



Synthesis and Anticancer Activities of Water Soluble Schiff Base Metal Complexes

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Abstract

In this study, we synthesized the water-soluble Schiff Base containing S, O, N heteroatoms by the condensation reaction from 4-amino-3-hydroxynaphthalene-1-sulfonic acid and 2-hydroxy-3-methoxybenzaldehyde. In addition, Cu (II), Zn (II) and Ni (II) complexes of the ligand were synthesized and characterized by elemental analysis, magnetic susceptibility and spectroscopic techniques such as FT-IR, ¹H-NMR and ¹³C-NMR. The anticancer activities of ligand and it's three metal complexes on A549 lung cancer cell lines were evaluated. Cell viability experiments revealed that Ligand (L) and nickel (II) complex (L-Ni) did not able to inhibit A549 cell proliferation while L-Cu and L-Zn had 12 and 80 μ M of IC₅₀ values respectively. These last two complexes also induced apoptosis and suppressed cell migration on A549 cells.

Keywords: Schiff base; Metal complexes; Lung cancer; Cell culture.

Suda Çözünebilir Schiff Bazı Metal Komplekslerinin Sentezi ve Antikanser Aktivitesi

Öz



Bu çalışmada, 4-amino-3-hidroksinaftalin-1-sülfonik asit ve 2-hidroksi-3-metoksibenzaldehitten kondensasyon reaksiyonu ile S, O, N hetero atomları içeren suda çözünebilir bir Schiff Bazı elde edildi. Ek olarak, ligandın Cu (II), Zn (II) ve Ni (II) kompleksleri sentezlendi ve elementel analiz, manyetik duyarlılık ve FT-IR, ¹H-NMR ve ¹³C-NMR gibi spektroskopik tekniklerle karakterize edildi. Ligandın ve üç metal kompleksinin A549 akciğer kanseri hücre hattında antikanser aktiviteleri değerlendirildi. L-Cu ve L-Zn olarak etiketlenmiş Bakır (II) ve Çinko (II) komplekslerinin IC₅₀ değerleri sırasıyla 12 ve 80 µM olarak bulundu. L-Cu ve L-Zn komplekslerinin A549 hücrelerinde apoptozu indüklediği ve hücre göçünü inhibe ettiği görüldü. Ligand (L) ve Nikel (II) bileşiklerinin (L-Ni) A549 hücre proliferasyonu üzerinde herhangi bir etki göstermediği belirlendi.

Anahtar Kelimeler: Schiff bazı; Metal kompleksleri; Akciğer kanseri; Hücre kültürü.

1. Introduction

Due to the in vitro cytotoxic effect of metal-based compounds, the interest in these compounds is increasing day by day in cancer treatment. The electronic nature of metals, modifications in ligands and conformational changes in functional groups give rise to the discovery of drugs with different cytotoxic and pharmacokinetic properties. Schiff bases and their metal complexes are the most studied coordination compounds and their uses as anticancer agents are becoming increasingly important [1].

Schiff Bases attract the scientists because of its features containing donor atoms such as oxygen and sulfur and generation of high stability complexes with mono, di, tri and polydentate that can be coordinated to metal ions. Schiff bases are formed as a result of the condensation reaction of aldehydes and primary amines. When these ligands contain different functional groups such as sodium carboxylate and sulfonate in their structure, they acquire water-soluble properties and can be converted into structures with a wide range of chemical properties. [2, 3]. Water soluble compounds expected to have low toxicity because of their inability to bind sulfhydryl groups of proteins of kidney tubules. Therefore, water solubility provides a decrease in the adverse effects and leads improvement in cellular uptake of the drug.

In this study, the Schiff base ligand (L) was synthesized in the water-methanol medium using 4-amino-3-hydroxynaphthalene-1 sulfonic acid and 2-hydroxy-3-methoxy benzaldehyde, which is a water-soluble structure having -SO₃, -OH and -C=N hetero groups. After that, its Cu (II), Ni (II) and Zn (II) complexes were synthesized and characterized by elemental analysis and spectroscopic techniques (FT-IR, ¹H-NMR and ¹³C-NMR). And also in-vitro cytotoxic, apoptotic

and anti-metastatic properties of these compounds were investigated on non-small cell lung cancer cells.

2. Materials and Methods

2.1. Materials

All solvents were commercially purchased in high purity. 4-amino-3-hydroxynaphthalene-1-sulfonic acid ($C_{10}H_9NO_4S$), 2-Hydroxy-3-methoxybenzaldehyde ($CH_3OC_6H_3-2-(OH)CHO$), sodium hydroxide (NaOH), $Zn(CH_3COO)_2 \cdot 4H_2O$, $Ni(CH_3COO)_2 \cdot 4H_2O$ and $Cu(CH_3COO)_2$ were purchased from Sigma-Aldrich Co.. All chemicals were analytical grade and used without further purification. Elemental analysis was carried out on LECO CHNS (model 932) instrument. FT-IR spectra of KBr discs were recorded on a Perkin-Elmer RX-1 $4000-400cm^{-1}$ FT-IR spectrometer. 1H -NMR and ^{13}C -NMR spectra were recorded a Bruker 600 MHz Ultrashield Spectrometer using TMS as an internal standard and DMSO- d_6 as a solvent. Elemental analysis was carried out by use of LECO CHNS (model 932).

A549 cells were obtained from Bingöl University, Department of Molecular Biology and Genetic and BEAS2B cells were obtained from Gaziantep University, Faculty of Medicine. MTT (Thiazolyl Blue Tetrazolium Bromide) used in the study was purchased BioFroxx. DMSO was purchased from Sigma-Aldrich Co. Biolegend APC-Annexin-V/PI apoptosis detection kit was used in the apoptosis analysis. All cell culture materials were purchased from Hyclone. The brand of the microplate reader used in the study is Biochrom EZ Read 400, and the flow cytometry analysis were performed on Beckman Coulter/CytoFLEX, (United States). Prism V 8.2.1 (GraphPad Software, Inc, CA, USA) was used for statistical analysis.

2.2. Methods

2.2.1. Synthesis of imine ligand: sodium 3-hydroxy-4 - ((2-hydroxybenzylidene amino) naphthalene-1-sulfonate (NaH_2L))

4-amino-3-hydroxynaphthalene-1-sulfonic acid (10 mmol, 2.3 g), NaOH (10 mmol, 0.4 g) and ethanol (50 ml) solution were added in a reaction flask than stirred to the soluble state were refluxed. Then 2-hydroxy-3-methoxybenzaldehyde (1.52 g, 10 mmol) was dissolved in 10 mL ethanol and added dropwise to the reaction flask [2, 4, 5]. The mixture was refluxed for 3 hours under $Ar_{(g)}$. A dark orange precipitate was obtained after cooling to room temperature, then filtered, washed several times with cold ethanol and dried in the vacuum desiccator. The structure of sodium 3-hydroxy-4-((2-hydroxy-3-methoxybenzylidene) amino) naphthalene-1-sulfonate (Ligand NaH_2L : Abbreviated as L) formed as a result of the condensation reaction is given in Fig. 1.

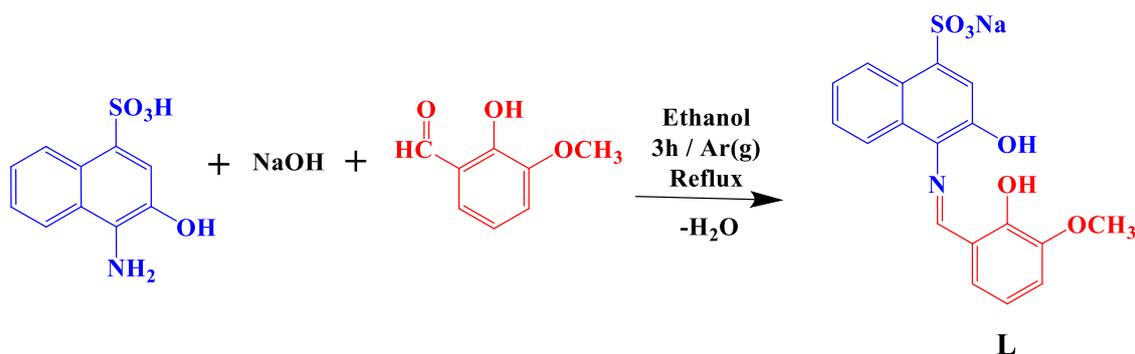


Figure 1: The structure and the reaction conditions of L

L: Yield 75% (2.96 g), m.p. >350 °C, formula wt; 395.36 g/mol. Anal. calcd. for $C_{18}H_{14}NNaO_6S$: C, 54.68; H, 3.57; N, 3.54; S, 8.11; Na, 5.8; found C, 53.92; H, 3.46; N, 3.52; S, 7.94; Na, 5.78. FT-IR (KBr pellet, cm^{-1}): 3279 ($\nu_{(OH)}$, m), 3076 ($\nu_{(OCH_3)}$, v), (s), 1613 ($\nu_{(C=N)}$, s), 1165-1046 ($\nu_{(SO_3)}$, s). 1H -NMR (600 MHz, $DMSO_4-d_6$) σ ppm: 13.77 (s, 1H) 10.16 (s, 1H); 9.13 (s, 1H); 8.78 (d, $J=8.40$ Hz, 1H); 7.92 (s, 1H); 7.91 (d, $J=9.00$ Hz, 1H); 7.49-7.47 (m, 1H); 7.39-7.36(m, 1H); 7.27-7.25 (m, 1H); 7.17 – 7.16 (m, 1H); 6.94 (t, $J=8.40$ Hz, 1H); 3.86 (s, 3H). ^{13}C -NMR (150 MHz, $DMSO_4-d_6$) σ ppm: 168.75; 151.21; 148.44; 143.69; 143.21; 130.25; 128.53; 128.26; 126.75; 124.16; 123.45; 121.80; 119.93; 119.07; 118.47; 115.92.

2.2.2. Synthesis of metal complexes

The three mmol L was dissolved in hot 30 ml 2: 1 methanol: water mixture. Then three mmol of metal salt dissolved in 10 ml of 1: 1 methanol: water mixture was added to the medium and left under reflux for 4 hours. All metal complexes were synthesized by the same method using the appropriate amount of metal salt. Finally, half of the solvent was evaporated to leave the precipitate. The precipitate formed was filtered, washed several times with cold ethanol and allowed to dry overnight at 60 °C in a vacuum oven. The synthesized compounds were characterized by analytical and spectroscopic methods. The proposed structures for the synthesized complexes are given in Fig. 2.

Na[CuL(H₂O)]H₂O (L-Cu): Yield %75 (1.05 g), dark brown, m.p. >350 °C, formula wt; 492,92 g/mol. Paramagnetic, 1.54 BM. Anal. calcd. for $NaC_{18}H_{16}NO_8SCu$: C, 43.86; H, 3.27; N, 2.84; S, 6.50; Na, 4.66; Cu, 12.89; found C, 43.91; H, 3.54; N, 2.97; S, 6.81; Na, 4.74; Cu, 13.01. FT-IR (KBr pellet, cm^{-1}): 3464 ($\nu_{(OH)}$, m), 1600 ($\nu_{(C=N)}$, s), 1220-1044 ($\nu_{(SO_3)}$, s), 550 ($\nu_{(M-N)}$, m), 486 ($\nu_{(M-O)}$, m).

Na[NiL(H₂O)]H₂O (L-Ni): Yield 65% (0.91 g), yellowish green, m.p. >350 °C, formula wt; 488.07 g/mol. Paramagnetic, 2.63 BM Anal. calcd. for NaC₁₈H₁₆NO₈SNi: C, 44.30; H, 3.30; N, 2.87; S, 6.57; Na, 4.71; Ni, 12.03. found C, 44.92; H, 3.41; N, 2.99; S, 6.28; Na, 4.82; Ni, 12.07. FT-IR (KBr pellet, cm⁻¹); 3223 (ν_(OH), m), 1612 (ν_(C=N), s), 1216-1042 (ν_(SO₃), s), 620 (ν_(M-N), m), 496 (ν_(M-O), m).

Na[ZnL(H₂O)]H₂O (L-Zn): Yield %66 (0.94 g), dark orange, m.p. >350 °C, formula wt; 494.75 g/mol. Diamagnetic. Anal. calcd. For NaC₁₈H₁₆NO₈SZn: C, 43.20; H, 3.26; N, 2.83; S, 6.48; Na, 4.65; Zn, 13.21 found C, 43.36; H, 3.31; N, 2.98; S, 6.54; Na, 4.73, Zn; 13.12. FT-IR (KBr pellet, cm⁻¹); 3428 (ν_(OH), m), 1610 (ν_(C=N), s), 1228-1049 (ν_(SO₃), s), 530 (ν_(M-N), m), 499 (ν_(M-O), m).

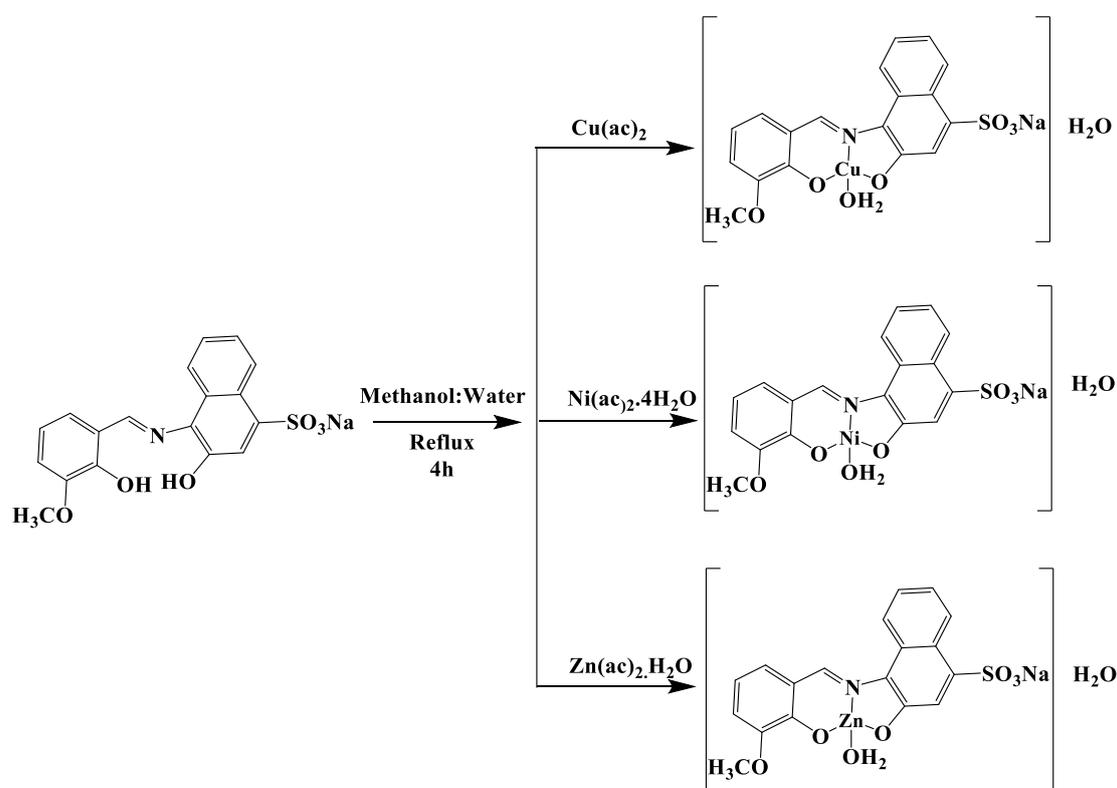


Figure 2: The proposed structures for the synthesized complexes

2.2.3. Cell visibility assay

A549 (lung cancer) cells were treated with different concentrations of the compounds to investigate the cytotoxic effect by using MTT assay according to the Mosmann's procedure [6]. Optimization experiments were performed to decide the number of cells per well to reach 80% confluency. 104 cells/well were cultured in a 96-well plate for overnight at 37 °C, 5% CO₂ in RPMI medium containing 1% antibiotics and 10% FBS. Then, the old medium was removed and

fresh medium containing compounds at (0, 0.1, 0.3, 1, 3, 10, 30, 100) μM concentrations were added in triplicate and the cells were incubated for 24 more hours. Water was used as a vehicle control. At the end of the incubation time, MTT solution was added to the wells to reach a final concentration of 0.5 mg/mL, and the cells were incubated for 4 hrs to metabolize MTT. The medium was removed and 50 μL DMSO was added per well to dissolve the formazan crystals. Lastly, the absorbance was measured at 570 nm in a microplate reader. The same procedure was applied to the BEAS2B normal bronchial epithelial cells to check whether the compounds were selective against the lung cancer cells.

2.2.4. Flow cytometry assay

The apoptotic effects of L-Cu (20 μM) and of L-Zn (100 μM) were tested on A549 cells via flow cytometry assay. The ratio of apoptotic cells were determined according to Biolegend APC-Annexin-V/PI apoptosis detection kit protocol [7]. Briefly, 10^7 cells/mL in 6 well-plates (cell number was optimized to reach 80% confluency per well) were maintained in RPMI medium, containing either drug or vehicle and incubated in the CO_2 incubator at 37 °C for 24 hours. At the end of the incubation period, the cancer cells were harvested and incubated with APC-Annexin V and PI. The fluorescence emission of APC- Annexin-V stained cells was measured at 633 nm (Red laser) in the flow cytometer instrument. The instrument software provided the result of the analysis as living cells (APC- / PI-); early apoptotic cells (APC + / PI-); necrotic cells (APC- / PI +); and the late apoptotic cells (APC + / PI +).

2.2.5. Wound healing assay

A549 cells were plated in 6 cm plates in regular growth medium containing 10% FBS. After 24 hrs, a straight-line scratch was made on cell layers using a sterile 200 μL disposable pipette tip and washed with PBS. Then, the cells treated either with IC_{50} concentration of the compounds (12 μM L-Cu 80 μM L-Zn) and with water as a control. Images of cell migration were taken using an inverted microscope at 0 hrs and 24 hrs after the scratch. Gap lengths of the cells were measured using Image J.

2.2.6. Statistical analysis

Prism V 8.2.1 (GraphPad) was used to calculate IC_{50} 's of the compounds. The measured spectrophotometric values were first normalized, concentrations were converted to log forms and Non-linear regression (curve fit) was used to generate log (inhibitor) vs. response curves to determine IC_{50} values.

3. Results and Discussion

3.1. Synthesis and characterization of ligand and its metal complexes

Experimental percentages of the elements for all synthesized compounds were determined by using ICP-OES and elemental analysis devices. The percentages of carbon (C), hydrogen (H), sulfur (S), nitrogen (N), metal (Cu, Ni, Zn, Fe) and sodium (Na) obtained were compatible with theoretical values, which are given in the synthesis section.

3.1.1. FT-IR spectra

The data of the FT-IR spectra are listed in Table 1 and Fig. S1 in Supporting information (SI). The sharp peak observed around 1613 cm^{-1} in the spectrum of the ligand is the stretching vibrations of the azomethine group (Ar-C=N). This spectrum supports the successful condensation reaction. In addition, the peaks obtained at 3279 cm^{-1} and 1165-1046 cm^{-1} support the presence of Ar-OH and $-\text{SO}_3$ groups in the molecular structure, respectively [8].

Table 1. FT-IR absorption bands of L and its metal complexes

Compounds	$\nu(\text{OH})$	$\nu(\text{C=N})$	$\nu(\text{SO}_3)$	$\nu(\text{M-N})$	$\nu(\text{M-O})$
H₂L	3279	1613	1165-1046	-	-
L-Cu	3464	1600	1220-1044	550	486
L-Ni	3223	1612	1216-1042	620	496
L-Zn	3428	1610	1220-1049	530	499

In the FT-IR spectra of Cu (II), Zn (II) and Ni (II) complexes, the azomethine peak shifted from 1620 to 1600, 1612 and 1611 cm^{-1} , respectively. This shift supports the formation of the metal complex. In addition, new peaks were observed in the range of 620-486 cm^{-1} after complex formation [8, 9]. The novel peaks support the presence of M-N and M-O bonds.

3.1.2. ¹H-NMR and ¹³C-NMR

¹H-NMR and ¹³C-NMR spectra of NaH₂L structure recorded in DMSO-d₆ were given in Fig. 3. According to the ¹H-NMR spectrum the peak and its integrations of the expected structure were found to be consistent. The spectrum shows the peak of the phenolic (-OH, b) was observed at δ 13.77 ppm (s, 1H); the naphthalic hydroxy (-OH, c) at δ 10.16 ppm (s, 1H); azomethine (-CH=N) at δ 9.13 (s, 1H); methoxy protons (-OCH₃, d) at δ 3.86 ppm (s, 3H); aromatic protons at δ 8.78 (d, 1H); δ 7.92 (s, 1H); δ 7.91(d, 1H); δ 7.49-7.47 (m, 1H); δ 7.39-7.36 (m, 1H); δ 7.27-7.25 (m, 1H); δ 7.17-7.16 (m, 1H); δ 6.94 (t, 1H). In accordance with ¹³C-NMR spectrum, the observation of 18 peaks with different chemical environments as follows 168.75; 151.21; 148.44; 143.69; 143.21; 130.25; 128.53; 128.26; 126.75; 124.16; 123.45; 121.80; 119.93; 119.07; 118.47; 115.92. The ¹³C-NMR spectrum of the NaH₂L confirms functional groups in the structure.



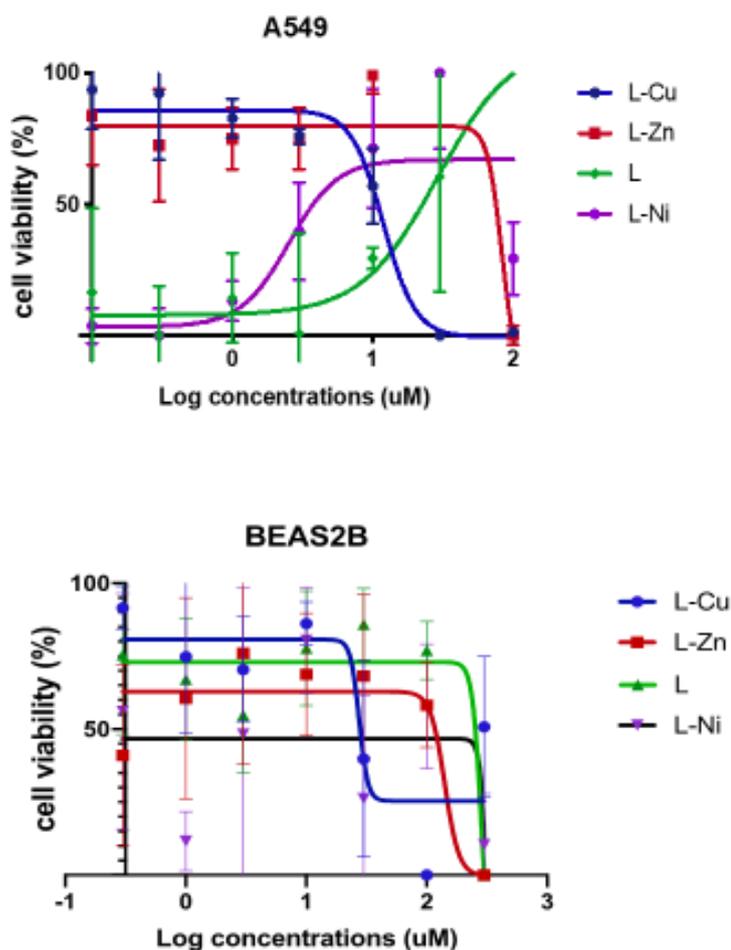
Figure 3: ¹H-NMR and ¹³C-NMR spectra of L in DMSO-d₆

3.1.3. Magnetic susceptibility

The magnetic susceptibility results of metal complexes can give information about the structure. The values of Cu (II) and Ni (II) complexes taken from magnetic susceptibility measurements were 1.54 B.M and 2.63 B.M, respectively. Based on these data, we found that the complexes synthesized were paramagnetic as expected. As a result of the calculations, it is determined that there is one unpaired electron for Cu (II) and two for Ni (II). It is known that for the Cu (II) complex it may prefer both sp^3 or dsp^2 (tetrahedral or square plane) hybrids [10]. The presence of two unpaired electrons in the Ni (II) complex indicates that it preferred tetrahedral geometry by sp^3 hybridization [11]. In addition, the Zn (II) complex was found to have diamagnetic properties. Considering the proposed structure, it is thought that four coordinated complexes are formed and have tetrahedral geometry by sp^3 hybridization [12].

3.2. Anticancer studies

To evaluate the cytotoxic activities of the compounds in A549 cells, we incubated the cells with L, L-Ni, L-Cu and L-Zn compounds in the 0-100 μ M concentration range for 24 hrs. The examined compounds are considered as not effective (IC_{50} values $> 100 \mu$ M) and effective ($IC_{50} < 99 \mu$ M). All experiments were independently repeated for three times. As shown in the dose-response curves (Fig. 4), L-Cu and L-Zn complexes exert an effective cytotoxic effect on A549 cells. L-Cu complex had the best cytotoxic effect on A549 cells with an average IC_{50} value of 12 μ M at 24 hrs. L-Zn complex was the second best complex with an IC_{50} value of 80 μ M at 24 hrs (Fig. 5). Both ligand and L-Ni complex showed no significant cytotoxic activity on A549 cells ($IC_{50} > 100 \mu$ M). Cytotoxicity studies on the normal bronchial epithelial BEAS2B cell line showed that the L-Cu and L-Zn complexes had cytotoxic effects only at high concentrations that IC_{50} values could not be calculated (Fig. 5). The findings of the experiment indicated that these two compounds had a specific cytotoxic effect against the cancer cells. Further, comparing with the literature, the L-Cu complex was found slightly more potent than cisplatin, which is a current therapy agent against lung cancer, where an IC_{50} of 17.2 μ M was reported for the same cell line (A549) and same treatment period [13, 14]. In addition, the water solubility of this compound might provide a great advantage in terms of toxicity.



Compounds	IC ₅₀ values (μM)	
	A549	BEAS 2B
L-Cu	12	Very wide
L-Zn	80	Very wide
L	>100	Very wide
L-Ni	>100	Very wide

Figure 4: The cells were treated with various concentrations of the compounds for 24 hrs. The cell viability assay was achieved with MTT protocol. IC₅₀ values were calculated. While L-Cu and L-Zn complexes had cytotoxic effects on A549 lung cancer cells, they had no cytotoxic effect on BEAS2B normal lung cells at low concentration

Compounds may kill cells in apoptotic or toxic ways. In order to understand the cell killing mechanism of these novel compounds, we examined apoptosis induction effects for L-Cu and L-Zn complexes. After incubating the cells with the compounds at 20 μM and 80 μM concentrations respectively, we performed APC Annexin-V / PI flow cytometry analysis to determine the rate of

apoptotic cells. According to the results, the total (early and late) apoptotic cell rate of A549 cells, after normalization with water treated cells as a control, treated with L-Cu and L-Zn at 24 hours were 42.27% and 68.86% respectively, (Fig. 5). The obtained results reveal that both complexes have cytotoxic effects in an apoptotic pathway.

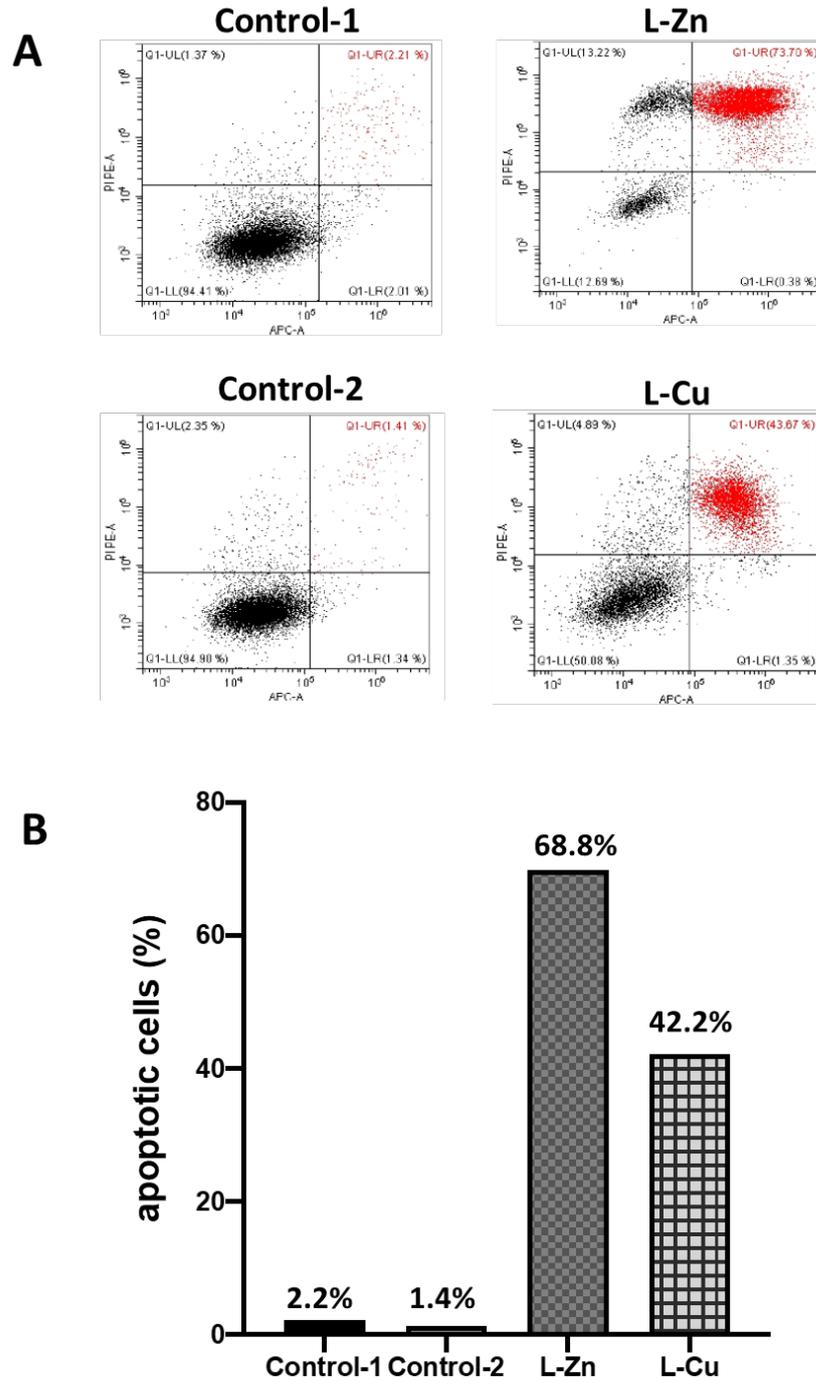


Figure 5: A549 Cells were treated with 20 μ M L-Cu and 80 μ M L-Zn complexes for 24 hours, cell viability was measured by flow cytometry. Upper (late) and lower (early) right quadrants show the apoptotic cells of the population (A). Apoptotic cell percentages are given in bar graphs (B)

We examined the effect of L-Cu and L-Zn on the metastasis of A549 cells. It is clearly seen in Figure 6 that the motility of the cells treated with both complexes for 24 hours is reduced. At the end of 24 hours, the gap between the cells did not close compared to the control for L-Cu ($12 \mu\text{M}$) and L-Zn ($80 \mu\text{M}$). Therefore, we concluded that these two complexes had ability to decrease the migration rate of the cells (Fig. 6).

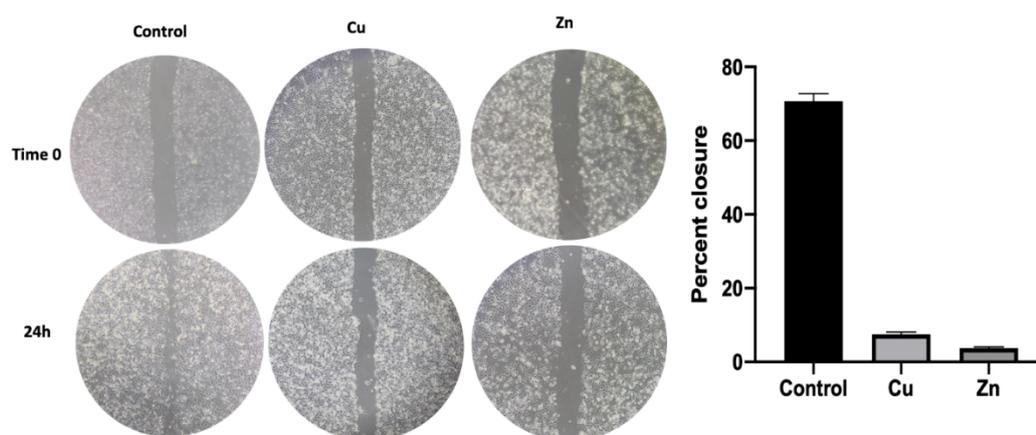


Figure 6: Representative images from the wound healing (scratch) assay using water as control are shown. L-Cu ($12 \mu\text{M}$) and L-Zn ($80 \mu\text{M}$) suppressed cell migration. Closure levels of A549 cells were analyzed after multiple measurements of the void area using image J. Statistical analysis was done using Prism V 8.2.1 and results are given in the bar graph

It is frequently seen in the literature that different metal complexes belong to the same ligand show different anticancer activity [15-17]. This may be related to the binding properties of the metal complex to DNA as well as the ligand. For example, in a study, the anticancer activity of complexes belong to the same ligand shows quite a difference according to the geometry of the complex. The activity sequence of Cu (II), Zn (II) and Ni (II) metals chelated with a ligand is given as $\text{Cu} > \text{Zn} > \text{Ni}$, may not be the same for another ligand [16]. Similarly, in another study, the anticancer activities of the metal complexes mentioned in the same ligand are very close to each other [17]. In this study, the activity order was determined as $\text{Cu} > \text{Zn} > \text{Ni}$. L-Cu, L-Zn and L-Ni complexes are tetra-coordinated. While L-Zn and L-Ni have tetrahedral geometry, L-Cu may have square plane geometry unlike others. The superiority of anticancer activity compared to others may be due to the difference in the geometry of the complex of covalent or non-covalent attachments with DNA. Sometimes Schiff base ligands are effective in killing cancer cells alone,

but other times accompanying with a metal may increase the anticancer activity. In a study with Schiff bases, the cytotoxic activity of most ligands in different cell lines are found to be at the level of $IC_{50} > 100 \mu M$ [15]. In our study, while the ligand was not effective alone ($IC_{50} > 100 \mu M$), antitumor activity was increased when Cu (II) and Zn (II) metals were added to the structure. A number of Cu (II) chelate complexes and Zn (II) Schiff base complexes were synthesized and evaluated for their anticancer effects on various cell lines in the literature [16, 18] Comparing to those, the complexes in this study can be considered superior due to their water solubility property.

4. Conclusion

This study aimed to develop an alternative drug to non-small cell lung cancer. For this purposes a water-soluble Schiff base ligand containing S, O, N heteroatoms and its Ni (II), Cu (II), Zn (II) complexes were synthesized and characterized. The advantages of these synthesized complexes over existing anticancer agents are that they are water-soluble, easily and cheaply obtainable. Moreover, while L-Cu and L-Zn complexes had good cytotoxic effect on A549 cell line, they were not effective even in higher concentrations on healthy lung cell line BEAS2B. These compounds, which act selectively against the A549 cancer cells, have the potential to be good antitumor agents when evaluated with their other advantages.

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