Synthesis of Cu(II)-Hydrazone Complexes Based on Benzohydrazide and Benzaldehyde/Salicylaldehyde as Chemotherapic Agent

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**Abstract.** Cancer is a global problem with the prevalence and mortality increased every year. Currently, cancer treatment is limited by drug resistance and the use of platinum metal that negative impact on organs and body systems. The use of copper complexes could be an alternative solution of chemotherapy drugs. Copper is a transitional series micronutrient that is very important for the body and plays a central role in angiogenesis defects. On the other hand, the determination of properties of the ligands has a big influence on the biological activity, especially anticancer. In this research, Cu(II)- hydrazone complexes was successfully synthesized with orange (**2ASR**) and brown (**2BSR**). The hydrazone ligand was obtained from the reaction of benzohydrazide with benzaldehyde(**1A**)/salicylaldehyde(**1B**). FTIR analysis revealed the presence of metal-ligand bonds in the form of Cu-N and Cu-O at wavenumber 445-470 cm-1 and 553-588 cm-1. The UV- Vis analysis indicates a batochromic shift in complex compounds that involve LMCT. Fragmentation mass spectra showed a 1:1 molar ratio of metal to ligand. These results were supported by TGA data. Powder XRD interpretation shows that complexes was polycrystalline, with similarity to the reference material. Cervical cancer cell “HeLa” cancer test using PrestoBlue (PB) method. The **2BSR** complexes optimally inhibits cancer with an IC50 of 0.54 μg/mL.

# INTRODUCTION

Cancer is a global health issue and a cause of reduced life expectancy in many countries [1]. It is one of the leading causes of death worldwide [2, 3], particularly in women, such as those with cervical cancer. Cervical cancer is a malignant gynecological tumor with a mortality rate of 59.4% among Indonesian women out of nearly 350,000 cases [4]. Alarmingly, the average age of patients is 15.7 years, the youngest among ASEAN countries [5]. Additionally, cervical cancer cells are resistant to the chemotherapy agent cisplatin [6].

Conventional therapeutic approaches, including surgery, radiotherapy, and chemotherapy, have been intensively pursued for decades. Chemotherapy is the best option for patients with cancer to extend survival rates, especially at the metastatic stage [7-10]. Chemotherapy uses specific compounds that directly target cancer cells, block essential cell division elements, initiate apoptosis, and have systemic effects on both organic compounds (Doxorubicin, Paclitaxel, and Vinorelbine) and complex compounds (Cisplatin and Oxaliplatin) [11, 12].

Commercial cytostatic agents based on complex compounds show good efficacy, but multidrug resistance (MDR) mechanisms reduce sensitivity and selectivity [13]. Additionally, platinum-based cytostatic drugs, as shown in Figure 1, negatively impact organs and body systems, including kidney and nerve damage, hearing loss, and

hemolytic anemia [14]. Therefore, researchers have sought to identify new therapeutic targets that minimize side effects, enhance pharmacological properties, and directly target diseases [15].

An alternative approach to chemotherapy agents is the use of complex compounds from bioessential metals, such as copper. Copper is a micronutrient found in all organisms, functioning as a catalytic cofactor for various metalloenzymes, a reactive oxygen species (ROS) activator, and playing a central role in angiogenesis deficiency [14, 16-19]. Furthermore, the effectiveness of complex compound-based drugs also depends on ligand selection. Ligand characteristics are crucial for reactivity, lipophilicity, metal oxidation state stabilization, and anticancer activity [20]. One example of a suitable ligand is hydrazone.

Hydrazone is a Schiff-base azomethine (–NHN=CR–) known for its various bioactivity profiles, including anticancer properties [21-23]. Hydrazones act as inhibitors of the estrogen receptor "aromatase" in breast cancer cells "MCF-7", and tyrosine kinases "ErbB-2/EGFR" and "EGFR" in breast cancer cells "SK-BR-3" and "BT474", gastric cancer cells "N87", cervical cancer cells "HeLa", colon cancer cells "HT-29", and lung cancer cells "A549" [24-27]. When a carbonyl group is added to hydrazone, forming an acylhydrazone, the mitotic inhibitor role remains the same, while hydrolytic stability and protein-binding ability increase [28]. Acylhydrazones are capable of acting as androgen receptor inhibitors in prostate cancer cells "LnCaP" [29] as well as cholinesterase enzymes "ACHE" and "BuCHE" in liver cancer cells "HepG2" [30]. The reported materials are shown in **Figure 1**.

**Figure 1**. Reported hydrazone compounds [24-27, 28-30]

The therapeutic activity of hydrazones increases when they are bonded to a metal to form a complex compound, as shown in **Figure 2**. Cu(II)-hydrazone complexes based on acetylpyridine and benzohydrazide exhibit inhibitory activity against cervical cancer cells "HeLa" and breast cancer cells "MCF-7" with IC50 values of 4.87−5.28 µM. These values are superior to the bioactivity of the ligand alone. Furthermore, cisplatin-resistant cancer cells "A549 cisR" can also be inhibited by these materials [16]. Other studies have shown similar results in objects such as liver cancer cells "HepG2", lung cancer cells "A549", colon cancer cells "HCT116", and spinal cancer cells "SF295" [31- 34]. Cu(II)-hydrazone complex compounds can be utilized as chemotherapeutic agents because they are bioessential [19], act as kinase inhibitors [24, 30], and are effective against cisplatin-resistant cancer cells [16, 18].

**Figure 2**. Reported Cu(II)-hydrazone complex compounds [16, 31-34]

Cu(II)-hydrazone complex compounds were previously reported by Mo et al. [18], where the ligand used was based on acetohydrazide. This complex showed bioactivity (IC50) on lung cancer cells "A549" and breast cancer cells "MCF-7" of 47.36±3.81 µM and 18.61±2.18 µM, respectively. These values are categorized as moderate toxicity. Therefore, Mo et al. attempted to replace the methyl substituent on the hydrazide with a phenyl group (–CH3→–Ph). As a result, the replacement with a more polar phenyl group increased the compound's toxicity to

5.27±0.45 µM and 3.66±0.38 µM. Therefore, this study synthesizes a hydrazone ligand based on benzohydrazide. Benzohydrazide is reacted with benzaldehyde (–H) and salicylaldehyde (–OH). The addition of descriptive groups influences the anticancer activity due to changes in bonding (distance, charge, angle, intramolecular, intermolecular, etc.), atomic groups (inductive, mesomeric, steric effects, etc.), conformation, lipophilicity, and reactivity [35, 36], allowing a quantitative relationship between structural changes and bioactivity to be established. The product will be tested for its anticancer effect on cervical cancer cells "HeLa"

# METHODOLOGY

## Materials and Instrumentations

All chemicals were obtained from commercial suppliers (Sigma Aldrich/Merck/Fulltime). Instruments used in this study included a UV-Vis spectrophotometer (Genesys 10S), FTIR spectrophotometer (8400S Shimadzu), ESI- MS (Q-TOF XEVO), 1H dan 13C NMR Delta2\_NMR JEOL RESONANCE 400 MHz, X-Ray Diffraction (XRD)

(PanAnalytical E’xpert Pro), TG/DSC 449 F3 Jupitert, Biosafety Cabinet (BSC) (Thermo scientific 1300 series a2), *centrifuge* (Thermo scientific microCL17), CO2 incubator (Thermo scientific series 8000DH), microscope (Thermo scientific EVOS XL Core), and *multimode reader* (Tecan Infinite M200 Pro).

## Synthesis of ligands and Copper(II) complexes

The ligands (E)-*N’*-benzylidenebenzohydrazide (**1A**) and (E)-*N’*-(2-hydroxybenzylidenebenzohydrazide (**1B**) were synthesized according to literature procedures [37]. The complex compounds were obtained by reacting the ligands with CuCl2·2 H2O at equal ratios in ethanol solvent. The mixture was refluxed for 2 hours, and then CH3COONa 1M was added until the pH reached 7-9. The mixture was filtered, washed several times with ethanol, and dried in a dessicator to a constant weight.

## Anticancer testing using PrestoBlue(PB) method

Anticancer testing was conducted at the Central Laboratory, Padjadjaran University using cervical cancer cells (HeLa). The testing procedure is outlined as follows previous studies [38]:

1. Preparation of Media/Positive Control/Samples

Complete Roswell Park Memorial Institute Medium (RPMI) containing 10% Fetal Bovine Serum (FBS) and 50 µL/50mL antibiotics, positive control (cisplatin), and PrestoBlue reagent were prepared. Test samples were prepared at specific concentrations as stock solutions in a non-toxic solvent, DMSO, in this study.

1. Cell Preparation

Confluent cells with at least 70% were used. The cell media was discarded, and cells were rinsed twice with 1 mL PBS solution. Next, 1 mL Trypsin-EDTA solution was added to the cells and incubated for 5 min to disperse the cell layer (cells appeared floating when observed under an inverted microscope). The cells were then transferred into a tube containing RPMI media and centrifuged at 3000 rpm for 5 min. The centrifuged cells were resuspended in RPMI medium in a tube.

1. Seeding Cells into a 96-Well Plate

The seeding process began by determining cell counts and viability using the trypan blue exclusion method and by resuspending the cells (final cell density of 170,000 cells/mL in media). Ten µL of trypan blue were placed into a sterile microtube, and 10 µL of cell suspension was added and mixed. Subsequently, 10 µL of the cell suspension was placed in a hemacytometer chamber sterilized with 70% ethanol, and the number of viable cells per mL was counted. The cell culture was seeded into a 96-well plate and incubated for 24 h (or until the cell confluency was at least 70%) at 37°C in an atmosphere containing 5% CO2.

1. Cell Treatment with Samples, Positive and Negative Control

Cells were treated with samples, and the positive and negative controls were examined in a 96-well plate. Test stock samples were diluted in a 1.5 mL microtube into eight concentration variants. 100 µL of the test solution and the positive control were added to the wells containing the cells and incubated for 48 h.

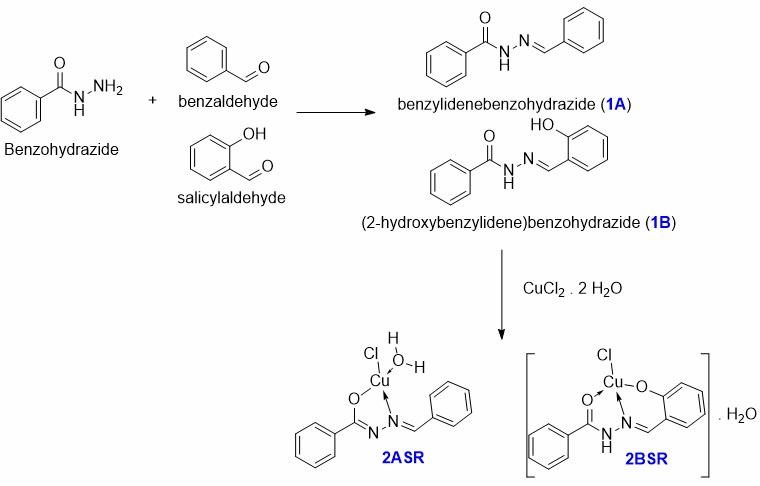
1. Testing with PrestoBlue Reagent and Absorbance Measurement

The testing began with the preparation of PrestoBlue reagent (PB) in a tube. 9 mL of RPMI media was added to the tube along with 1 mL of “PrestoBlue™ Cell Viability Reagent” (100 µL reagent for 900 µL media) and mixed. Then, 100 µL of the solution was added to each well where the RPMI media had been removed and incubated for 1- 2 hours until a color change was observed (Upon entering live cells, the PrestoBlue® reagent was reduced from the non-fluorescent blue compound resazurin to the red, highly fluorescent compound resorufin. This conversion is proportional to the number of metabolically active cells and can be measured quantitatively. The absorbance spectra of resazurin and resorufin were used for measurement. Finally, absorbance was measured at a wavelength of 570 nm (reference: 600 nm) using a multimode reader.

# RESULTS AND DISCUSSION

## Elucidation of Cu(II)-Hydrazone complexes

The hydrazone ligands was prepared by the equimolar condensation reaction of benzohydrazide and benzaldehyde (**1A**)/salicylaldehyde (**1B**) in ethanol with the presence of few drops of acetic acid glacial under reflux for 2 hours. Structure of the hydrazone ligands was confirmed in our previous studies [37]. The copper(II) complex was obtained as orange (**2ASR**) and brown (**2BSR**) solid from the equimolar ratio ligands with CuCl2. 2 H2O in ethanol with reflux for 2 hours. The synthetic route of ligands and copper(II) complexes are presented in **Figure 3**.



**Figure 3**. Synthetic route of hydrazone ligands and copper(II) complexes

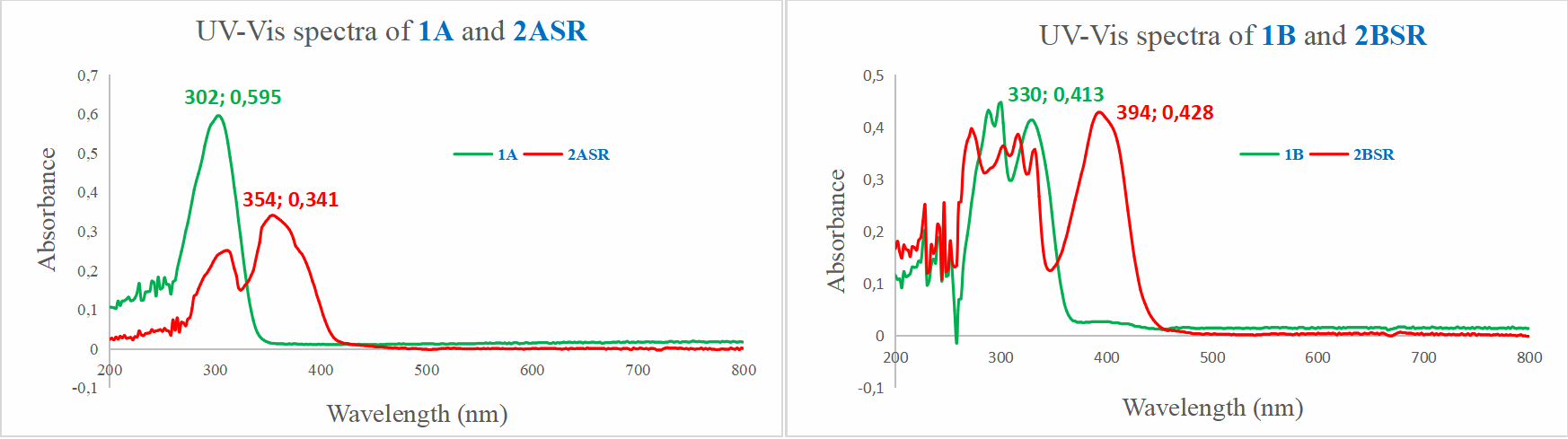
FTIR spectroscopy is used to determine the functional groups and coordination bonds between metals and ligands [39]. The infrared spectrum of the complex compounds and ligands shows similarities in absorption patterns, but there is a shift in some peaks. This indicates that the metal is bonded to the Lewis base site of the ligand. Both spectra exhibit absorption bands at wavenumber 445-470 cm-1 and 553-588 cm-1, indicating Cu–N and Cu–O coordination [16, 40-42]. However, the stereoisomer profiles of the two complexes are different.

The secondary N-H absorption band of the ligand at 3180.72 cm-1 present in ligand **1A** is not visible in the FTIR spectrum of **2ASR**. This indicates that the amide ligand was converted to a tertiary amine has occurred [43]. This evidence is supported by the loss of carbonyl stretching [16, 42] and strong absorption at wavenumbers 1599.04 and 1541.18 cm-1 due to –C=N–N=C–O– resonance [44, 45]. So, **2ASR** exhibits enol tautomerism. On the other hand, the **2BSR** complex compound shows a secondary N–H stretching vibration at 3240.52 cm-1 [46]. Additionally, the presence of the C=O peak proves that **2BSR** exhibits keto tautomerism [40]. The detailed absorption bands are listed in **Table 1**.

**Tabel 1.** Comparison of FTIR characterization of ligands and Cu(II) complexes

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Entry Functional** | | **Wavenumber of Wavenumber of complexes (cm-1) Reference** | | |
|  | **groups** | **ligands (cm-1)** |  |  |
|  |  | **1A** = 3180,72 | **2ASR** = - | 3179 [16] |
| 1 | -NH | **1B** = 3271,38 | **2BSR** = 3240,52 | 3141-3285 [32] |
|  |  |  |  | 3098-3100 [46] |
|  |  | **1A** = 1643,41 | **2ASR** = - | 1654 [16] |
| 2 | -C=O | **1B** = 1674,27 | **2BSR** = 1641,48 | 1650-1700 [31] |
|  |  |  |  | 1639 [42] |
|  |  | **1A** = 1548,89 | **2ASR** = 1599,04 dan 1541,18 | 1526-1575 [32] |
| 3 | -C=N | **1B** = 1539,25 | **2BSR** = 1539,25 | 1525-1531 [33] |
|  |  |  |  | 1591-1593 [45] |
|  |  | **1A** = - | **2ASR** = 445,57 | 456 [40] |
| 4 | -Cu-N | **1B** = - | **2BSR** = 470,65 | 461 [42] |
|  |  |  |  | 485 [47] |
| 5 -Cu-O **1A** = - | | | **2ASR** = 553,59 | 599 [40] |
| **1B** = - | | | **2BSR** = 588,31 | 502 [41] |

**Figure 4** shows that the maximum absorbance of ligand **1A** at 302 nm shifts to 354 nm (28249 cm-1) after forming the **2ASR** complexes, while the λmax peak of ligand **1B** also shifts from 330 nm to 394 nm (25381 cm-1) when bound to the metal (**2BSR**). The LMCT energy in cm-1 is calculated from the inverse of the maximum wavelength (cm-1=107/λ in nm). This bathochromic shift is due to charge transfer from the ligand to the metal (LMCT) [48-50]. These values are similar to those of previously reported studies as shown in **Table 2**. The LMCT energy indicates that the complexes have a square-planar geometry. The LMCT energy for square planar Cu(II)- hydrazone complex compounds is more than 25000 cm-1, while for tetrahedral geometry, it ranges from 21000- 25000 cm-1 [51-55]. On the other hand, the peaks at wavenumbers 228-270 nm and 302-316 nm indicate intraligand transitions of n→π\* and π→π\* [56].



**Figure 4**. UV-Vis spectra of ligand and copper(II) complexes

**Table 2.** LMCT analysis of hydrazone complexes have been reported

**Entry Compound Hydrazone base Geometry**

**λmax (nm) [energy**

**(cm-1)] LMCT**

**Reference**

* 1. [Cu(L)Cl] 6-nitro-benzothiazole-2-yl-hydrazine

+ 5-chloro salicylaldehyde

* 1. [Cu(L)Cl] 6-nitro-benzothiazole-2-yl-hydrazine

+ 3,5-dichloro salicylaldehyde

Tetrahedral 475

[21052] [51]

Tetrahedral 466

[21459]

* 1. [Cu(L) ].H O 2-hydrazinyl-4,8-dimethyl quinoline

Tetrahedral 453

[52]

2 2 + isatin [22075]

1. [Cu(L)(Oac)] Cyanoacylhydrazine + furfuraldehyde
2. [Cu(L)2] Cyanoacylhydrazine + acetophenone
3. [Cu(L)(N3)]- Acetohydrazide + o-vanillin
4. [Cu(L)(NCS) 3-methylbenzohydrazide + salicylaldehyde
5. [Cu(L)(C H OH)] Cyanoacylhydrazine +

|  |  |  |
| --- | --- | --- |
| Square  planar | 390  [25641] | [55] |
| Square  planar | 338  [29590] | [53] |

[53]

[53]

|  |  |
| --- | --- |
| Tetrahedral | 446  [22420] |
| Square | 398 |
| planar | [25120] |
| Square | 397 |
| planar | [25189] |

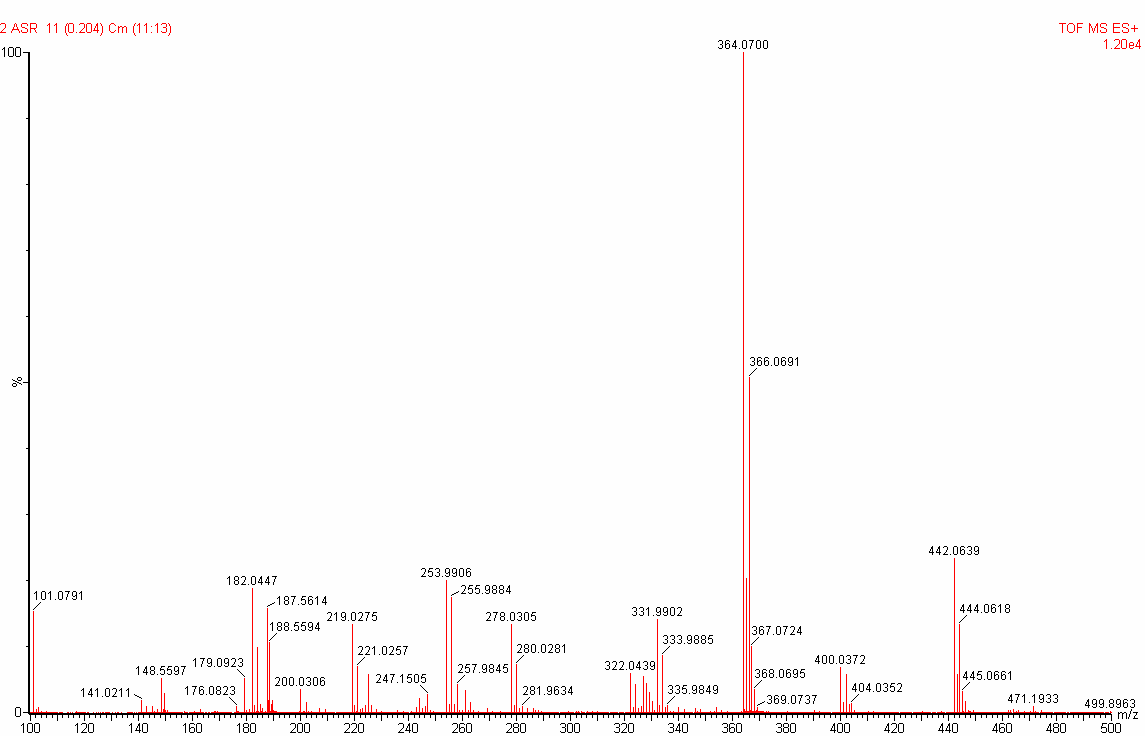
[54]

2 5 salicylaldehyde

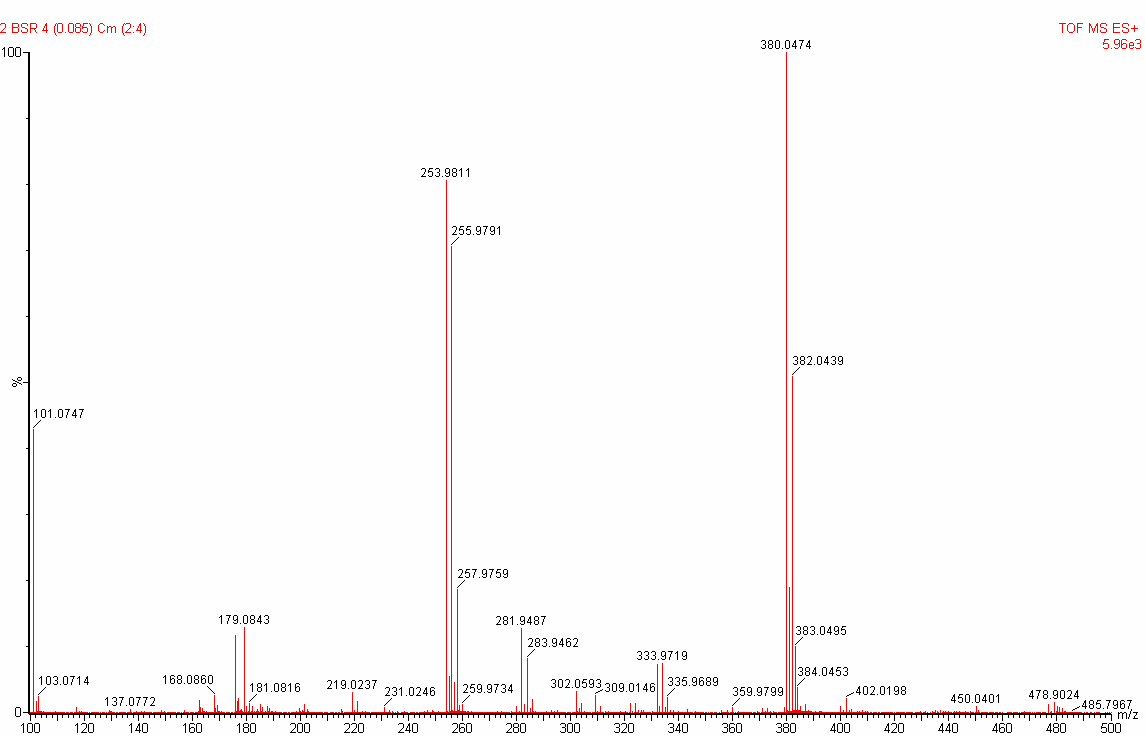
|  |  |  |
| --- | --- | --- |
| 9 | **2ASR** | Benzohyrazide + benzaldehyde |
| 10 | **2BSR** | Benzohydrazide + salicylaldehyde |

|  |  |  |
| --- | --- | --- |
| Square | 354 |  |
| planar | [28249] | This |
| Square | 394 | research |
| planar | [25381] |  |

MS characterization was performed to determine the molecular weight (m/z) of the Cu(II)-hydrazone complex compounds [57, 58]. In **Figure 5** and **Figure 6**, the **2ASR** and **2BSR** spectra shows the highest peak at m/z 364.0700 and 380.0474, corresponding to fragmentation [M + L + Cl + H2O + Na + H]+. The effects of alkali ions and solvents is consistent with those previously reported. Thermionic emission from both species provides strong affinity through physical bonding with the Lewis base of the compounds [59-62]. The MS characterization results indicate that the synthesized complex compounds have a metal-to-ligand ratio of 1:1.



**Figure 5**. ESI-MS spectra of **2ASR**



**Figure 6**. ESI-MS spectra of **2BSR**

The thermal behavior of the copper(II)-hydrazone complexes was studied using thermogravimetric analysis (TG) technique. The thermogram results indicate that the complex compounds undergo two thermal degradations. In the **2ASR** complex, the first degradation occurs at 190-300°C, indicating the release of one molecule of the ligand C14H11N2O. Subsequently, in the range of 300-400°C, there is a mass loss of 0.1353 mg (12.4673%), which is estimated to arise from H2Cl. On the other hand, the **2BSR** complex experienced a sample mass loss of 6.9797% at 210-260°C, which was identified as the dehydration of one molecule of crystal water. The second decomposition occurs at 260-400°C, corresponding to the mass loss of C14H11ClN2O. The final residues produced by both complexes is copper(II) oxide, with respective amounts of 24.1749% and 21.3766%. Copper(II) oxide is the residual compound of the Cu(II) complex, consistent with previously reported studies [63-65]. The decomposition stages of the complex compounds are summarized in **Table 3**.

**Table 3.** Thermal degradation of complexes

**Weight Loss (%)**

**No Compound T (°C) Moieties**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | | | **Found** | **Theoretical** |  |
| 1 190-300 | | | 63,0766 | 65,5882 | C14H11N2O |
| 2 300-400  3 > 400 | | | 12,4673  24,1749 | 11,0294  23,3824 | H2Cl  CuO (Residue) |
|  |  |  | (Residue) |  | |
| 4 |  | 210-260 | 6,9797 | H2O  5,0562  Mr = 18 g/mol | |
| 5 | **2BSR** | 260-400 | 71,6659 | C14H11ClN2O  72,6123  Mr = 257 g/mol | |
| 6 |  | > 400 | 21,3766 | CuO (Residue)  22,3315 | |
| (Residue) | | | | Mr = 79,5 g/mol | |

**2ASR**

14000



Intensity (counts)

12000

10000

8000

6000

4000

2000

0

10 20 30 40 50 60 70 80

**Figure 7**. XRD of **2ASR**

2Theta (°)

XRD characterization aims to determine the structure and crystal size of the synthesized Cu(II)-hydrazone complex compounds. The powder XRD patterns of the complex compounds are shown in **Figure 7** and **Figure 8**. The diffractogram results indicated that the generated peaks tended to be sharp, suggesting that the test material was in a polycrystalline phase [66]. The average crystal sizes of the **2ASR** and **2BSR** complex compounds were 64.24 and 66.92 nm, respectively. These values were obtained using the Debye-Scherrer formula, as shown in Equation 1 [67]. Nanocrystalline complexes can easily penetrate cell membranes [68].

(equation 1)

Note :

D = average crystal size

K = Debye-Scherrer constant (0.9)

λ = wavelength of light (Cu Kα = 1.5406Å) β = FHWM

3000



Intensity (counts)

2000

1000

0

10 20 30 40 50 60 70 80

**Figure 8**. XRD of **2BSR**

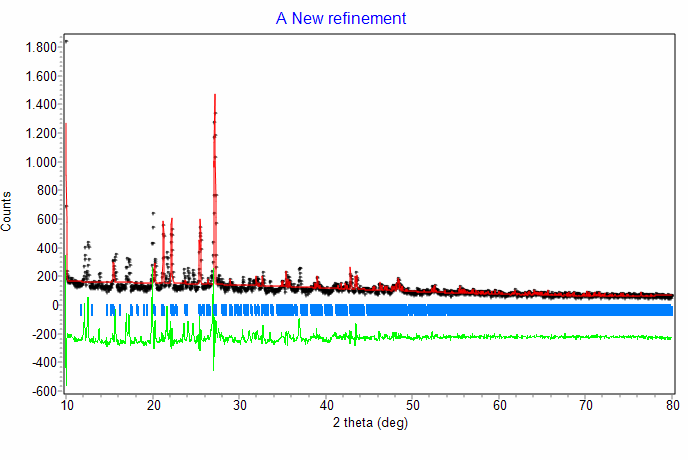
2Theta (°)

The diffractogram results were refined using the Rietveld method through the Rietica application. The Rietveld method is a nonlinear matching process between the calculated and measured diffraction pattern curves based on the crystal structure data using the least squares method [69]. The refinement process was performed only

on the **2BSR** complex because its predicted structure was similar to the reference structure, as shown in **Figure 9**. The reference was obtained from the IUCR electronic archives (SU2089) or CCDC 606531 data [70].

**Figure 9**. Reference structure [64]

The refinement results are shown in **Figure 10**. The red, black, and green peaks represent the reference data, sample, and difference between the reference and sample, respectively. Based on the refinement process performed using the Rietica application, the obtained values of Rp, Rwp, and χ2 are 17.1, 32.9, and 6.521, respectively. The chi-square index (χ2) and Rwp play a significant role in the match between the sample material and the reference. Referring to these values, the chi-square index of the refinement process approaches 4 [71], indicating a high match with the reference data. Additionally, the sample peaks resemble the reference peaks at 2θ values of 21.43°, 22.42°, 25.55°, 27.30°, and 43.075°. Thus, the **2BSR** complex has a four-coordinate geometry. The crystal system of the **2BSR** complex is triclinic, with side lengths of 6.78, 9.01, and 10.29 and angles of 76.64, 84.40, and 81.89.



**Figure 10**. Refinement **2BSR** in Rietica

## PrestoBlue (PB) test of Ligand and Cu(II)-Hydrazone Complexes

The chemotherapeutic potential of Cu(II)-hydrazone ligands and complex compounds was evaluated in vitro using the PrestoBlue (PB) method on HeLa cervical cancer cells. This method measures cell viability by involving direct contact between the sample and target cells using the PB reagent. PB works by reducing resazurin (**blue**, λmax = 600 nm) to resorufin (**pink**, λmax = 570 nm) [72-77]. The extent of conversion is directly proportional to the number of metabolically active cells (living cells) [78]. The therapeutic success potential of the sample is expressed as IC50, which is the minimum concentration required to inhibit 50% of the biological function of the total objects. The lower the IC50 value, the higher the activity of the compound [79, 80].

Cytological evaluation of the cells was conducted using Rosswell Park Memorial Institute (RPMI 1640) medium, which functions to grow and maintain cell morphology [81]. Positive and negative controls were cisplatin and DMSO solvent, respectively. The concentrations of the test solutions used for all samples were 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 μg/mL, with each variation repeated twice. Specifically for the 2BSR sample, the test solution concentration was reduced to 0.78, 0.39, and 0.20 μg/mL to normalize the % live cell data. All test sample data and well plate visualization results are shown in **Table 4** – **Table 7** and **Figure 11** – **Figure 14**.

**Table 4.** Absorbance of **1A** on “HeLa”

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Media** | **Media**  **+ cell** | **Cisplatin** | **Solvent** | **1,56** | **3,13** | **6,25** | **12,50** | **Concentration (µg/mL)**  **25,00** | **50,00** | **100,00** | **200,00** |
| **Absorbance** 0,3641 | 0,5735 | 0,4361 | 0,5829 | 0,5960 | 0,5830 | 0,5722 | 0,5820 | 0,5738 | 0,5722 | 0,6146 | 0,6627 |
| **570nm** 0,3735 | 0,5763 | 0,4408 | 0,5790 | 0,5836 | 0,5801 | 0,5923 | 0,5867 | 0,5882 | 0,5920 | 0,6101 | 0,6584 |
| **Absorbance** 0,4386 | 0,1754 | 0,3816 | 0,1855 | 0,2006 | 0,1901 | 0,1792 | 0,1865 | 0,1838 | 0,2029 | 0,2487 | 0,2833 |
| **600nm** 0,4499 | 0,1766 | 0,3841 | 0,1835 | 0,1954 | 0,1869 | 0,1907 | 0,1899 | 0,1844 | 0,2059 | 0,2345 | 0,2767 |
| **Difference in** -0,0745 | 0,3981 | 0,0545 | 0,3974 | 0,3954 | 0,3929 | 0,3930 | 0,3955 | 0,3900 | 0,3693 | 0,3659 | 0,3794 |
| **Absorbance** -0,0764 | 0,3997 | 0,0567 | 0,3955 | 0,3882 | 0,3932 | 0,4016 | 0,3968 | 0,4038 | 0,3861 | 0,3756 | 0,3817 |
|  | 100,35 | 27,54 | 100,20 | 99,78 | 99,25 | 99,27 | 99,80 | 98,63 | 94,25 | 93,53 | 96,39 |
| **% viability cell** | 100,69 | 28,00 | 99,80 | 98,25 | 99,31 | 101,09 | 100,07 | 101,56 | 97,81 | 95,58 | 96,87 |
| **Average %**  **viability cell** | 100,52 | 27,77 | 100,00 | 99,01 | 99,28 | 100,18 | 99,94 | 100,10 | 96,03 | 94,55 | 96,63 |
| **SEM** | 0,17 | 0,23 | 0,20 | 0,76 | 0,03 | 0,91 | 0,14 | 1,46 | 1,78 | 1,03 | 0,24 |
| **Normalization data % viability** | 100,52 | 27,77 | 100,00 | 99,01 | 99,28 | 100,18 | 99,94 | 100,10 | 96,03 | 94,55 | 96,63 |

**cell**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Media | Media  + cell | Cisplatin | Solvent | Concentration (µg/mL) | | | | | | | |
| 1,56 | 3,13 | 6,25 | 12,50 | 25,00 | 50,00 | 100,00 | 200,00 |

**Figure 11. 1A** well plate visualization of “HeLa” cells

**Table 5.** Absorbance of **1B** on “HeLa”

**Media**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Media** | | | | | **1,56** | **3,13** | **6,25** | **12,50** | **Concentration (µg/mL)**  **25,00** | **50,00** | **100,00** | **200,00** |
| **Absorbance** | 0,3359 | 0,5527 | 0,4095 | 0,5304 | 0,5362 | 0,4986 | 0,4913 | 0,4825 | 0,4857 | 0,4786 | 0,4672 | 0,4748 |
| **570nm** | 0,3520 | 0,5481 | 0,4172 | 0,5400 | 0,5354 | 0,5374 | 0,4928 | 0,5115 | 0,4969 | 0,5059 | 0,4615 | 0,4888 |
| **Absorbance** | 0,4000 | 0,1701 | 0,3573 | 0,1860 | 0,2072 | 0,2673 | 0,2865 | 0,2940 | 0,2920 | 0,2915 | 0,3133 | 0,3258 |
| **600nm** | 0,4179 | 0,1654 | 0,3654 | 0,1924 | 0,2048 | 0,2121 | 0,2814 | 0,2900 | 0,2988 | 0,2908 | 0,3146 | 0,3465 |
| **Difference in** | -0,0641 | 0,3826 | 0,0522 | 0,3444 | 0,3290 | 0,2313 | 0,2048 | 0,1885 | 0,1937 | 0,1871 | 0,1539 | 0,1490 |
| **Absorbance** | -0,0659 | 0,3827 | 0,0518 | 0,3476 | 0,3306 | 0,3253 | 0,2114 | 0,2215 | 0,1981 | 0,2151 | 0,1469 | 0,1423 |

**+ cell Cisplatin Solvent**

108,91 28,52 99,61 95,86 72,09 65,64 61,68 62,94 61,34 53,26 52,07

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **% viability cell**  **Average %** | 108,93  108,92 | 28,42  28,47 | 100,39  100,00 | 96,25  96,06 | 94,96  83,53 | 67,25  66,45 | 69,71  65,69 | 64,01  63,48 | 68,15  64,74 | 51,56  52,41 | 50,44  51,25 |
| **viability cell** |  |  |  |  |  |  |  |  |  |  |  |
| **SEM** | 0,01 | 0,05 | 0,39 | 0,19 | 11,44 | 0,80 | 4,01 | 0,54 | 3,41 | 0,85 | 0,82 |
| **Normalization** |  |  |  |  |  |  |  |  |  |  |  |
| **data % viability** | 108,92 | 28,47 | 100,00 | 96,06 | 83,53 | 66,45 | 65,69 | 63,48 | 64,74 | 52,41 | 51,25 |
| **cell** |  |  |  |  |  |  |  |  |  |  |  |

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Media | Media  + cell | Cisplatin | Solvent | Concentration (µg/mL) | | | | | | | |
| 1,56 | 3,13 | 6,25 | 12,50 | 25,00 | 50,00 | 100,00 | 200,00 |

**Figure 12. 1B** well plate visualization of “HeLa” cells

**Table 6.** Absorbance of **2ASR** on “HeLa”

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Media** | **Media**  **+ cell** | **Cisplatin** | **Solvent** | **1,56** | **3,13** | **6,25** | **12,50** | **Concentration (µg/mL) 25,00** | **50,00** | **100,00** | **200,00** |
| **Absorbance** | 0,3432 | 0,5368 | 0,4354 | 0,5300 | 0,5466 | 0,5337 | 0,4908 | 0,3994 | 0,3799 | 0,3778 | 0,3751 | 0,3868 |
| **570nm** | 0,3530 | 0,5337 | 0,4315 | 0,5184 | 0,5419 | 0,5308 | 0,4972 | 0,3925 | 0,3984 | 0,3865 | 0,3802 | 0,3834 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Absorbance** | 0,4072 | 0,1654 | 0,3404 | 0,1861 | 0,1758 | 0,1965 | 0,2449 | 0,3803 | 0,3902 | 0,3831 | 0,3839 | 0,4164 |
| **600nm** | 0,4185 | 0,1637 | 0,3392 | 0,2085 | 0,1755 | 0,1956 | 0,2479 | 0,3780 | 0,4009 | 0,3953 | 0,3891 | 0,4049 |
| **Difference in** | -0,0640 | 0,3714 | 0,0950 | 0,3439 | 0,3708 | 0,3372 | 0,2459 | 0,0191 | -0,0103 | -0,0053 | -0,0088 | -0,0296 |
| **Absorbance** | -0,0655 | 0,3700 | 0,0923 | 0,3099 | 0,3664 | 0,3352 | 0,2493 | 0,0145 | -0,0025 | -0,0088 | -0,0089 | -0,0215 |
|  |  | 111,36 | 40,79 | 104,34 | 111,21 | 102,63 | 79,32 | 21,41 | 13,90 | 15,18 | 14,29 | 8,97 |
| **% viability cell** |  | 111,00 | 40,10 | 95,66 | 110,09 | 102,12 | 80,19 | 20,23 | 15,89 | 14,29 | 14,26 | 11,04 |
| **Average %**  **viability cell** |  | 111,18 | 40,44 | 100,00 | 110,65 | 102,37 | 79,75 | 20,82 | 14,90 | 14,73 | 14,27 | 10,01 |
| **SEM** |  | 0,18 | 0,34 | 4,34 | 0,56 | 0,26 | 0,43 | 0,59 | 1,00 | 0,45 | 0,01 | 1,03 |
| **Normalization data % viability cell** |  | 111,18 | 40,44 | 100,00 | 110,65 | 102,37 | 79,75 | 20,82 | 14,90 | 14,73 | 14,27 | 10,01 |

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Media | Media  + cell | Cisplatin | Solvent | Concentration (µg/mL) | | | | | | | |
| 1,56 | 3,13 | 6,25 | 12,50 | 25,00 | 50,00 | 100,00 | 200,00 |

**Figure 13. 2ASR** well plate visualization of “HeLa” cells

**Table 7.** Absorbance of **2BSR** on “HeLa”

**Media**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | | | | **0,20** | **0,39** | **0,78** | **1,56** | **3,13** | **6,25** | **12,50** | **25,00** |
| **Absorbance** | 0,4785 | 0,8112 | 0,5657 | 0,7752 | 0,8006 | 0,7415 | 0,5487 | 0,4991 | 0,4965 | 0,5032 | 0,5043 | 0,5000 |
| **570nm** | 0,4857 | 0,8120 | 0,5850 | 0,7833 | 0,7985 | 0,7849 | 0,5283 | 0,4888 | 0,5075 | 0,5119 | 0,5121 | 0,4967 |
| **Absorbance** | 0,6054 | 0,2234 | 0,5469 | 0,2558 | 0,2300 | 0,3006 | 0,5315 | 0,6135 | 0,6055 | 0,6124 | 0,6151 | 0,6116 |
| **600nm** | 0,6127 | 0,2106 | 0,5579 | 0,2406 | 0,2149 | 0,2955 | 0,5573 | 0,6049 | 0,6152 | 0,6237 | 0,6188 | 0,6158 |
| **Difference in** | -0,1269 | 0,5878 | 0,0188 | 0,5194 | 0,5706 | 0,4409 | 0,0172 | -0,1144 | -0,1090 | -0,1092 | -0,1108 | -0,1116 |
| **Absorbance** | -0,1270 | 0,6014 | 0,0271 | 0,5427 | 0,5836 | 0,4894 | -0,0290 | -0,1161 | -0,1077 | -0,1118 | -0,1067 | -0,1191 |

**Concentration (µg/mL)**

**Media**

**+ cell Cisplatin Solvent**

108,62 22,15 98,23 106,01 86,30 21,91 1,91 2,73 2,70 2,45 2,33

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **% viability cell**  **Average %** | 110,69  109,66 | 23,41  22,78 | 101,77  100,00 | 107,99  107,00 | 93,67  89,98 | 14,89  18,40 | 1,65  1,78 | 2,93  2,83 | 2,30  2,50 | 3,08  2,77 | 1,19  1,76 |
| **viability cell** |  |  |  |  |  |  |  |  |  |  |  |
| **SEM** | 1,03 | 0,63 | 1,77 | 0,99 | 3,69 | 3,51 | 0,13 | 0,10 | 0,20 | 0,31 | 0,57 |
| **Normalization** |  |  |  |  |  |  |  |  |  |  |  |
| **data % viability** | 109,66 | 22,78 | 100,00 | 107,00 | 89,98 | 18,40 | 1,78 | 2,83 | 2,50 | 2,77 | 1,76 |
| **cell** |  |  |  |  |  |  |  |  |  |  |  |

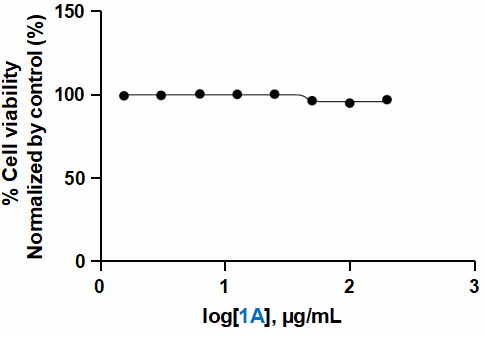
|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Media | Media  + cell | Cisplatin | Solvent | Concentration (µg/mL) | | | | | | | |
| 0,20 | 0,39 | 0,78 | 1,56 | 3,13 | 6,25 | 12,50 | 25,00 |

**Figure 14. 2BSR** well plate visualization of “HeLa” cells

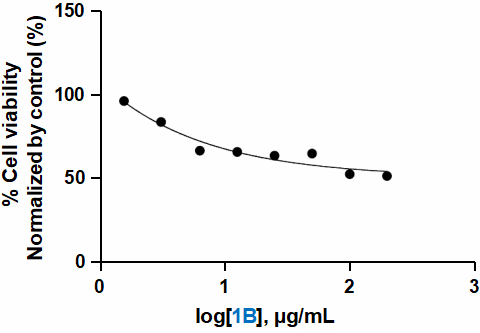
The well plate visualization results show two colors in all samples: **blue** and **pink**. **Blue** in media indicates the absence of living cells, whereas **blue** in the positive control indicates cell death due to cisplatin toxicity. On the other hand, the **pink** color in the media containing the test objects (HeLa) and samples at certain concentrations indicates the viability of cancer cells. When a compound successfully inhibits the viability of test cells, an oxidation reaction occurs, converting resorufin to resazurin, which is indicated by a **blue** color [82].

For ligand **1A**, cell viability tends to stagnate as the test concentration increases. This indicates that the IC50 was greater than 200 μg/mL or that the material was nontoxic. For ligand **1B**, increasing the concentration reduced the probability of cell survival; however, inhibition did not reach 50% at the highest tested concentration. Thus, ligands **1A** and **1B** have low or inactive toxicity since the IC50 is greater than 200 μg/mL.

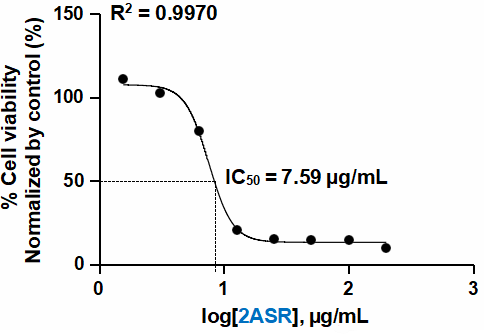
The test material, in the form of complex compounds, can extremely inhibit test cells, with IC50 values of 0.54 μg/mL for **2BSR** and 7.59 μg/mL for **2ASR**. These bioactivity values are better than those of the ligands, indicating that adding a metal can enhance therapeutic effects [16, 18, 44]. The increased inhibitory properties of complex compounds are due to the chelation of ligands with metal ions and increased planarity [35]. Additionally, the presence of copper ions can activate reactive oxygen species (ROS) [17], leading to DNA damage. DNA damage reduces the levels of NADH, FADH, NADPH, and cytochrome proteins, preventing cancer cells from metabolizing [75]. Based on the pharmacophore review of the compounds, the addition of hydroxyl groups decreased the IC50 value. This result is due to the π → π\* conjugation effect or electron delocalization from the hydroxyl groups [45, 83], making **2BSR** more polar than **2ASR**. This result is consistent with previous studies indicating that the presence of electron-donating groups enhances anticancer activity [84]. The half-maximal inhibitory concentration (IC50) values of the ligand samples and Cu(II)-hydrazone complex compounds are shown in **Figure 15** - **Figure 18** and **Table 8**.



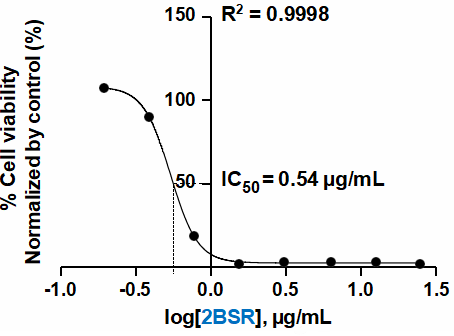
**Figure 15.** Curve of **1A** test results on "HeLa" cells



**Figure 16.** Curve of **1B** test results on "HeLa" cells



**Figure 17.** Curve of **2ASR** test results on "HeLa" cells



**Figure 18.** Curve of **2BSR** test results on "HeLa" cells

**Table 8.** IC50 ligands and Cu(II)-hydrazone complex compounds against “HeLa” cells

**Compound IC50 in HeLa(µg/mL) 1A** > 200

**1B** > 200

**2ASR** 7,59

**2BSR** 0,54

The IC50 levels of complex compounds were obtained from the non-linear dose-inhibition regression equation shown in **Equation 2** [85, 86]. This mathematical model interprets a sigmoidal response pattern (S curve) and is used in determining the IC50 dose of a medicinal material [87, 88]. The non-linear regression equation was obtained through the GraphPad Prism application and the results shown in **Table 9** were obtained

(equation 2)

Note :

Y = response (cell viability) X = concentration

Min = smallest asymptote on a curve Max = largest asymptote on a curve

**Table 9** Sigmoidal curve equation data for complex compound materials

|  |  |  |
| --- | --- | --- |
| Sample | Equation | R2 |
| **2ASR** |  | 0,9971 |
| **2BSR** |  | 0,9998 |

# CONCLUSION

Two complex compounds of copper(II) with the ligands (E)-*N'-benzylidenebenzohydrazide* and (E)-*N'-(2- hydroxybenzylidene)benzohydrazide* have been successfully synthesized. The molecular formulas of **2ASR** and **2BSR** obtained are [Cu(**1A**)(Cl)(H2O)] and [Cu(**1B**)Cl]H2O, respectively. Ligand and complex compound samples were tested for bioactivity as chemotherapy agents using the prestoblue method against cervical cancer cells (HeLa). As a result, complex compounds have better inhibition values than ligands. The **2BSR** sample with hydroxy substituents had the lowest IC50 of 0.54 μg/mL.

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