

Article

Experimental and Theoretical Studies on DNA Binding and Anticancer Activity of Nickel(II) and Zinc(II) Complexes with *N*-(8-Quinolyl) Salicylaldimine Schiff Base Ligands

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Abstract: Transition metal complexes of nickel(II) with 5-bromo-*N*-(8-quinolyl)salicylaldimine (HqsalBr, HL¹); [Ni(qsalBr)₂] (1) and 3,5-dibromo-*N*-(8-quinolyl)salicylaldimine (HqsalBr₂, HL²); [Ni(qsalBr₂)₂] (3) including zinc(II) complex with HL¹, [Zn(qsalBr)₂] (2), have been synthesized and successfully characterized using various techniques, namely IR, NMR, mass spectrometry, thermogravimetric analysis (TGA), and single crystal X-ray crystallography. DFT calculations were employed to examine the structural and electronic parameters of the complexes at their optimized geometries. The complexes showed strong DNA-binding activities, assessed by UV-Vis and fluorescence spectroscopy, primarily through intercalation. Molecular docking investigations were carried out to provide profound insights into the interaction mechanisms of these complexes with DNA and lung cancer cells. These computational studies revealed that [Ni(qsalBr₂)₂] (3) exhibits the most favorable negative binding energies, −9.1 kcal/mol with DNA and −9.3 kcal/mol with cancer cells, facilitated by hydrogen bonding and hydrophobic interactions. Furthermore, the in vitro anticancer activity was evaluated against the A549 human lung adenocarcinoma cell line, with [Zn(qsalBr)₂] (2) exhibiting the highest potency against this cancer cell line.

Keywords: schiff base; DNA binding; ANTI-lung cancer; molecular docking



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1. Introduction

Schiff bases, known for their versatility as ligands through the azomethine N-atom, are synthesized via the reaction of amines with substituted salicylaldehydes. Additionally, these N-containing ligands have been intensively used as chelating ligands in coordination chemistry, serving as bidentate N,O-, tridentate N,O,O-, N,O,N-, and tetradentate N,N,O,O- donors [1–8]. Consequently, transition metal complexes of these ligands have found applications in various fields, including sensors [9,10], catalysts for organic synthesis [11,12], and some biological activities [13,14]. The biological and physical properties of transition metal Schiff base complexes are influenced by the coordinating characteristics of the ligands towards the transition metal centers. A wide range of biological activities, including antibacterial, antifungal, antimicrobial, and anticancer activities, have been exhibited by different families of nickel(II) and zinc(II) complexes. Schiff base metal complexes also aid in the design of more effective anticancer agents, potentially with fewer side effects than traditional platinum-based drugs [15,16]. DNA serves as a valuable tool in studying the interaction of transition metal complexes with biological molecules. Literature reviews have indicated that protein and enzyme synthesis involved in the replication and

transcription of hereditary information carried by DNA [17]. Hence, nucleic acids can be utilized to design the effective anticancer agents [18], as DNA is the primary target for these complexes to bind with. The interaction between DNA and these substrates causes DNA damage, preventing cell division and leading to cell death [19,20]. Several attempts have been made to investigate the use of Schiff base complexes for inhibiting cancer cell growth, especially Schiff base metal complexes in anticancer and antibacterial evaluations [21]. In Thailand, lung cancer incidence has been rising, reflecting global trends. This makes it a critical area for research within the country. Apart from smoking, which is a primary risk factor for lung cancer, the increase in industrialization in Thailand has led to broader exposure to pollutants and hazards which contribute to lung cancer risk [22].

In the present study, 5-bromo-*N*-(8-quinolyl)salicylaldimine (HqsalBr, HL¹) and 3,5-dibromo-*N*-(8-quinolyl)salicylaldimine (HqsalBr₂, HL²), derived from the condensation of salicylaldehyde with 8-aminoquinoline, along with their complexes (**1**, **2**, and **3**) where the metal atoms are nickel(II) and zinc(II), were synthesized and characterized using spectroscopic techniques and X-ray crystallography. The biological study with calf thymus DNA (CT-DNA), the minimum inhibitory concentration (IC₅₀) in anticancer activity, and the molecular docking of Schiff base ligands and their complexes were also reported.

2. Materials and Methods

2.1. Materials

All chemicals used were of analytical grade and of the highest purity available. They included 8-aminoquinoline (Sigma, Singapore), 5-bromosalicylaldehyde (Sigma, Singapore), and 3,5-dibromosalicylaldehyde (Sigma, Singapore). The metal salts used were Ni(OAc)₂·4H₂O (Sigma, Singapore) and Zn(NO₃)₂·6H₂O (Sigma, Singapore). Organic solvents, namely diisopropyl ether, dichloromethane (DCM), tetrahydrofuran (THF), methanol, and ethanol, were used without further purification unless otherwise stated. Protein-free calf thymus DNA (CT-DNA), sourced from Invitrogen, was kept at temperatures between 0 and 4 °C. The purity of this DNA was verified by assessing its absorbance at 260 nm. Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) buffer (5 mM Tris-HCl, 50 mM NaCl, pH of 7.2) was used for preparing DNA stock solutions. Ethidium bromide (EB) was purchased from Loba Chemie and used without further purification. The A549 cell line (human lung carcinoma) was kindly provided by National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.. Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma-Aldrich and Tokyo Chemical Industry (Bangkok, Thailand), respectively. Minimum essential medium Eagle (MEM), Trypsin-EDTA, and penicillin-streptomycin (Pen-Strep) were purchased from Gibco (Bangkok, Thailand).

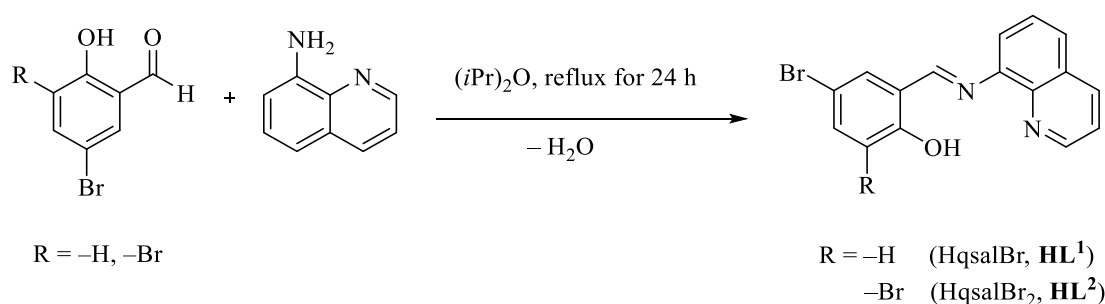
2.2. Instruments

IR spectra were obtained through the ATR technique using a Perkin-Elmer FT-IR spectrophotometer, model 1650, covering the wavenumber range of 4000–400 cm^{−1}. ¹H NMR spectra were collected with a 400 MHz Bruker instrument. Spectrophotometric measurements in solution were conducted using a UV-Vis spectrophotometer (SPECORD® 200 PLUS, Analytik Jena, Jena, Germany). Mass spectrometry was conducted using an electrospray ionization (ESI) method on an Agilent 6540 LC/MS system (United States). Thermogravimetric analysis was carried out using simultaneous thermogravimetry and differential scanning calorimetry (LINSEIS, STA PT 1600, Selb, Germany). The binding study with CT-DNA was conducted by absorption titration using a UV-Vis spectrophotometer (SPECORD® 200 PLUS, Analytik Jena, Jena, Germany) and fluorescence titration using a spectrofluorometer FluoroMax-4 (HORIBA Scientific, Kyoto, Japan). The absorbance of forming formazan at 570 nm was measured using an EnSpire Multimode Plate Reader (PerkinElmer, MA, USA).

The molecular structure of $[\text{Ni}(\text{qsalBr}_2)_2]$ (**3**) was determined by single crystal X-ray diffraction using a Bruker D8 VENTURE diffractometer. The crystal was irradiated with MoK_α radiation ($\lambda = 0.71073 \text{ \AA}$). The structure was solved by Olex2 1.5 with ShelXS and the anisotropic thermal parameters were refined to all non-hydrogen atoms. Hydrogen atoms were inserted at calculated positions using a riding model. The Mercury 3.10 programs were utilized to prepare the materials and molecular graphics for publication.

2.3. Synthesis of Schiff Base Ligands

5-bromo-*N*-(8-quinolyl)salicylaldehyde (HqsalBr , HL^1) was synthesized according to the literature (68.0%) [23]. 3,5-dibromo-*N*-(8-quinolyl)salicylaldehyde (HqsalBr_2 , HL^2) was synthesized from condensing 8-aminoquinoline (4 mmol, 579.1 mg) dissolved in hot di-isopropyl ether (20 mL, 60°C) with 3,5-dibromosalicylaldehyde (4 mmol, 578.0 mg). The reaction mixture was refluxed for 24 h. The slow evaporation of the solution to approximately 5 mL yielded red crystals of the desired product. The product was filtered, washed with ethanol several times, and dried at room temperature (86.9%). The reactions are presented in Scheme 1.



Scheme 1. Preparation of Schiff base ligands.

2.4. Synthesis of Complexes

2.4.1. Synthesis of Complex $[\text{Ni}(\text{qsalBr})_2]$ (**1**)

The metal salt $\text{Ni}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ (0.25 mmol, 62.7 mg) dissolved in methanol (1.5 mL) was added dropwise to a stirred solution of HL^1 (0.50 mmol, 163.8 mg) in DCM (3.5 mL). The mixture was refluxed for 4 h, leading to the formation of a precipitate. The product was separated by filtration, washed with ethanol several times, and dried at room temperature (96.7%).

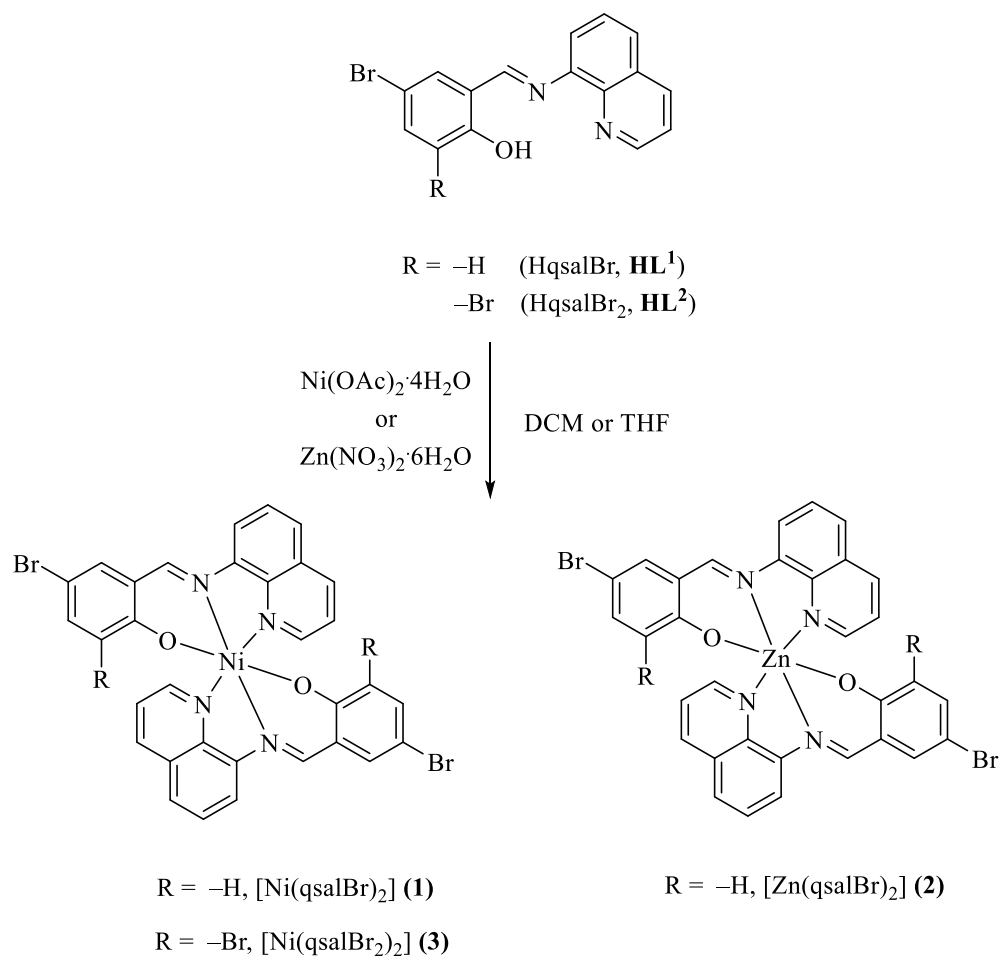
2.4.2. Synthesis of Complex $[\text{Zn}(\text{qsalBr})_2]$ (**2**)

Complex $[\text{Zn}(\text{qsalBr})_2]$ was synthesized using the same procedure as complex **1**, with $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.25 mmol, 74.6 mg) substituted for $\text{Ni}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ (85.9%).

2.4.3. Synthesis of Complex $[\text{Ni}(\text{qsalBr}_2)_2]$ (**3**)

The metal salt of $\text{Ni}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ (0.25 mmol, 62.3 mg) dissolved in methanol (1.5 mL) was added dropwise to a stirred solution of HL^2 (0.50 mmol, 203.2 mg) in THF (5.5 mL). The mixture was refluxed for 4 h, resulting in the formation of a precipitate. The product was separated by filtration, washed with ethanol several times, and dried at room temperature (94.4%). Single crystals of complex **3** were obtained in a reddish-yellow color from recrystallization of the product in DCM [24].

The reactions for the synthesis of all complexes are presented in Scheme 2.



Scheme 2. Preparation of zinc(II) and nickel(II) complexes.

2.5. DFT Calculations

The optimized geometries and molecular properties of ligands HL^1 and HL^2 , along with their corresponding complexes with nickel(II) and zinc(II) metal ions, were investigated. All structures were fully optimized using the hybrid DFT (B3LYP) [25,26] functional with the 6-311G(d,p) [27] basis set for the H, C, N, O, and Br atoms. The Los Alamos effective core potential (ECP) including relativistic effects plus double atomic orbitals (LANL2DZ) [28–30] basis set was employed for the metal center (Ni and Zn atoms). All calculations were performed using the Gaussian 09 suite of programs [31]. The following equations were used to compute the reactivity descriptors: HOMO–LUMO energy gap (ΔE), absolute hardness (η), softness (S), chemical potentials (μ), absolute electronegativity (χ), and global electrophilicity (ω). These were computed using Equations (1)–(6) below [29,30,32], as follows:

$$\Delta E = (E_L - E_H) \quad (1)$$

$$\eta = (E_L - E_H)/2 \quad (2)$$

$$S = 1/2\eta \quad (3)$$

$$\mu = (E_L + E_H)/2 \quad (4)$$

$$\chi = -\mu \quad (5)$$

$$\omega = \mu^2/2\eta \quad (6)$$

2.6. DNA Binding Study

2.6.1. Electronic Absorption Titration

The interaction between the complexes and CT-DNA was assessed using a UV-Vis spectrophotometer, with the absorption spectrum set to scan from 280 to 600 nm. The experimental solutions were prepared by combining a fixed concentration of 50 μM of the complex dissolved in DMSO with different concentrations of CT-DNA, successively added in the presence of a buffer. The range of CT-DNA concentration was 0–10 μM . After each addition, the solution was mixed and allowed to equilibrate for 10 min. A reference cell containing DNA and solvent (at the same concentration as DMSO in the complex solution) was used simultaneously to nullify the absorbance of DNA at the measured wavelength. The binding constant (K_b) of the complexes with CT-DNA was determined from the absorption titration data using the Wolfe–Shimer equation [33].

$$[\text{DNA}]/\epsilon_a - \epsilon_f = [\text{DNA}]/\epsilon_b - \epsilon_f + 1/K_b (\epsilon_b - \epsilon_f) \quad (7)$$

where $[\text{DNA}]$ denotes the concentration of DNA in base pairs and ϵ_a , ϵ_f , and ϵ_b represent the observed absorbance (A_{obs}) per [complex], the extinction coefficient for unbound Schiff base complexes, and the extinction coefficient for the complexes when fully bound to DNA, respectively. A graph of $[\text{DNA}]/(\epsilon_a - \epsilon_f)$ plotted against $[\text{DNA}]$ allows for the calculation of the binding constant (K_b), determined by the ratio of the graph's slope to its intercept.

2.6.2. Fluorescence Titration

Emission intensity measurements were recorded on a fluorescence spectrometer with excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}) ranges set at 525 nm and 545–700 nm, respectively. Successive additions in fluorescence experiments were performed by adding Schiff base complexes ranging from 0 to 10 μM to the sample containing 10 μM of CT-DNA in buffer and 4 μM of ethidium bromide. After each addition, the solution was mixed and allowed to equilibrate for 15 min. The quenching efficiency was determined by the linear Stern–Volmer equation [34].

$$I_0/I = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (8)$$

$$K_{sv} = K_q\tau_0 \quad (9)$$

where I and I_0 represent the emission intensities of the EB–DNA solution in the presence and absence of the quencher (Schiff base complex), respectively; meanwhile, $[Q]$ denotes the concentration of the complex. The τ_0 represents the average lifetime of the emitting system without quencher (3 ns), and K_q is the quenching constant. K_{sv} is calculated from the Stern–Volmer plots by the slope of the diagram F_0/F versus $[Q]$, with τ_0 [35]. The quenching constants and Stern–Volmer constants of the compounds can be determined according to Equations (8) and (9). Additionally, the number of binding sites (n) were calculated from the Scatchard Equation (10).

$$\log[(F_0 - F)/F] = \log K + n \log[Q] \quad (10)$$

2.7. Anticancer Study

A549 (lung carcinoma) cells were cultured in modified Eagle's medium (MEM) supplement with 10% fetal bovine serum (FBS) and 100 unit/mL penicillin-streptomycin, and then incubated in a standard tissue culture incubator at 37 °C and 5% CO_2 . The culture medium was changed twice a week. Subsequently, 96-well culture plates were seeded with A549 cell at a density of 10,000 cells/100 μL /well and incubated for 12 h to allow cell adherence. The cells were then treated with different concentrations of ligands and complexes (0–100 μM). After a 48 h incubation period, the medium in each well was replaced with 100 μL of MTT solution (0.5 mg/mL in culture medium) and further incubated at 37 °C for 3 h. Following this, the MTT solution was aspirated, and the formed formazan crystals

were suspended in 100 μL of DMSO. The absorbance at 570 nm was measured, and the half maximal inhibitory concentration (IC_{50}) was calculated from the plot between %inhibition and $\log[\text{concentration}]$ using GraphPad Prism8 software. The experiment was performed in triplicate.

2.8. Molecular Docking

To gain insights into the molecular interactions of the synthesized complexes with DNA and lung cancer cells, the targets of our docking study were downloaded from the Protein Data Bank. These targets included the synthetic DNA dodecamer (PDB id: 1bna) [36] and the epidermal growth factor receptor (EGFR) kinase domain, implicated in non-small cell lung cancer (PDB id: 7ukv) [37]. The docking studies were performed using AutoDock Vina (v.1.1.2), with protein and compound structures prepared using AutoDockTools (ADT v.1.5.7) to convert the PDB files to PDBQT formats containing all information about atom types and charges for both compounds and protein. All docking protocols were executed on the Windows 10 operating system.

3. Results

3.1. IR Spectra

The infrared spectra of ligands and complexes displayed several modes of vibrations with variable intensities in the region of $4000\text{--}400\text{ cm}^{-1}$. The main stretching frequencies of the ligands and their complexes are shown in Table 1.

Table 1. IR spectroscopic data of HL^1 , HL^2 , and Complexes 1–3.

Compounds	IR (cm^{-1})				
	$\nu(\text{C}=\text{N})$	$\nu(\text{C}=\text{O})$	$\nu(\text{C}=\text{N})$ (Quinoline)	$\nu(\text{M}=\text{O})$	$\nu(\text{M}=\text{N})$
HqsalBr (HL^1)	1606	1282	1423	-	-
HqsalBr ₂ (HL^2)	1607	1283	1425	-	-
[Ni(qsalBr) ₂] (1)	1603	1231	1417	470	419
[Zn(qsalBr) ₂] (2)	1605	1234	1415	468	416
[Ni(qsalBr ₂) ₂] (3)	1604	1240	1413	489	426

The stretching of azomethine and quinoline in free Schiff base ligand HL^2 was observed at 1607 cm^{-1} and 1425 cm^{-1} , respectively [23]. These numbers were found at lower frequencies for [Ni(qsalBr₂)₂] (3) (1604 cm^{-1} and 1413 cm^{-1}). The observed wavenumbers indicated the coordination of these functional groups towards the metal centers for the complex formation. Additionally, the band at 1283 cm^{-1} belongs to C=O of the Schiff base ligand and also shifts to 1240 cm^{-1} in complex 3. The changes in wavenumbers observed in complex 1 and complex 2 with ligand HL^1 are also similar to those in the complex of ligand HL^2 . Moreover, new bands appeared in the spectra of all the complexes in the region of $468\text{--}489\text{ cm}^{-1}$ and $416\text{--}426\text{ cm}^{-1}$ which were assigned to the vibrations of $\nu(\text{M}=\text{O})$ and $\nu(\text{M}=\text{N})$, respectively [38].

3.2. UV–Vis Absorption Spectra

The UV–Vis absorption spectra of Schiff base ligands and their complexes with a concentration of $1.0 \times 10^{-3}\text{ M}$ were recorded in THF solvent within the wavelength range of $300\text{--}600\text{ nm}$ at room temperature. The spectra of ligands and complexes are presented in Figure 1.

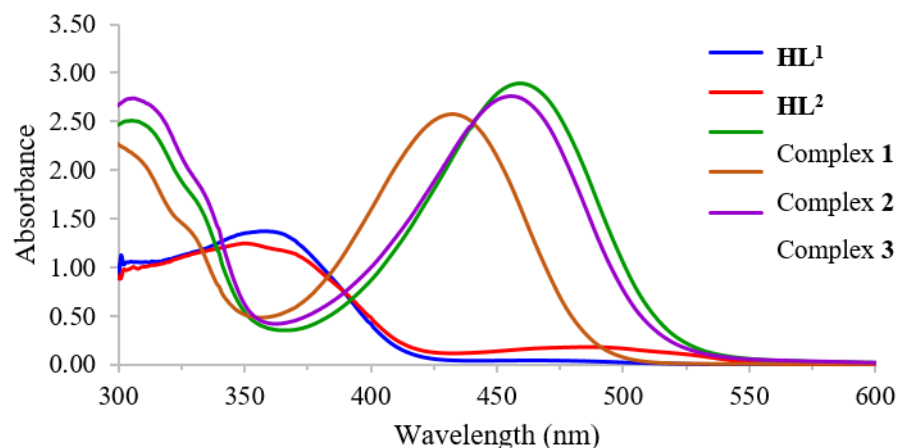


Figure 1. UV-Vis spectra of HL¹, HL², and complexes 1–3 in THF.

The free Schiff base ligands HL¹ and HL² displayed moderated absorption at 357 nm and 351 nm, respectively, which can be contributed to the $n-\pi^*$ transition of the azomethine group, whereas the $d-d$ transition also appeared as a broad peak at approximately 475 nm [39]. The stoichiometric ratio between metal ions and Schiff base ligand was determined using Job's method. All measurements were made at different wavelengths according to the observed $\lambda_{\max}$ (1; 484 nm, 2; 460 nm and 3; 487 nm). The results revealed the mole fraction of 1:2 for metal to ligands. The stability constants of the synthesized complexes were evaluated, and the results are shown in Table 2. Among the synthesized complexes, nickel(II) with d^8 electron configuration provided a higher formation constant compared to zinc(II) with d^{10} electron configuration. Furthermore, the mono-substituted Schiff base ligand also contributed a greater formation constant for the nickel(II) complex. This might be attributed to the steric and electronic effects caused by the bromo-substituent at the meta-position of the azomethine. Such a group might withdraw electron density from the metal to $-O, N, N$ bond, leading to a weaker coordination binding and consequently reducing the formation constant.

Table 2. Appearance, maximum wavelength, and formation constant of complexes.

Compounds	Color (Solid)	Color (Solution)	$\lambda_{\max}$ (nm), $\epsilon(M^{-1}cm^{-1})$	Formation Constant
HqsalBr (HL ¹)	Orange	Pale yellow	357 (12,428)	-
HqsalBr ₂ (HL ²)	Red	Pale yellow	351 (13,732)	-
[Ni(qsalBr) ₂] (1)	Brown	Deep orange	484 (20,545)	2.52×10^6
[Zn(qsalBr) ₂] (2)	Orange	Light orange	460 (15,690)	5.67×10^5
[Ni(qsalBr ₂) ₂] (3)	Brown	Deep orange	487 (14,810)	8.69×10^5

3.3. Thermogravimetric Analysis (TGA)

The thermal properties of complexes were characterized using the thermogravimetric analysis (TGA) method in the temperature range of 30–1000 °C (Figure 2). The effects of temperature and the percentage of mass loss are listed in Table 3.

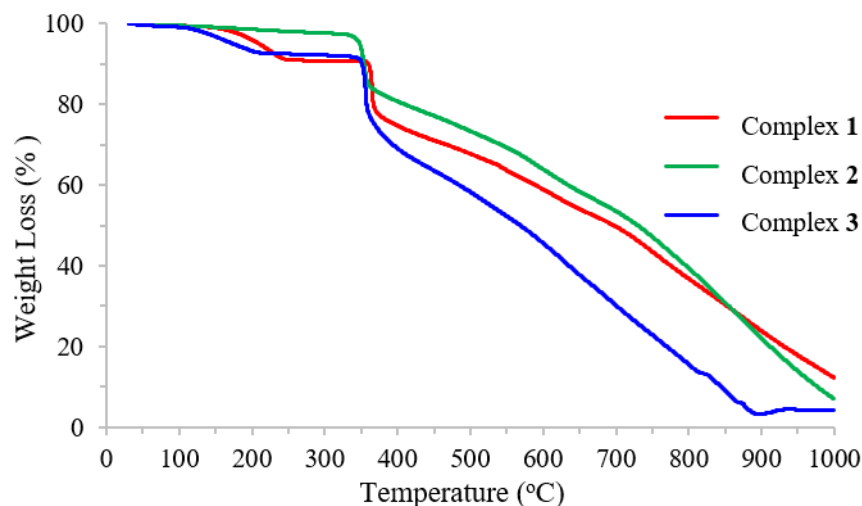


Figure 2. Thermogravimetric analysis (TGA) of Schiff base ligand and complexes.

Table 3. Thermogravimetric analytical results of Schiff base ligand and complexes.

Compounds	TG Range (°C)	Weight Loss Exp. (Calcd.)%	Total Weight Loss Exp. (Calcd.)%	Assignment	Residue
[Ni(qsalBr) ₂] (1) C ₃₂ H ₂₀ Br ₂ N ₄ NiO ₂	138–467	22.64 (22.89)	86.49 (85.07)	Br ₂	NiO, C atom
	467–685	20.48 (19.78)		C ₉ H ₂ NO	
	685–1000	43.37 (42.40)		C ₂₀ H ₁₈ N ₃	
[Zn(qsalBr) ₂] (2) C ₃₂ H ₂₀ Br ₂ N ₄ ZnO ₂	137–466	22.22 (22.11)	84.64 (83.81)	Br ₂	ZnO, C atom
	466–682	20.83 (19.62)		C ₉ H ₂ NO	
	682–1000	41.59 (42.08)		C ₂₀ H ₁₈ N ₃	
[Ni(qsalBr ₂) ₂] (3) C ₃₂ H ₁₈ Br ₄ N ₄ NiO ₂	137–467	36.33 (36.54)	100 (100)	Br ₄	-
	467–575	27.92 (28.72)		C ₁₈ H ₄ N ₂	
	575–1000	35.75 (34.74)		C ₁₄ H ₁₄ N ₂ O ₂ Ni	

The TGA curve of complex 1 shows the following three decomposition steps: the first stage at the temperature range of 138–467 °C related to the loss of two bromine atoms (found 22.64%; calc. 22.89%), the second stage at 467–685 °C related to the loss of C₉H₂NO (found 20.48%; calc. 19.78%), and the third stage at 685–1000 °C correlated with the elimination of C₂₀H₁₈N₃ (found 43.37%; calc. 42.40%), leaving carbon atoms and NiO as residue (found 13.51%; calc. 14.93%). Complex 2 also exhibited similar decomposition as shown in the TGA curve. Like complexes 1 and 2, complex 3 also exhibited three stages of decomposition. The first step involved the loss of four bromine substitutions on the aromatic ring at the temperature range of 137–467 °C (found 36.33%; calc. 36.54%). The second stage at 467–575 °C was related to the elimination of C₁₈H₄N₂ (found 27.92%; calc. 28.72%), and the last stage was associated with the loss of the remaining (found 35.75%; calc. 34.74%). Notably, no residue was observed for this compound.

3.4. ¹H NMR Spectra

The ¹H NMR spectra of HL¹ and its zinc(II) complex 2 were determined. In the free ligand, the distinct peak of the azomethine HC=N was observed at 9.10 ppm, and a significant downfield shift of this proton to 9.36 ppm (Figure S7) was detected in complex 2. These results indicated the coordination of the nitrogen of the azomethine to the metal center. The chemical shift at 14.07 ppm, corresponding to the proton of the hydroxyl group (–OH) of HL¹, disappeared upon complex formation. The deprotonation of this proton leads to the coordination of the oxygen atom toward the zinc(II) ion. Moreover, chemical shifts in the range of 8.98–6.98 ppm, assigned to protons of the quinoline ring of ligand HL¹,

also presented significant shifts upon the formation of complex **2**. This result could affirm the binding of the nitrogen donor of this heterocyclic aromatic ring to such a metal center.

3.5. Mass Spectra

The mass spectra of the synthesized Schiff bases and their complexes were determined by relative intensity molecular ion peaks at 100 eV. The mass spectra of HL¹, HL², and complexes **1–3** provided good evidence for the molecular formula, with well-defined signals at m/z (cal.) = 327.01 (327.18) [M]⁺, 406.91 (406.07) [M]⁺ (HL¹ and HL²), 710.93 (711.13) [M]⁺, 716.95 (717.71) [M]⁺, and 868.74 (868.82) [M]⁺ (complexes **1–3**), respectively.

3.6. Description of the Molecular Structure

Complex **3** was characterized by single-crystal X-ray crystallography. The experimental details of the X-ray data of the synthesized complex are presented in Table 4.

Table 4. Crystal data and structure refinement for complex **3**.

Crystal Data	Complex 3
Compound	[Ni(qsalBr ₂) ₂]
Empirical formula	C ₃₂ H ₁₈ Br ₄ N ₄ NiO ₂
Formula weight	868.83
Temperature/K	100.01
Crystal system	monoclinic
Space group	P2 ₁ /c
a/Å	21.8302 (12)
b/Å	10.1217 (6)
c/Å	13.4878 (8)
α/°	90
β/°	103.808 (2)
γ/°	90
Volume/Å ³	2894.1 (3)
Z	4
ρ _{calc} g/cm ³	1.9939
μ/mm ^{−1}	6.232
F (000)	1685.8
Crystal size/mm ³	0.63 × 0.62 × 0.13
Radiation	MoK _α (λ = 0.71073)
2θ range for data collection/°	4.46 to 51.56
Index ranges	−26 ≤ h ≤ 26, −12 ≤ k ≤ 12, −16 ≤ l ≤ 16
Reflections collected	54,704
Independent reflections	5542 [R _{int} = 0.1105, R _{sigma} = 0.0519]
Data/restraints/parameters	5542/0/388
Goodness-of-fit on F ²	1.096
Final R indexes [I ≥ 2σ (I)]	R ₁ = 0.0532, wR ₂ = 0.0942
Final R indexes [all data]	R ₁ = 0.0744, wR ₂ = 0.1008
Largest diff. peak/hole/e Å ^{−3}	1.27/−1.27

The structure of complex **3** (Figure 3) reveals that the nickel(II) metal center is coordinated with two molecules of ligand HL² in a tridentate fashion, involving a deprotonated oxygen atom and two atoms of nitrogen from the quinoline and azomethine functional groups, respectively. The two nitrogen atoms (N1 and N3) of the quinoline ring and two oxygen atoms (O1 and O2) are located within the square plane, while the two azomethine nitrogen atoms (N2 and N4) occupy the axial positions. The two oxygen atoms are positioned in a cis configuration to each other. Additionally, the complex adopts a distorted octahedral geometry.

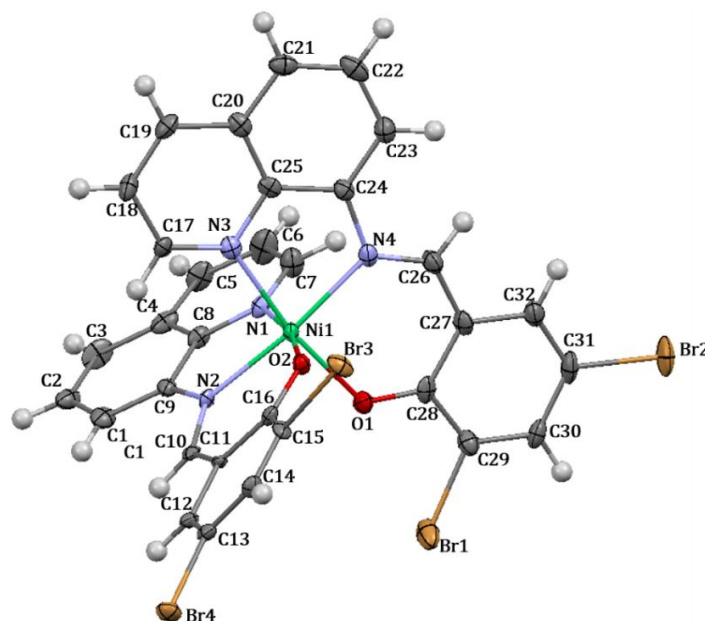


Figure 3. Crystal structure with thermal ellipsoid of complex 3.

Selected bond lengths, bond angles, and torsion angles are shown in Table 5. The C–N(H) bond of HL² with a distance of 2.312 (2) (Table S1) is shortened to 1.316 (8) in complex 3, specifically the N2–C10 bond, upon the coordination of the nitrogen atom towards the metal center. Typically, the C–N distance of a Schiff base compound represents the double bond of the azomethine functional group; therefore, after complex formation, this bond is normally lengthened. However, in this study, the obtained crystal structure of HL² displays an enamine form (Figure S8, Scheme S1), in which after the formation of a coordinative covalent bond, such a ligand has converted to an imine form, leading to the lower bond distance. Other relevant bonds in complex formation also exhibit similar tendencies to the aforementioned C–N bond, all indicating changes in bond lengths upon coordination.

Table 5. Selected bond lengths [Å], bond angles [°], and torsion angles [°] for complex 3.

Bond Lengths [Å]	Ligand HL ²	Complex 3	Bond Angles [°]	Ligand HL ²	Complex 3	Torsion Angles [°]	Ligand HL ²	Complex 3
C8–C9	1.431 (3)	1.437 (8)	C15–C16–C11	115.0 (2)	114.3 (6)	C11–C10–N2–C9	178.0 (2)	168.7 (6)
C11–C16	1.447 (2)	1.427 (9)	C16–C11–C10	120.4 (2)	124.9 (6)	C9–C8–N1–C7	−178.6 (2)	176.8 (6)
C15–C16	1.437 (3)	1.43 (1)	C12–C11–C10	118.1 (2)	114.1 (5)	N1–C8–C4–C3	178.4 (2)	176.8 (6)
O2–C16	1.280 (2)	1.275 (7)	C10–N2–C9	127.1 (2)	120.0 (5)	C13–C12–C11–C10	177.2 (2)	175.1 (6)
N2–C9	1.416 (3)	1.424 (9)	C7–N1–C8	117.2 (2)	119.0 (6)	C10–N2–C9–C1	3.8 (3)	15 (1)
N2–C10	2.312 (2)	1.316 (8)	N2–C9–C8	115.4 (2)	114.3 (5)	C15–C14–C13–C12	−0.3 (4)	−3 (1)
N1–C7	1.318 (3)	1.322 (8)	N1–C8–C4	118.2 (2)	118.3 (6)	N1–C8–C9–N2	1.8 (3)	−0.7 (8)
N1–C8	1.367 (3)	1.351 (9)	N2–C9–C1	123.7 (2)	126.3 (6)			
			O2–C16–C11	122.1 (2)	125.7 (6)			
			O2–C16–C15	123.0 (2)	119.9 (6)			

According to the orientation of complex 3, the nickel(II) ion exhibits a fairly repulsive distribution of electrons, as the two nitrogen atoms (N1 and N3) of the quinoline ring and two oxygen atoms (O1 and O2) are located in a cis geometry. This results in a reasonably adjustment in the bond angles and torsion angles of the Schiff base compound. Bond angles of C16–C11–C10, C12–C11–C10, and C10–N2–C9 with values of 118.1 (2), 120.4 (2), and 127.1 (2) for ligand HL² (Table S1) have shifted by around six or seven degrees to become C12–C11–C10; 124.9 (6), C16–C11–C10; 114.1 (5), and C10–N2–C8; 120.0 (5) in complex 3. The angles of the complex display more deviation from planarity with the torsion angles of C11–C10–N2–C8; 168.7 (6), C13–C12–C11–C10; 175.1 (6), and C10–N2–C9–C1; 15 (1). This appearance is a result of the steric hindrance generated from two molecules of Schiff base compounds being in a cis conformation. Thus, each molecule undergoes

a repulsive orientation in which all N,N,O atoms could properly donate their electrons towards the nickel(II) metal center.

3.7. DFT Investigation

3.7.1. Geometry Optimization of Ligands

Figure 4 displays the optimized structures of nickel(II) and zinc(II) complexes. These findings align with X-ray crystallographic results, indicating the *trans* conformation with a tridentate fashion of the ligand upon the formation of complex 3. Furthermore, the optimized structure geometry confirms that the two nitrogen atoms (N1 and N3) of the quinoline ring and two oxygen atoms (O1 and O2) are located in a *cis* orientation. The detailed geometric parameters, including bond lengths and bond angles, are presented in Table S3.

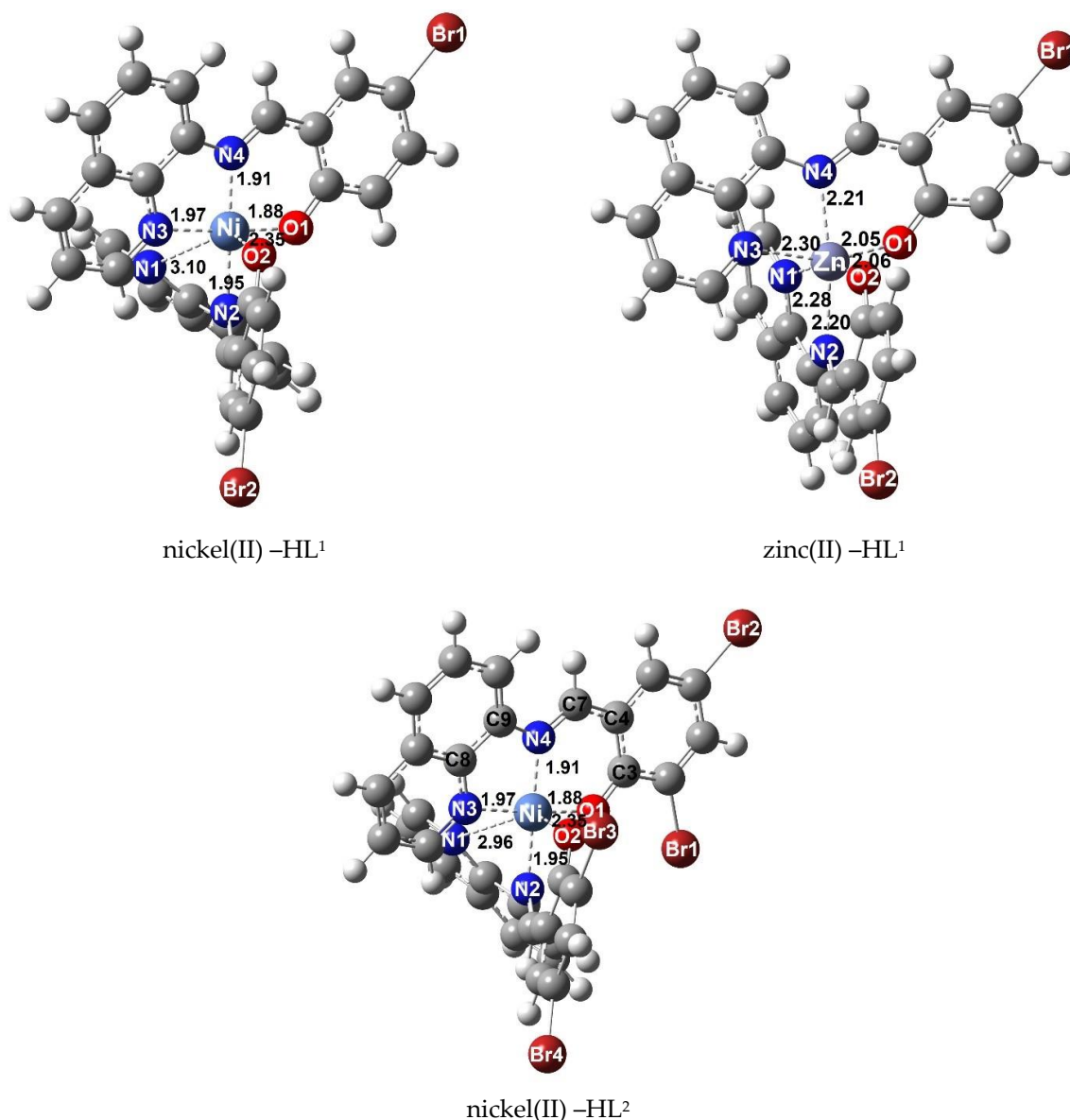


Figure 4. Optimized structures of HL¹ and HL² binding with nickel(II) and zinc(II).

3.7.2. Global Reactivity Descriptors

The chemical reactivity of compounds was estimated by calculating quantum chemical parameters using the density functional theory (DFT) approach. These parameters describe stability and reactivity and suggest the most bioactive compound. Therefore, the lowest

unoccupied molecular orbital (LUMO) and the highest occupied molecular orbital (HOMO) are presented in (Figures 5 and 6 and Table 6) along with the difference between their energies (ΔE). The narrowing of the HOMO (electron donor) and LUMO (electron acceptor) energy gaps indicates that an intramolecular charge transfer (ICT) is occurring within the complex molecule and is responsible for the molecular activity. For the ligands, the HOMO and LUMO orbitals are distributed around the whole molecule with energy gaps of 3.98 and 3.91 eV for HL¹ and HL², respectively. The interaction of the metal ions with the ligands has lowered the energy gap, suggesting that these compounds have the capacity for electron transitions, demonstrating favorable reactivity and stability. The synthesized compounds were organized based on their reactivities as follows: HL–Ni > HL–Zn > HL. Small ΔE values indicate the low stability and high chemical reactivity of the compounds. From the results presented in Table 6, the energy gaps of the complexes are increasing in the sequence of [Ni(qsalBr)₂] (1) < [Ni(qsalBr₂)₂] (3) < [Zn(qsalBr)₂] (2), respectively. Therefore, [Ni(qsalBr)₂] (1) defined as HL1–Ni has the smallest energy gap, which means it is the most bioactive compound and has the highest reactivity compared with other synthesized complexes.

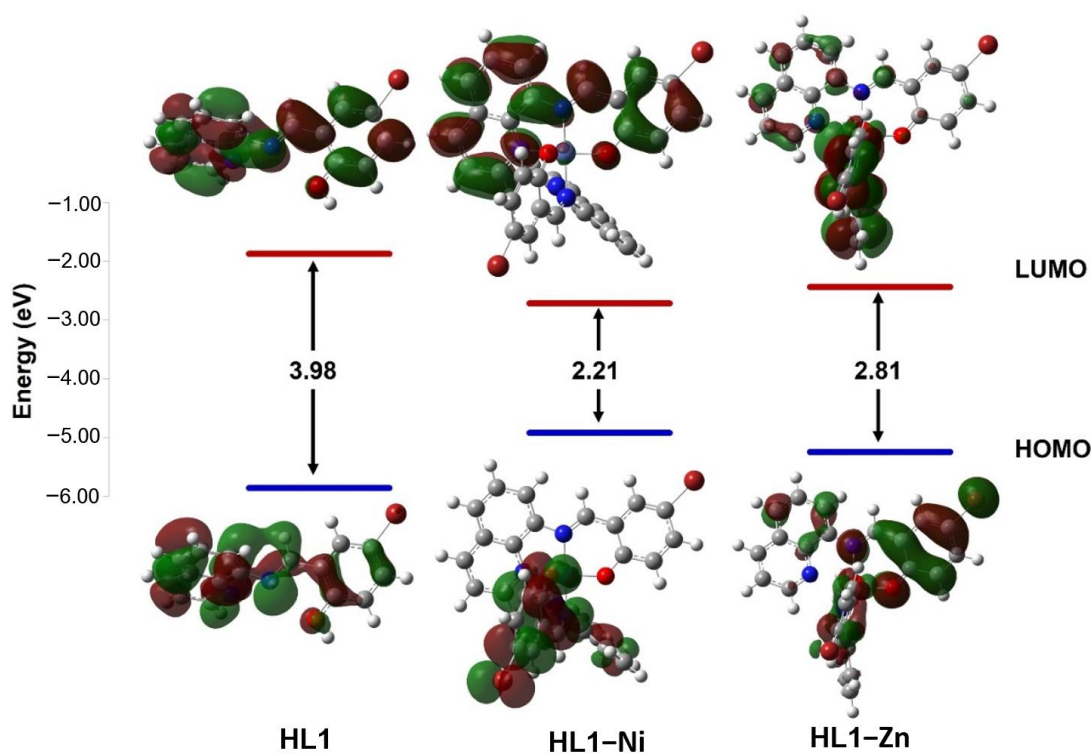


Figure 5. HOMO and LUMO charge density maps and the energy gaps (ΔE) of the HL¹ ligand and its nickel(II) and zinc(II) complexes.

Table 6. HOMO and LUMO energy (eV) and quantum parameters are calculated based on energy gaps for the ligands and their metal complexes.

eV	E_H	E_L	ΔE	η	S	μ	χ	ω
HL1	−5.86	−1.87	3.98	1.99	185.82	−3.87	3.87	3.75
HL1–Ni	−4.92	−2.71	2.21	1.10	335.41	−3.82	3.82	6.60
HL1–Zn	−5.25	−2.44	2.81	1.41	263.50	−3.84	3.84	5.25
HL2	−5.97	−2.06	3.91	1.95	189.49	−4.01	4.01	4.12
HL2–Ni	−5.10	−2.88	2.22	1.11	332.91	−3.99	3.99	7.15

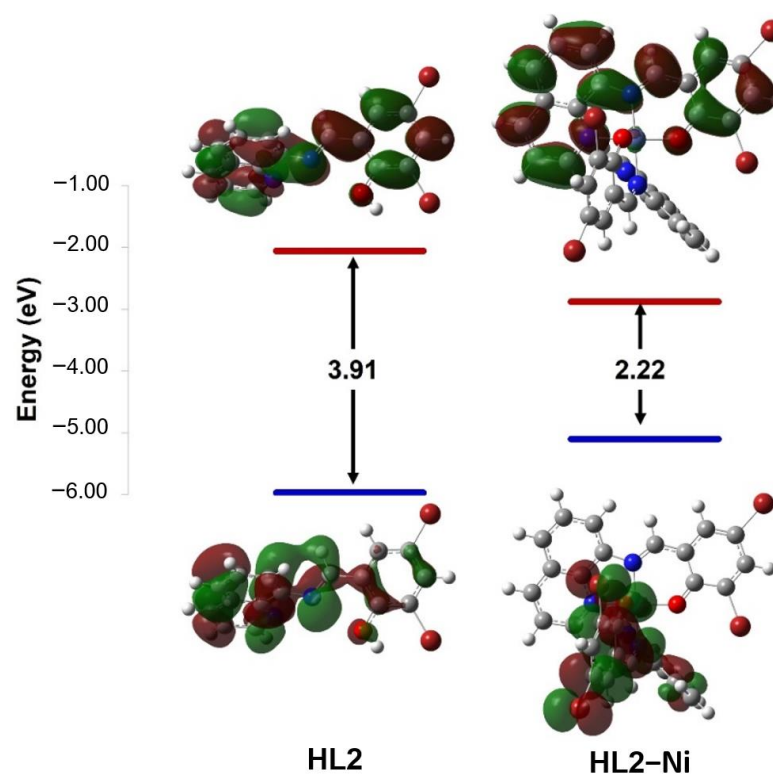


Figure 6. HOMO and LUMO charge density maps and the energy gaps (ΔE) of the HL^2 ligand and its nickel(II) complex.

Absolute hardness (η), softness (S), chemical potentials (μ), absolute electronegativity (χ), and global electrophilicity (ω) are important tools in the study of the stability order of molecular systems.

3.8. DNA Binding Study

3.8.1. Electronic Absorption Titrations

UV–Vis absorption spectroscopy is a typical method employed to determine the binding characteristics of metal complexes with DNA [40]. Generally, hyperchromism and hypochromism are the spectral characteristics of DNA regarding the changes in its double helix structure. In addition, a red shift in spectroscopic studies indicates a change in the electronic environment of the DNA base pairs, suggesting that the synthesized complexes contribute to the stabilization of the DNA structure [41].

Figure 7 presents the absorption spectra of the Schiff base with and without progressively increasing amounts of CT–DNA by successive addition. The arrows indicate the changes accompanying the increasing amounts of CT–DNA, demonstrating a hypochromic effect, and spectra of the complexes also exhibit a red shift around 2–8 nm. These changes could indicate the interaction between the synthesized complexes and the added CT–DNA. The interaction observed between the synthesized complexes 1–3 and CT–DNA is likely an indication of the intercalation mode of binding [42,43]. To quantify the extent of binding of the complexes to CT–DNA, the binding constant (K_b) of each complex is determined by the ratio of the slope to the intercept in plots employing the Wolfe–Shimmer equation [44], and the results are presented in Table 7. The K_b values of the complexes are $1.13 \times 10^6 \text{ M}^{-1}$, $4.82 \times 10^6 \text{ M}^{-1}$, and $6.02 \times 10^5 \text{ M}^{-1}$ for complexes 1–3, respectively. It is clearly seen that the zinc(II) complex shows superior binding to the CT–DNA than the binding of the nickel(II) complexes for both mono- and di-substituted ligands. This might be related to the formation constant of such complexes in which nickel(II) complexes with higher stability (2.52×10^6 and 8.69×10^5 for complexes 1 and 3) could weakly interact with CT–DNA in comparison to zinc(II) complex with the formation constant of 5.67×10^5 . Furthermore, the

zinc(II) complex also provides a higher K_b value than the classical intercalator, ethidium bromide (EB), with a value of $K_b = 1.40 \times 10^6 \text{ M}^{-1}$ [45].

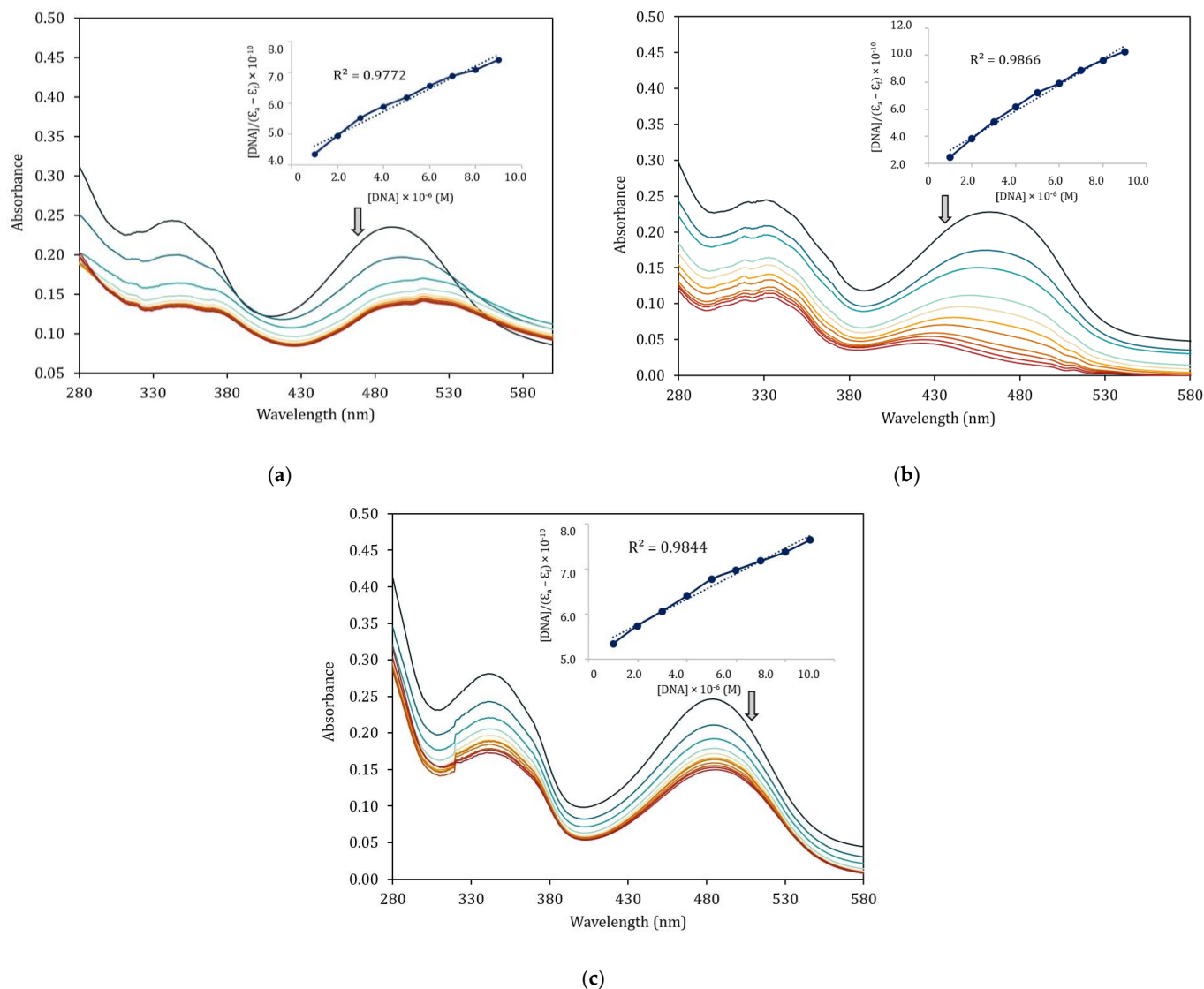


Figure 7. Electronic absorption spectra of Schiff base complexes in the absence and presence of increasing amount of CT-DNA. (a) $[\text{Ni}(\text{qsalBr})_2]$ (1); (b) $[\text{Zn}(\text{qsalBr})_2]$ (2); (c) $[\text{Ni}(\text{qsalBr}_2)_2]$ (3).

Table 7. The binding constant (K_b), the Stern–Volmer constant (K_{sv}), and the quenching constant (K_q) between the complexes with CT-DNA.

Complexes	$K_b \text{ (M}^{-1}\text{)}$	$K_{sv} \text{ (M}^{-1}\text{)}$	$K_q \text{ (M}^{-1} \text{ s}^{-1}\text{)}$
$[\text{Ni}(\text{qsalBr})_2]$ (1)	1.13×10^6	1.78×10^4	7.74×10^{11}
$[\text{Zn}(\text{qsalBr})_2]$ (2)	4.82×10^6	2.00×10^4	8.70×10^{11}
$[\text{Ni}(\text{qsalBr}_2)_2]$ (3)	6.02×10^5	1.66×10^4	7.22×10^{11}

3.8.2. Fluorescence Titration

Fluorescence spectroscopy has been helpful for characterizing the binding mode of complex–DNA interactions [46]. Ethidium bromide (EB) is a favorable tool to examine the change in fluorescent spectra upon interaction with DNA [47]. Typically, EB has a planar conjugate structure and fluoresces weakly, while its solution with DNA emits sharply at 610 nm. This is due to the strong stacking of its planar ring and base pairs on the DNA via

an intercalation mode of binding [48]. The emission intensities for EB–DNA in the absence and presence of the complexes in the range from 545 to 700 nm were measured. When the additive complexes displace the bound ethidium bromide (EB), competitive binding to DNA occurs, leading to the quenching of the fluorescence from the EB–DNA complex.

Figure 8 presents the effect of the complexes at different concentrations on the fluorescence intensity of the EB–DNA system, with the arrows indicating the changes upon increasing concentrations of complexes. It is obvious that the emission intensity of EB–DNA gradually decreases with the increasing concentration of the complexes. The findings suggest that the complexes are capable of quenching the fluorescence of EB–DNA by displacing EB with the synthesized complexes. This is owing to the interaction between the aromatic chromophore of the ligand and the base pairs of the DNA [48].

Furthermore, the quenching ability was evaluated using the Stern–Volmer equation, with the quenching constant (K_q) derived as the slope from the plot of I_0/I against the concentration of the complexes. The K_q values for the complexes were found to be $7.74 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$, $8.70 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$, and $7.22 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$, respectively. The obtained K_q constant of the complexes is also consistent with the UV–Vis titration results, which indicates that the zinc(II) complex intercalated more strongly than the nickel(II) complexes. From the results presented in Table 7, DNA-binding activities observed by both titration methods are increasing in the order of $[\text{Ni}(\text{qsalBr}_2)_2]$ (3) < $[\text{Ni}(\text{qsalBr})_2]$ (1) < $[\text{Zn}(\text{qsalBr})_2]$ (2). These results are also associated with the formation constants of the complexes in which $[\text{Zn}(\text{qsalBr})_2]$ (2) with the least formation constant provides the highest activity in binding with CT–DNA. Additionally, the number of binding sites (n) was also calculated from the linear plots according to the Scatchard equation (10). The values of n for complexes 1 and 2 suggest the one binding site available on the CT–DNA. On the contrary, complex 3 gives a smaller ligand–binding site value of n of about a half (Figure S10).

According to the ability of such complexes in the interaction of CT–DNA via intercalation, the complexes are supposed to perform their ability in the inhibition of cell growth. Therefore, the anticancer activities of the synthesized complexes were further investigated.

3.9. In Vitro Anticancer Activity

The cytotoxicity against A549 lung cancer cells of Schiff base ligands, nickel(II), and zinc(II) complexes obtained from MTT assays are summarized in Table 8. Media (DMSO/media) and etoposide were used as controls under the same experimental conditions. The results indicated that all the complexes exhibited lower activity against lung cancer cells than the standard etoposide and cisplatin. Categorized by types of complexes, zinc(II) complex 2 with IC_{50} of 88.59 ppm provided better results than nickel(II) complexes 1 and 3 with IC_{50} values of 258.43 and 315.00 ppm, respectively. These results are precisely associated with the capability of such complexes in the interaction with CT–DNA which are in the sequence of $[\text{Ni}(\text{qsalBr}_2)_2]$ (3) < $[\text{Ni}(\text{qsalBr})_2]$ (1) < $[\text{Zn}(\text{qsalBr})_2]$ (2). While the compounds tested did not inhibit lung cancer cell growth as effectively as standard treatments like etoposide and cisplatin, it is still necessary to further investigate their anticancer activities against other cancer cell types, along with their pharmacological and toxicological properties, through in vivo studies.

Table 8. IC_{50} values for A549 cell line.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
HL ¹	N/A ^a	$[\text{Ni}(\text{qsalBr})_2]$ (1)	258.43 ± 6.25
HL ²	N/A	$[\text{Zn}(\text{qsalBr})_2]$ (2)	88.59 ± 5.90
Etoposide	18.61 ± 1.25	$[\text{Ni}(\text{qsalBr}_2)_2]$ (3)	315.00 ± 9.20
Cisplatin [49]	21.3		

^a IC_{50} value could not be determined.

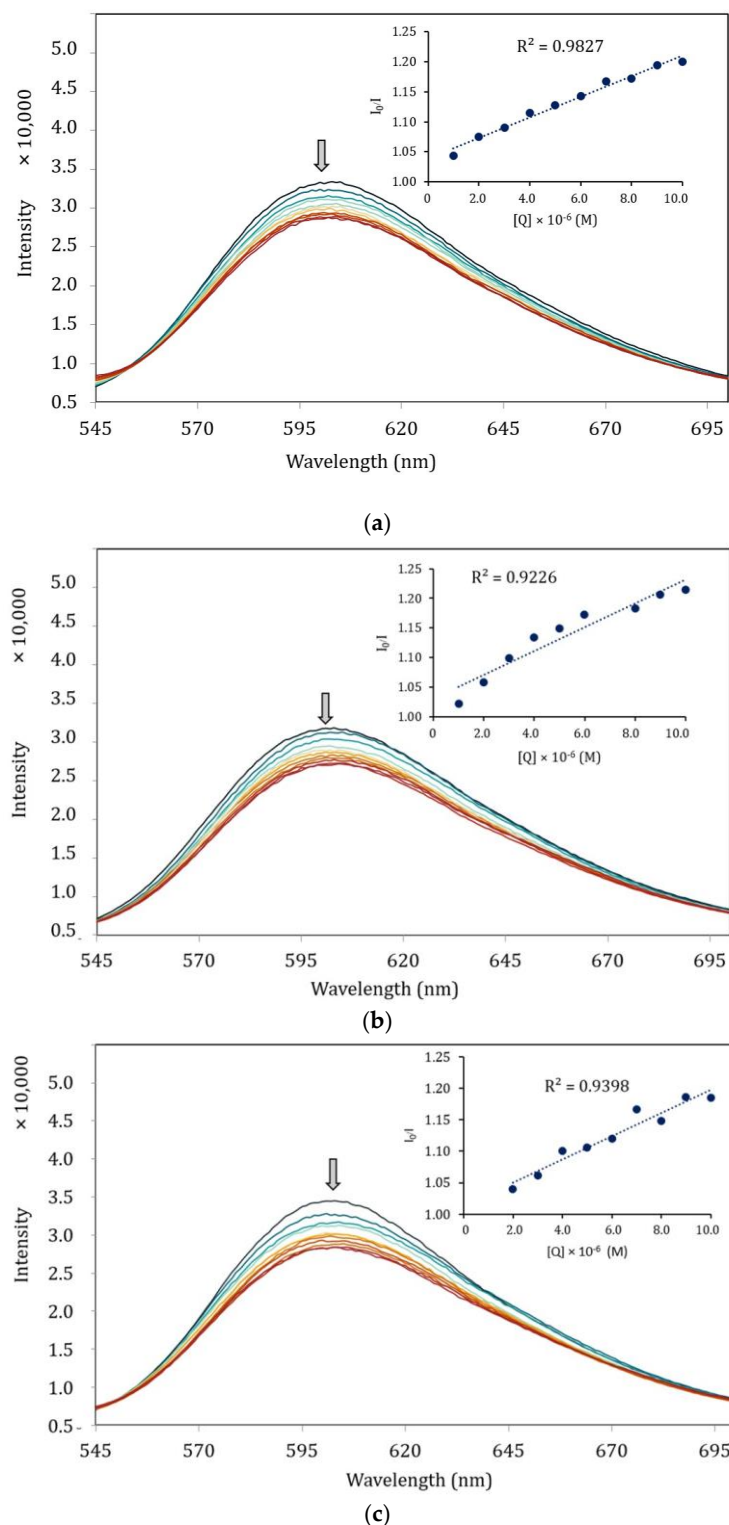


Figure 8. Fluorescence quenching spectra ($\lambda_{\text{ex}} = 525 \text{ nm}$) of EB–DNA in the absence and presence of increasing amounts of the complexes. (a) $[\text{Ni}(\text{qsalBr})_2]$ (1); (b) $[\text{Zn}(\text{qsalBr})_2]$ (2); (c) $[\text{Ni}(\text{qsalBr})_2]$ (3).

Nevertheless, there are discrepancies in the reactivity between experimental and theoretical studies. DFT calculation exhibits the highest energy gap of $[\text{Zn}(\text{qsalBr})_2]$ (2), suggesting the least chemical reactivity of such compound. On the contrary, DNA binding and anticancer studies reveal the highest capability of this zinc(II) complex among the tested compounds. This may arise from subtle differences in the geometric structures of the complexes. As previously reported by our group, the zinc(II) complex bearing HqsalBr_2

exhibited a more planar orientation of its Schiff base ligands, resulting in a less distorted octahedral structure compared to the nickel(II) complex **3**. This enhanced planarity likely contributes to the superior reactivity of the zinc(II) complex **2** in interactions with CT-DNA and its anticancer activity [24].

Furthermore, our findings indicate that complex **1**, featuring a mono-substituted Schiff base ligand, demonstrates reduced steric repulsion in comparison to complex **3**, as evidenced by the lower torsion angles reported in Table S3. Notably, the torsion angles of complex **1** and **3** from the crystallographic determination show significant differences; however, these angles were similar in computational studies (Table S4). The minor disparity between the theoretical and experimental outcomes suggest that the orientation of ligands might influence the observed reactivity among the complexes.

3.10. Molecular Docking

To validate the experimental findings regarding how the complexes interact with DNA, molecular docking simulations focusing on the binding site and modes of interaction with 1bna were conducted. It was observed that HL¹ and HL² bound to B-DNA (PDB id: 1bna) at the minor groove (Figure S11), while the complexes bound to DNA at the major groove are as depicted in Figure 9. Among the complexes examined, [Ni(qsalBr₂)₂] (**3**) exhibited the most favorable binding energies towards DNA (Table 9). The results also indicate that the tested complexes are capable of engaging in both major groove binding and intercalation. However, a comparison of binding energies calculated using the AutoDock program suggests a preference for major groove binding in which the stability primarily relies on the hydrogen bonds and hydrophobic interactions. The relative binding energies of the docked ligands and complexes with DNA fall within the range of -7.7 to -9.1 kcal/mol.

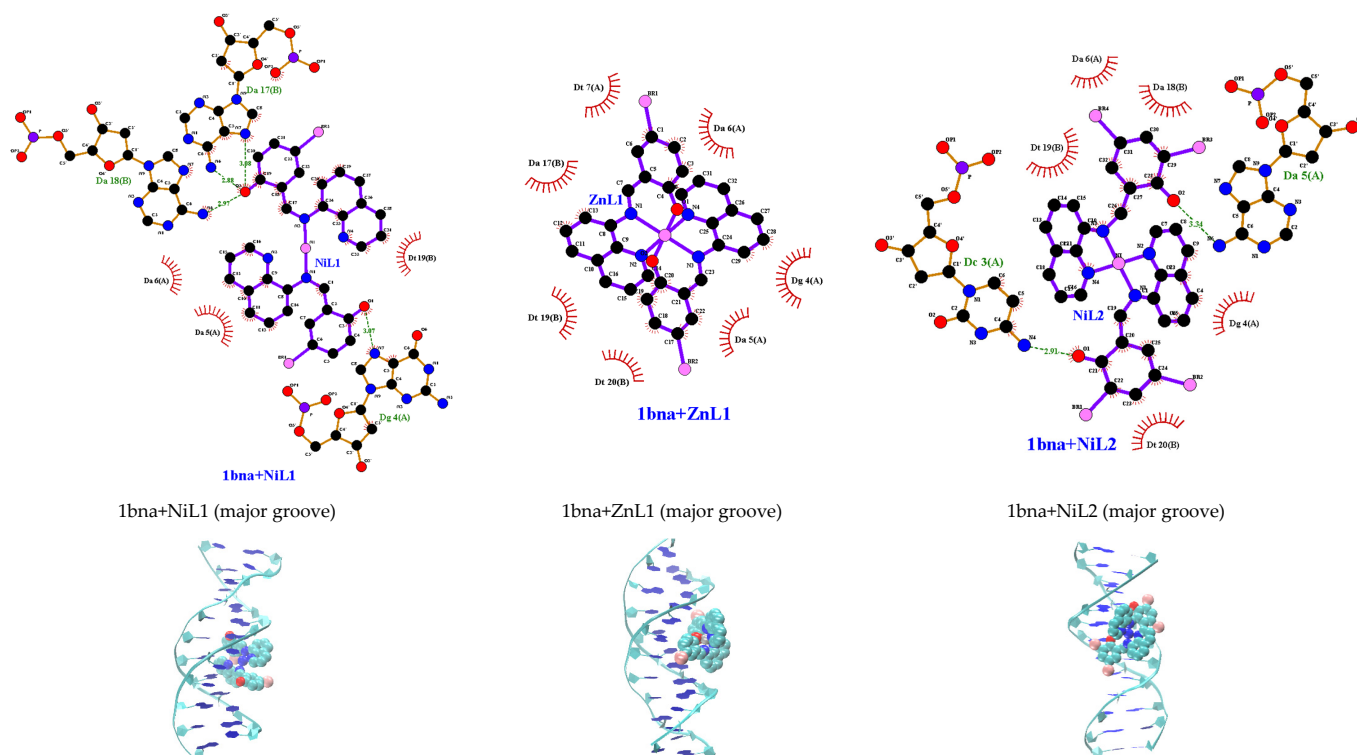


Figure 9. Molecular docking with 2D and 3D interactions of the complexes into the B-DNA (PDB id: 1bna).

Table 9. Binding energy into the B-DNA and the EGFR kinase domain.

Compound	Binding Energy (kcal/mol)	
	1bna	7ukv
HL ¹	−8.0	7ukv
HL ²	−7.7	−7.8
[Ni(qsalBr) ₂] (1)	−7.8	−7.2
[Zn(qsalBr) ₂] (2)	−8.7	−9.0
[Ni(qsalBr) ₂] (3)	−9.1	−8.6
ZRT (Lazertinib)	−	−9.3

The molecular docking investigation involving the EGFR kinase domain (PDB id: 7ukv) and the complexes depicted in Figure 10 revealed that all interactions predominantly occurred via hydrophobic bonds, except for HL¹ and HL², which formed hydrogen bonds with MET793 (for HL¹) and THR790 (for HL²) (Figure S12). The binding energies of the docked ligands and complexes with lung cancer cells range from −7.2 to −9.3 kcal/mol. Interestingly, despite this variation in interaction types, all studied complexes exhibited more negative binding energies compared to ZRT, the native compound in the EGFR kinase domain structure.

In our study, the molecular docking performed for [Ni(qsalBr)₂] (3) indicated the highest binding affinity and more favorable interaction. However, investigations involving DNA binding and anticancer activity showed that this complex demonstrated the least effectiveness compared to the other compounds. The difference occurring between theoretical studies and experimental results may arise from omitting the solvent from the molecular docking calculations.

Solvent molecules can affect the conformation of both the complexes and the receptors. The nickel(II) complexes display the dissociated bonds of the nickel(II) center with the N,O-donor atoms during the synergy in a biological environment, as presented in Figures 9 and 10. In the presence of solvent in an experiment, the zinc(II) complex of [Zn(qsalBr)₂] (2) might perform an orientation with some unbound Zn–O and Zn–N bonds that favor optimal interaction with biological targets. These experimental conditions could facilitate the interaction of the zinc complex with 1bna and 7ukv via the hydrogen bonds and hydrophobic interactions as aforementioned for nickel(II) complexes, leading to the highest potency of [Zn(qsalBr)₂] (2). Although the incorporation of solvent should be executed to improve the reliability of the molecular docking studies, the obtained results in this study certainly demonstrate potential binding targets of all synthesized complexes with DNA and lung cancer cells.

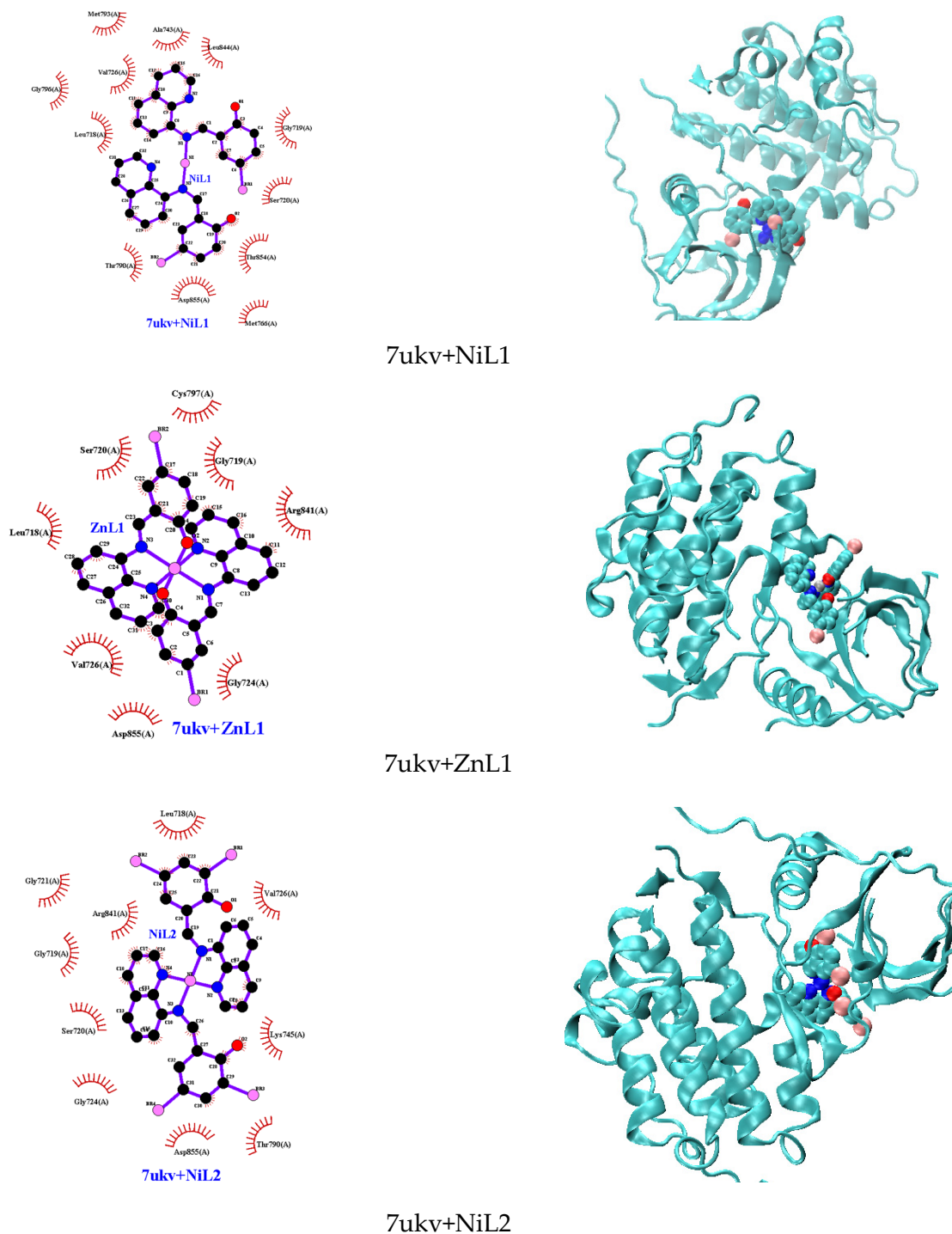


Figure 10. Molecular docking with 2D and 3D interactions of the complexes into the EGFR kinase domain (PDB id: 7ukv).

4. Conclusions

This research involved the synthesis of nickel(II) and zinc(II) complexes with 5-bromo-*N*-(8-quinolyl)salicylaldehyde (HqsalBr, HL¹) and 3,5-dibromo-*N*-(8-quinolyl)salicylaldehyde (HqsalBr₂, HL²) via simple condensation reaction. All the ligands and complexes underwent structural characterization using spectroscopic techniques, confirming the formation of the desired products. The complexes were utilized to study the interaction with calf thymus DNA (CT-DNA). Electronic absorption and fluorescence titration tests indicate that the complexes bind to DNA via intercalation. The experimental results re-

vealed that complex $[\text{Zn}(\text{qsalBr})_2]$ (2) with a higher binding constant (K_b) and quenching constant (K_q) values exhibited superior efficacy in inhibiting cancer cells growth compared to nickel(II) complexes. However, the computational studies revealed that $[\text{Ni}(\text{qsalBr}_2)_2]$ (3) exhibits the most favorable negative binding energies, -9.1 kcal/mol with DNA and -9.3 kcal/mol with cancer cells, facilitated by hydrogen bonding and hydrophobic interactions. Despite the dissimilarity in biological activity through molecular docking, this exploratory study suggests potential binding targets of the synthesized complexes with DNA and lung cancer cells.

Supplementary Materials: CCDC 1918638 contains the supplementary crystallographic data for complex 3. These data can be obtained via <http://www.ccdc.cam.ac.uk/conts/retrieving.html> (accessed on 29 June 2024). The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemistry6040037/s1>, Figure S1: The FT-IR spectrum of $[\text{Ni}(\text{qsalBr})_2]$ (1); Figure S2: The FT-IR spectrum of $[\text{Zn}(\text{qsalBr})_2]$ (2); Figure S3: The FT-IR spectrum of $[\text{Ni}(\text{qsalBr}_2)_2]$ (3); Figure S4: Job's plot for stability constant of $[\text{Ni}(\text{qsalBr})_2]$ (1); Figure S5: Job's plot for stability constant of $[\text{Zn}(\text{qsalBr})_2]$ (2); Figure S6: Job's plot for stability constant of $[\text{Ni}(\text{qsalBr}_2)_2]$ (3); Figure S7: ^1H NMR spectrum of $[\text{Zn}(\text{qsalBr})_2]$ (2) in $\text{DMF-}d_7$; Figure S8: Crystal structure with thermal ellipsoid of HqsalBr_2 (HL^2); Scheme S1: Tautomerization of HqsalBr_2 (HL^2); Table S1: Selected bond lengths [$\text{\AA}$], angles [$^\circ$] and torsion angles [$^\circ$] for HL^2 ; Table S2: Crystal data and structure refinement for HL^2 ; Table S3: Selected bond lengths [$\text{\AA}$], angles [$^\circ$] and torsion angles [$^\circ$] for complex 1; Table S4: Selected geometric bond lengths and torsion angles of the optimized ligand and its metal complexes; Figure S9: The optimized geometry with a numbering system for the HL^2 ligand and nickel(II)- HL^2 complex; Figure S10: Number of binding sites determined by Scatchard plot of EB–DNA; Figure S11: Molecular docking revealed 2D and 3D interactions into B-DNA (PDB id: 1bna); Figure S12: Molecular docking revealed 2D and 3D interactions into the EGFR kinase domain (PDB id: 7ukv).

Author Contributions: Conceptualization, B.P. and R.C.; methodology, B.P. and R.C.; software, M.P. and S.K.; validation, B.P. and R.C.; formal analysis, B.P. and R.C.; investigation, B.P., M.P., S.K., K.C., and T.K.; resources, K.C.; data curation, B.P.; writing—original draft preparation, B.P., M.P., S.K., and T.K.; writing—review and editing, B.P.; visualization, B.P. All authors have read and agreed to the published version of the manuscript.

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