a yellow color, whereas the copper (II) solution produced an orange color. For the copper (II) determination, a 1:1 ammonia solution was added until the formation of the dark-blue tetraamminecopper (II) complex, [Cu (NH₃)₄]²⁺. The corresponding metal solutions were then titrated with a standard 0.01 M EDTA solution until the appropriate endpoint color was obtained. The metal content was calculated from the volume of the standard EDTA solution consumed during titration.

Characterization of the L and Complexes:

The characterized using several analytical techniques to investigate their physicochemical properties and provide information about their chemical structures. The characterization included elemental (CHN) analysis, (FTIR), (¹H NMR), mass spectrometry, (UV–Vis), melting-point determination, and molar conductivity measurements. Table 1 shown, the instruments for the characterization.

Table 1 Instruments for the characterization

SN.	Instrument / Technique	Brand / Model
1	NMR spectrometer	Varian Gemini 200 MHz
2	Elemental analyser	Vison EA 1108
3	Fourier transform infrared (FTIR) spectrometer	Varian 3100 Excalibur
4	Conductivity meter	Inolab Digital
5	Mass spectrometer	Micro Autof Q86
6	Melting-point apparatus	Stuart SMP 30
7	UV–Vi's spectrophotometer	PerkinElmer Lambda 750

Comet assay procedure:

The alkaline comet assay was conducted with modifications to the procedure of Singh [22]. Heparinized blood cells were embedded in 0.5% low-melting-point agarose on microscope slides and subjected to lysis using a solution containing freshly added Triton X-100 (1%) and DMSO (10%) for at least 1 h. DNA was allowed to unwind in an alkaline buffer for approximately 20 min before electrophoresis at 25 V (0.90 V/cm) and 300 mA for 20 min. The slides were then neutralized with 0.4 M Tris buffer (pH 7.5), rinsed, dried overnight, stained, and examined by fluorescence microscopy. The TM and OTM values were calculated using the corresponding equations described below.

$$TM = \frac{\text{Tail Length} \times \text{Tail\%DNA}}{100}$$

$$OTM = \frac{\text{Tail mean} - \text{Head . mean}}{100} \times \text{Tail\%DNA}$$

Results and discussion:

The synthesized ligand was obtained as a colored solid in a yield of 60%. The compound exhibited a Mp 215 °C. Table 2 shows, the CHN analysis and physical properties. The ligands' elemental analysis findings match the computed values, and its melting point indicates that its purity was 95%

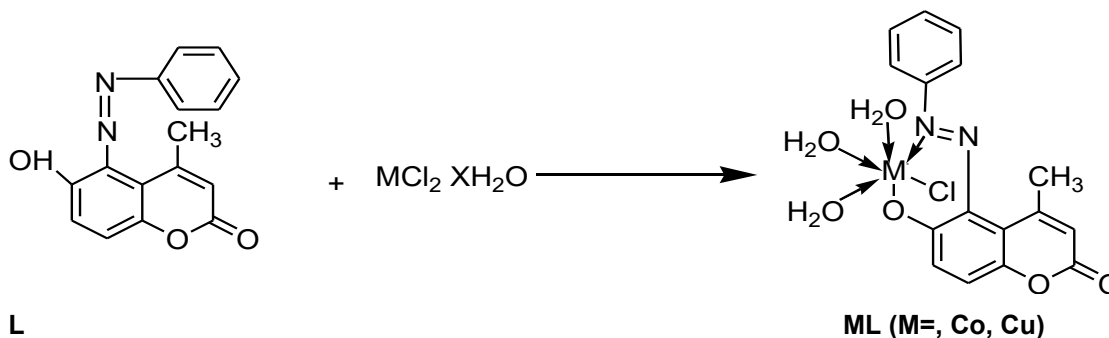
The formation of the ligand was carried out through two successive steps. First, coumarin was synthesized from hydroquinone and ethyl acetoacetate under acidic conditions. In the second step, the synthesized 6-hydroxy-4-methylcoumarin was used in an azo-coupling reaction with the diazonium salt prepared from aniline, leading to the formation of the 6-hydroxy-4-methyl-5-(phenylazo)coumarin ligand (L).

Table (2): Elemental analysis and physical properties of ligand and complexes

Ligand	Molecular formula	Color	%	Mp (o C)	Calculated was found%		
					C	H	N
L	C 16 H 12 O 3 N 2	Brown	60	215	66 (68.57)	5.9 (4.28)	11.9 (10.00)

Complex	Yield (%)	m.p. (°C)	C Found (%)	C Calcd. (%)	H Found (%)	H Calcd. (%)
CoL	85	653	44.50	44.92	3.16	3.97
CuL	80	899	45.30	44.44	3.14	3.93

Formation complexes: The reaction scheme for the formation of the metal complexes (ML) from the synthesized ligand (L).



Interpretation of the FTIR Spectrum of Synthesized Ligand and complexes":

Table 3 and Table 4 shows, principal IR bands of (L) and complexes, the FTIR of L and complexes show noticeable changes after complexation. FTIR of complexes, the band detected within the range 1410-1450 cm⁻¹ attributed to ν) N=N (in L is moved to range 1308-1337 cm⁻¹ after complexation, showing that N is involved in the chelation. Coordination via the hydroxyl group is shown by a shift in the frequency of the hydroxyl band from 3339-3200 cm⁻¹ in free ligands to 3411-3453 cm⁻¹.

In the range 3411-3453 cm^{-1} is attributed to $\delta(\text{O-H})$, whereas $\delta(\text{C=O})$ is discovered in the region 1628-1643 cm^{-1} [23].

Table (3): Principal FTIR Bands of the Ligand (L)

Band assignment	Ligand L (cm^{-1})
$\nu(\text{O-H})$	3398
$\nu(\text{C=O})$	1600
$\nu(\text{C=C})$	1525
$\nu(\text{N=N})$	1450
$\nu(\text{Ar-H})$	3000
$\nu(\text{C-N})$	1250
$\gamma(\text{C-H})$	899

Table (4): FTIR of the Complex Metal Ions (Co^{2+} , Cu^{2+})

Complexes	OH	$\nu \text{ C=O}$	$\nu \text{ N=N}$	M-N
CoL	3453	1643	1337	436-453
CuL	3453	1643	1337	436-453

Interpretation of NMR Spectra of the Synthesized Ligand and complexes:

Table 5 and Table 6 shown the NMR Spectra of ligand and complexes. The chemical shifts (^1H NMR) spectra of the compounds. The absence of hydroxyl (OH) group signals in complexes at 12.54 ppm in the free bonds indicates the loss of the hydroxyl group proton and the bonding of the metals via the hydroxyl group.

Table (5): ^1H -NMR for the Synthesized (L)

Chemical shift, δ (ppm)	Assignment
12.54	O-H
7.42-7.39	Aromatic C-H protons
7.14	Pyrone ring C-H
2.19	Ring CH_3

Table (6): Nuclear Magnetic Resonance Spectroscopy Data for Complexes

Complex M: L	Chemical shift	Assignment
CuL	7.58-7.36	Aromatic C-H protons
	7.14	Pyrone ring C-H
	2.41	CH_3 pyrone ring
CoL	7.30-7.16	Aromatic C-H protons
	6.65	Pyrone ring C-H
	2.63	CH_3 pyrone ring

Ultraviolet and visible spectroscopy:

UV-Vis spectra of ligand L and its metal complexes were recorded in ethanol (1×10^{-4} M) over 250–500 nm. Ligand L showed two prominent bands at about 270 and 330 nm (Table 8), assigned to $\pi \rightarrow \pi^*$ transitions and $n \rightarrow \pi^*$ transitions of the azo ($-\text{N}=\text{N}-$) group, respectively, with the latter associated with charge-transfer behavior.

When ligand L binds to copper (II) and cobalt (II) ions, significant changes in the position and intensity of these absorption bands are observed compared to the free ligand. These spectral changes indicate the involvement of the nitrogen atom in the azo group and the phenolic oxygen atom in ligand L in binding to the metal ions. The observed spectral behavior confirms the formation of metal-ligand complexes and supports the proposed binding pattern of ligand L with copper (II) and cobalt (II) ions.

Table 8 Ultraviolet and Visible Absorption Bands of the L.

Compound	Absorption, λ (nm)			
	Peak 1	Assignment	Peak 2	Assignment
L	270	$\pi-\pi^*$	330	$n-\pi^*$

Comet Assay Results:

Comparison of Treatments to Baseline (Negative Control):

Figure 1, illustrates the percentage of DNA damage (% Tail DNA) in healthy individuals using the comet test across various treatment groups: coumarin, (L) and CoL, and CuL complexes. Hydrogen peroxide (H₂O₂) served as (PC) and (NC). The findings show no significant DNA damage from the tested compounds compared to the negative control, whereas hydrogen peroxide treatment resulted in significant fragmentation.

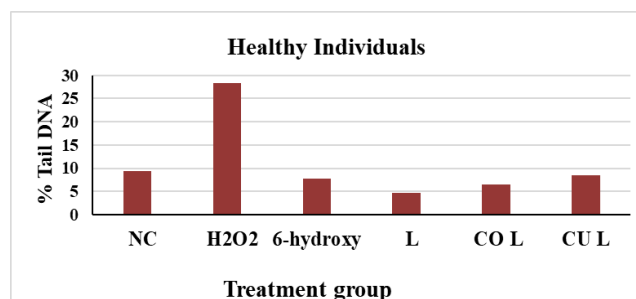


Figure 1: The percentage of DNA damage (% Tail DNA)

In prostate cancer patients:

The prostate cancer data demonstrated that the H₂O₂ group exhibited the highest average DNA damage at 32.04% as show in Figure 2. This level of damage was significantly elevated compared to all other experimental groups (6-hydroxy), (L), (CoL), and (CuL), with all pairwise comparisons producing $p < 0.005$.

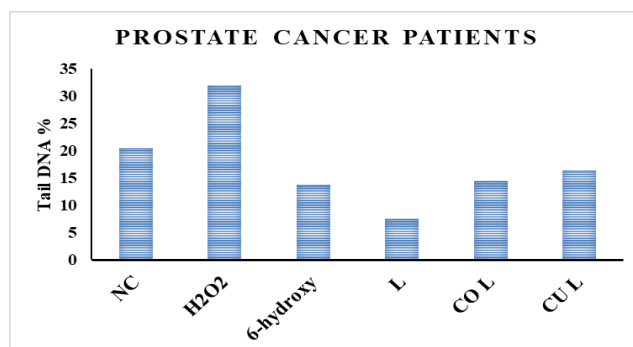


Figure (2): Genotoxicity assessment measured (Tail DNA %)

Figure 3 shows, the comparative genotoxicity assessment measured via the comet assay (% TAIL DNA) between healthy individuals (blue bars) and prostate cancer patients (orange bars) following exposure to various test compounds and controls. Higher % Tail DNA values indicate greater levels of DNA strand breaks.

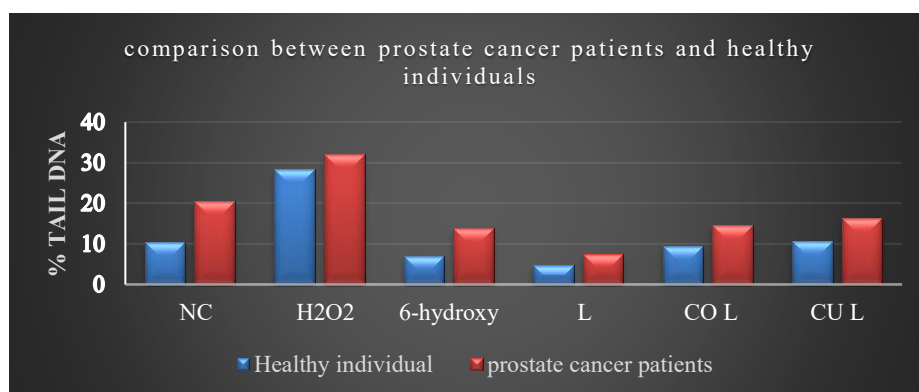


Figure (3): Comparison between prostate cancer patients and healthy individuals

Tail DNA%, parameters, offers a sensitive measurement of genomic DNA damage in prostate cancer cells subjected to oxidative and therapeutic conditions. The six treatment groups that were assessed in

this study (NC, H₂O₂) Ligand, Coumarin, Cobalt complex, and Copper complex) showed a highly significant variance ($p < 0.0001$). Compared to all other groups, the positive control group had the highest mean Tail DNA% (32.04%), indicating significant oxidative DNA strand breakage ($p < 0.005$). This finding aligns with prior research indicating that hydrogen peroxide induces significant single- and double-strand DNA breakage in prostate cancers. This occurs through the generation of hydroxyl free radicals via Fenton-type reactions [24, 25].

Remarkably, 6-hydroxy-4-methyl-5-phenylisocoumarin, the synthesized uncomplexed ligand, produced the lowest mean tail DNA % (7.51%) and showed the strongest antigenotoxic and protective profiles. In contrast, metal coordination frequently increases DNA breakage or cytotoxicity by redox cycling or intercalative binding in research on transition metal-isocoumarin complexes [26, 27]. The absence of increased genotoxicity in Co (II) and Cu(II) complexes in our prostate cancer experimental setup indicates that these coordination compounds maintain high biological safety toward DNA integrity at the tested doses, while the free ligand continues to be the most efficient direct ROS scavenger as can be seen in Figure 4.

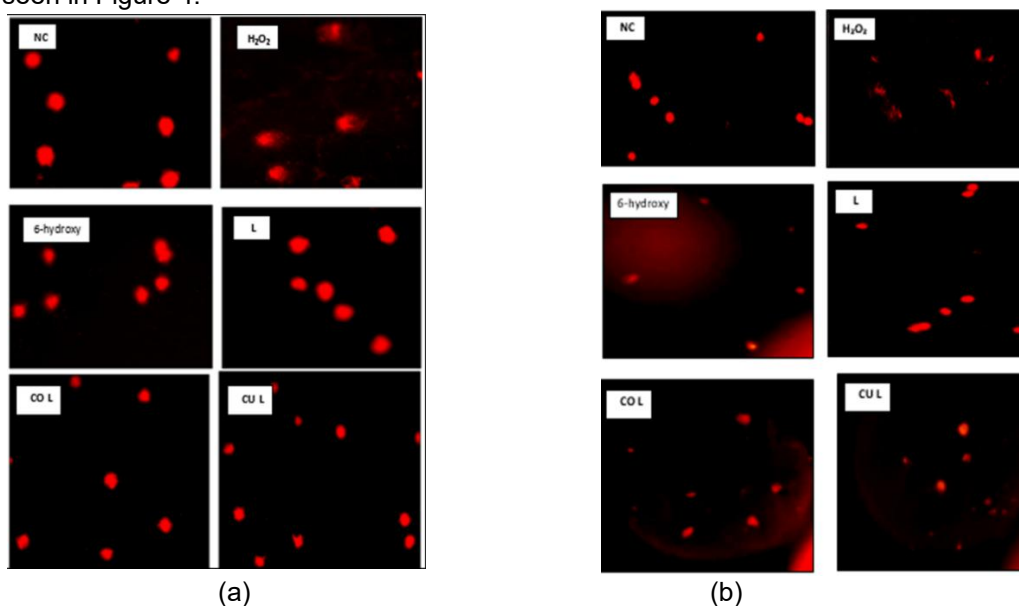


Figure (4): Representative Comet Assay Images of the Control and Treatment Groups on (a) healthy cells (b) unhealthy cells (Prostat cancer)

Conclusion:

In this study, (L) and its complexes were successfully synthesized and characterized using elemental analysis and spectroscopic techniques. The characterization results supported the formation of the proposed metal complexes. The alkaline comet assay showed that H₂O₂ produced the highest level of DNA damage, whereas L showed the lowest mean Tail DNA value (7.51%). The Cu(II) and Co(II) complexes did not significantly increase DNA damage compared with the negative control under the tested conditions. These findings suggest that L and its metal complexes have low genotoxic effects and warrant further biological investigation.

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