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RESEARCH ARTICLE

ROS-mediated Genotoxicity and Apoptosis Induced by a Novel Salicylaldimine Derivatives in Human Cervical Cancer Cells

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Abstract: ***Background*:** Cervical cancer is one of the most common types of cancer among women. Therefore, cancer studies are underway for a new chemo-agent with more effect on cancer cells and fewer side effects on normal human healthy cells. The currently studied novel ligand L_2b as a reduced salicylaldimine derivative was examined in seven cell lines, HeLa, DU-145, PC3, DLD-1, ECC, HT-29, and PNT1-A as a control.

***Aim*:** Because of the antiproliferative ability of L_2b, this study intends to look at the apoptotic, cytotoxic, and genotoxic activity of L_2b on HeLa.

***Methods*:** For this purpose, MTT assay is for screening cytotoxic effects, comet assay for looking for DNA damaging or genotoxicity levels, ELISA and DNA fragmentation for apoptotic measuring, AO/EB stain test for checking the rates of live, apoptotic and necrotic cells were performed. To reveal the oxidative state, OSI was assessed by total oxidant and antioxidant status ratios. FRAP assay was calculated for ferric-reducing antioxidant power, using total thiol and GSH assays to measure the antioxidant values of HeLa cells.

***Results*:** Of this result, we have found a tremendous effect of L_2b on HeLa cells, especially in raising the ROS rate, damaging their DNA, and causing a range of reactions leading to apoptosis.

***Conclusion*:** In conclusion, the data predict which ligand L_2b is capable of rising apoptosis *in vitro* cervical cancer cell line studied. Further cancer studies are needed to reveal the apoptosis pathways of the ligand L_2b in the HeLa cell line and its anticancer drug potency *in vivo* work.

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1. INTRODUCTION

Human cervical cancer is one of the most popular cancers among women of reproductive age worldwide [1]. It is one of the most common types of cancer in women in Türkiye [2]. Human papillomavirus (HPV) causes cervical cancer. The main factors responsible for cervical cancers are viral infection, inappropriate sexual behavior, multiple sexual partners, weak immune system, and smoking. The mechanism of cervical cancer blocks the tumor suppressor genes p53 and pRb by HPV virus genes or changes the base of DNA of cervical cells due to the effect of harmful chemicals and smoking that mutated the genetic compound of cells alternated with cancer [3]. In the early stages of cervical cancer, there is typically no symptom. The earliest finding is probably the abnormal pap smear detect-

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ed during routine gynecological examinations and used as a genetic test for screening HPV. PET/CT scans make it possible to diagnose cervical cancer in its developmental stages [4]. Cervical cancer develops quite slowly, so the symptomless period can last for years. In the advanced stage of cervical cancer, it has been seen that cancer metastasizes to other organs such as the bladder, intestines, lungs, and liver.

Blocking apoptosis, known as the cell death program, leads to the uncontrolled proliferation of cancer. Apoptosis has many inducers and pathways that lead cells to death. Firstly, there is a mitochondria-dependent internal pathway. The internal pathway is the most basic pathway for cell death, and it is active with effects such as growth hormone deficiency, heat shock, oncogene activation, DNA damage agents, reactive oxygen species (ROS), excessive cytosolic calcium, and other cellular stresses. The intrinsic pathway is followed by the extrinsic pathway (surface receptor-dependent). The Fas, TNF-like apoptosis-inducing ligand, interacts with the receptor of interest to promote appropriate alteration. Depending on these pathways and inducers, chemotherapeutic agents are designed to kill cancer cells and save normal cells in the human body. Chemotherapeutic drugs such as doxorubicin, etoposide, gemcitabine, and the three platinum drugs carboplatin, cisplatin, and oxaliplatin play a prominent role in killing cervical cancer cells in clinical settings [5-7]. Chemo medication targets apoptosis pathways to induce a cell death program that kills cancer cells. The cytotoxic effect of chemotherapy on cancer cells can be resulted in DNA damage, blocking genetic materials, and increased ROS in these cells that are affected by chemotherapeutic agents. These drugs, which cause an increase in ROS in cancer cells, also cause a decrease in the presence of antioxidants [8, 9]. However, these medications used to treat cervical cancer also cause numerous unwanted side effects such as drug resistance, severe kidney disorders, allergies, decreased immunity to infections, gastrointestinal disorders, bleeding, and hearing loss, especially in young patients [10]. Because of these points, the research and quest to discover pharmaceutical agents do not stop.

Researchers are constantly working towards developing new molecules that kill cancer cells and protect healthy cells with fewer side effects. Additionally, the molecules should generate a protective effect against toxic chemicals in healthy cells [11].

The present *in vitro* study aimed to demonstrate the apoptotic, cytotoxic, and genotoxic effect of a novel synthesis salicylaldimine derivative ligand (L_2b, Fig. **1**) as an anticancer agent on cervical cancer cell line HeLa and compared against normal PNT1-A cell line. For this purpose, we applied L_2b at different doses to HeLa and PNT1-A cell cultures for 24 hours.

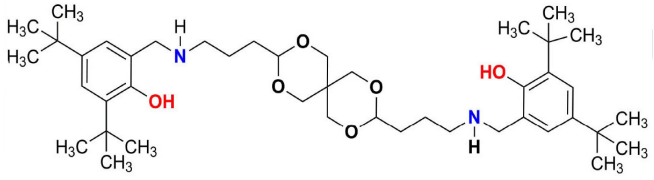


Fig. (1). Chemical structure of novel synthesized ligand L_2b [12].

2. MATERIALS AND METHODS

2.1. Cell Culture and Reagent

HeLa cells known as human cervical carcinoma and PNT1-A as normal prostate cell lines were obtained from the Laboratory of Medical Biology Department of Medicine Faculty, Van Yuzuncu Yil University (Van, Türkiye). Cells were routinely cultured in RPMI-1640 containing filter-sterilized fetal bovine serum 10% and penicillin/streptomycin (1% P/S 50 U/ml / 50 µg/ml) plus additives L-glutamine in a humidified atmosphere with 5% CO_2 in 37 °C. Since L-glutamine is an unstable amino acid, the medium was supplemented with L-glutamine just before use to a final concentration of 2 mM (0.292 g/L).

When cells were grown to confluence, they were allocated with sterilized trypsin/EDTA solution. As the cells were detached in a 37 °C incubator, trypsin activity was inhibited by adding a complete medium. Cells were counted with a hematocytometer by the following formula:

Cells/ml = average count per square × dilution factor × 10

Equal volumes of cell suspension were plated onto new dishes or flasks in appropriate densities.

2.2. Novel Ligand L_2b Salicylaldimine Derivative

This study synthesized a new type of ligand L_2b as an electron donor-acceptor and characterized it well by both analytical and spectroscopic methods [12].

The L_2b solution has been prepared by dissolving and diluting it in DMSO. In our studies, we used ligand L_2b to show its antiproliferation effect on HeLa cells and used MTT, comet, ELISA, DNA laddering, *etc*.

2.3. Cell Viability Assay (MTT Assay) and IC_{50} Measurement

The cytotoxicity and IC_{50} of ligand L_2b and Cisplatin were used in the MTT cytotoxicity assay, which is based on the cleavage of tetrazolium salts by mitochondrial succinate tetrazolium reductase in viable cells to form formazan dye [13]. HeLa cells and PNT1-A cells were grown in the final volume of 100 µL DMEM medium containing 10% FBS per well in 96 well plates at a 5 x 10^3 cells/ ml density and allowed to attach. After 24 h of incubation at 37°C in 5%, CO_2 added ligand L_2b was to it at various doses (2-200 µM). The medium was removed and the cells were slightly washed with PBS. Adding 5 mg/ml of MTT labeling reagent to each well. The plate was incubated for 3 h at 37°C, and DMSO was added for 3 h to dissolve the formazan crystals.

Finally, the optical density (OD) at 570 nm was measured using a microplate reader. Calculated the inhibition rate (IR) according to the following formula:

IR (%) = (OD treated / OD control) x 100%

The assay was performed at least three times. The half maximal inhibitory dose (IC_{50}) value was calculated by regression curve analysis of IR values at different doses. The IC_{50} value was the effective dose of ligand L_2b that killed half of the cells from different cell lines that experimented with it.

2.4. Measurement of DNA Damage by Comet Assay

Single-cell gel electrophoresis, called comet assay, was performed using the alkaline version described by some modifications [14, 15]. This technique version detects DNA migration caused by strand breaks, alkaline labile sites, and transient repair sites. Briefly, the slides were covered with the first layer of normal agarose; the second layer was low melting point agarose with slight modifications [16]. The trypan blue exclusion test measured cell viability above 95% for all treatments.

All experiments were repeated in triplicate cells and were classified according to their tail length in five categories, ranging from 0 on the visible tail to 4, still detectable head of the comet, but most of the DNA in the tail [17, 18].

2.5. Measurement of Apoptosis Level by ELISA

The ELISA assay was based on the quantitative sandwich enzyme two immunoassays. Principle of mouse monoclonal antibodies directed against DNA and histones, respectively [19].

The ELISA was developed and evaluated using a 20 µL sample and 80 µL immune reagent per microplate (MP) well. Cell equivalent: using 1x10^4 cells/well (200 µL), the sample analyzed (20 µL lysate corresponds to a cell equivalent of 1x 10^3 cells/well or 5x10^4 cells/ml). Measurement at 405 nm with ABTS solution (peroxidase substrate for ELISA reader + 100 µL ABTS step solution) as a blank (reference wavelength approx. 490 nm).

2.6. Apoptotic DNA Fragmentation Assay

According to the manufacturer's protocol, the Apoptotic DNA ladder kit (Roche Diagnostics GmbH, Mannheim, Germany) was used with DNA isolating to monitor apoptosis derived from HeLa cells. Briefly, human cervical carcinoma cells (5x10^6 cells) were incubated alone or with L_2b (10-100 µM) for 24 h and then collected. DNA was isolated after cells were homogenized in a lysis buffer. Finally, the extracted DNA was electrophoresed using 1% agarose gel and visualized by ultraviolet light following ethidium bromide staining, and the photograph was taken.

2.7. Measurement of Apoptosis, Necrosis, and Living Cells by AO/ EB

Acridine orange/ethidium bromide (AO/EB) fluorescent staining assay was performed to distinguish the living, apoptotic necrotic cells [20]. AO/EB is a technique to determine morphological changes in cells; the cells undergoing apoptosis are distinguished from the viable cells by the morphological changes of apoptotic nuclei. EB and AO are DNA intercalating dyes. AO is taken up by living and dead cells and stains double-stranded DNA.

AO is a nucleic acid selective metachromatic stain useful for living and apoptotic cell determination. AO interacts with DNA by intercalation and RNA by electrostatic attraction. DNA intercalated AO fluoresces green (525 nm); RNA electrostatically bound AO fluoresces red (>630 nm). AO stain staining living and apoptotic cells, and EB staining necrotic cells. Thus, quiescent and activated proliferating cells may be discriminated against and may also allow differential detection of multiple G1 compartments [21].

2.8. Determination of Intracellular TAS, TOS, and OSI

Total antioxidant status (TAS) and total oxidant status (TOS) were determined in cell homogenates using commercial colorimetric kits (Rel Assay, Türkiye) by the manufacturer's instructions. An autoanalyzer (Cobas Integra 800, Roche) colorimetrically measured

the resulting-colored products. With the TAS assay, it is possible to measure the antioxidative capacity of a sample in terms of inhibition of free radical reactions. In this method, the Fenton reaction produces the hydroxyl radical, which reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is a bright yellowish-brown colored compound. TAS results were expressed as mmol Trolox equivalent/mg protein. For the TOS assay, color intensity was associated with the total level of oxidant molecules present in the sample. Expressed TOS values were in mmol H_2O_2 equivalent/mg protein. In this method, the oxidants present in the sample oxidize the ferrous ion-O-dianisidine complex to ferric ions. To assess redox balance disorders, we have also used the oxidative stress index (OSI), which may be considered an indicator degree of oxidative stress in biological systems. The percentage ratio of the TOS to TAS is used to calculate OSI. OSI (Arbitrary Unit) = TOS / TAS X 10.

2.9. Ferric Reducing Antioxidant Power (FRAP) Assay

Reducing power capacity was determined using FRAP assay [22, 23]. HeLa cell line samples (0.1ml) treated with five various doses of ligand L_2b mixed with 3 ml of FRAP reagent (300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ), 20 mM $FeCl_3$ [10:1:1]. The mixture had incubated in a dark room for 20 min, and the absorbance was recorded at 593 nm using an ultraviolet-visible (UV/VIS) spectrophotometer. Six ascorbic acid concentrations were plotted in the standard preparation to determine the reducing power capacity. The following equation does the calculation:

FRAP value (μM) = [Abs. (sample) × FRAP value of Std (μM)] / Abs. of Std

2.10. Total Thiol Assay

A thiol is a compound containing a carbon-bonded sulfhydryl (–C–SH) group. The thiol kit protocol was used to analyze the total thiol of HeLa cells treated with different concentrations of L_2b and cisplatin. All reagents were prepared and mixed thoroughly before use. The absorbance of each well was read on a microplate reader using 450 nm as the primary wavelength.

2.11. Reduced Glutathione (GSH) Assay

Glutathione (GSH) is mammalian cells' major intracellular low defense against oxidative stress. For analysis of total glutathione or reduced glutathione, measurement was made in a microtiter plate reader using the colorimetric method. The assay is based on the glutathione recycling system by DTNB and glutathione reductase. The concentrations of GSH were determined in the sample solutions using the standard glutathione calibration curve [24, 25].

2.12. Statistical Analysis

Data are reported and evaluated statistically, and the final results were expressed as mean ± SD, and the analyzed concentrations of MTT, comet assay, ELISA, TAS, TOS, OSI, FRAP, total thiol, and GSH Data in all experiments were analyzed by using variance (one-way ANOVA) in Minitab 14® package program for Windows, and $p < 0.05$ was considered a statistically significant difference.

3. RESULTS

3.1. Measurement of IC_{50} and Cytotoxicity by MTT Assay

On various cancer cell lines; HeLa as cervical cancer, (DU-145 and PC3) prostate cancer, (DLD-1 and HT-29) colon cancer, ECC endometrial cancer, PNT1-A normal prostate epithelial cell line, the cytotoxic effect of new ligand L_2b is detected in triple repeat tests. This result revealed a higher effect of ligand L_2b on the HeLa cervical cancer cell line from 6 cancer cell lines. These data are recorded by estimating the concentration of the IC_{50} value that killed half of the cells. Depending on this experiment, we selected the HeLa cell line to test the activity of new synthesis ligand L_2b as an anticancer agent to discover a new anticancer drug agent in the future to cure cervical cancer, known as the second fatal cancer in the world in women. Table **1** summarized the effect of ligand L_2b on cancer cell lines and compared it with IC_{50} values of Cisplatin on HeLa and PNT1-A cell lines.

Table 1. IC_{50} values of various cell lines treated with ligand L_2b and Cisplatin.

Number	Cell lines	IC_{50} μM of L_2b	IC_{50} μM of CIS
1	HELA	24.53±1.31	15.18±0.78
2	DU-145	120.00±4.12	--
3	PC3	62.12±2.01	--
4	DLD-1	37.41±1.53	--
5	ECC	82.59±2.82	--
6	PNT1-A	30.20±1.38	5.84±0.26
7	HT-29	60.76±2.77	--

To determine the cytotoxicity of the novel synthesis ligand L_2b by MTT assay, we applied the ligand L_2b and Cisplatin at different concentrations (2, 5, 10, 25, 50, 100, 200 μM) on HeLa and PNT1-A cell lines. This result showed the cytotoxic effect of ligand L_2b

on cancer and normal cell lines and was compared with Cisplatin (an anti-cancer drug).

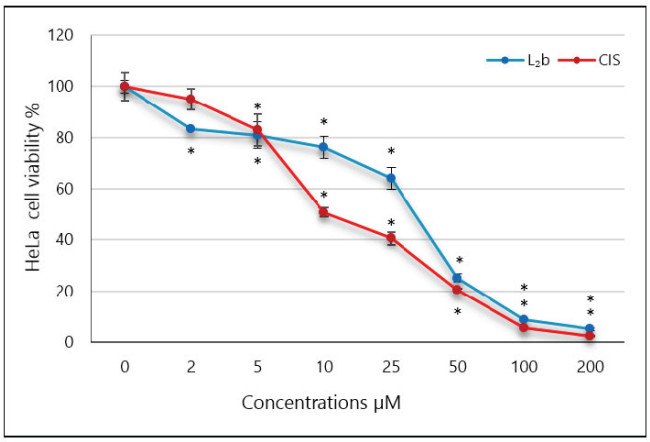


Fig. (2A). Cytotoxicity effect of L_2b and Cisplatin on HeLa-cells viability for 24 h. Data are expressed as the mean ± standard deviation of three independent experiments. (*) p-value < 0.05 compared with 0 µM dose negative control. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

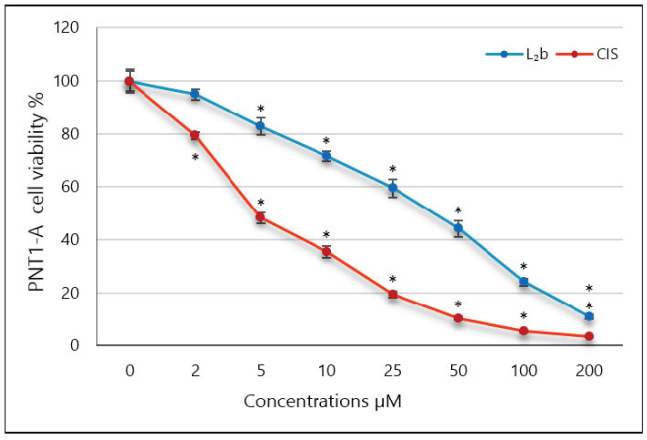


Fig. (2B). Cytotoxicity effect of L_2b and Cisplatin on PNT1-A cells viability for 24 h. Data are expressed as the mean ± standard deviation of three independent experiments. (*) p < 0.05 compared with 0 µM dose negative control. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Findings from the MTT assay of HeLa cells treated with various doses of L_2b ligand and Cisplatin demonstrate that the ligand L_2b, which showed a significant cytotoxic effect at all doses, augmented its activity even more due to the increasing concentration. At the 200 µM dose, the readings on cell viability in HeLa averaged higher cytotoxic activity. In another hand, applied HeLa cells were with cisplatin at various doses, and the result was a cytotoxic effect of cisplatin in all concentrations. The cytotoxic effect of the novel synthesis ligand and Cisplatin on Hela cervical cancer cells was observed where the ligand L_2b acted just like Cisplatin.

All results showed that L_2b significantly inhibited the proliferation of HeLa cells in a dose-dependent manner compared to the negative control (0 µM) at p-value < 0.05. Fig (**2A**) summarizes the novel ligand L_2b and Cisplatin cytotoxicity on HeLa cells.

According to the MTT assay, we obtained the cytotoxicity effect of novel ligands L_2b and Cisplatin on normal PNT1-A cells. In this context, the L_2b ligand showed its cytotoxic effect significantly after its higher dose than cisplatin. It has been noted that the new ligand L_2b has much less effect on normal healthy cells than cisplatin. All doses showed significantly higher data p-value < 0.05 except dose 2 µM. The overall measurement results are summarized in Fig. (**2B**). This result means the new synthesis ligand L_2b salicylaldimine derivative has less effect on normal cell lines and higher on cervical cancer cell lines.

3.2. Measurement of DNA Damage by Comet Assay

The comet assay, called single-cell gel electrophoresis (SCGE), utilizes the analysis of a fluorescent stain reflecting the enumeration of DNA breaks to determine DNA damage in the HeLa cell line administered with the reduced salicylaldimine derivative ligand L_2b.

In this test, we determined the high effect of ligand L_2b to break the DNA of HeLa cells, 25 and 50 µM doses of L_2b indicated the same effect of cisplatin and at 100 µM dose of L_2b got a result higher than cisplatin. Compared with 0 doses, all doses produced significant results with p < 0.05. (Fig. **3A** and **B**) presents the comet assay results measuring damaged DNA in the ligand L_2b-treated HeLa cell line compared with 15 µM Cisplatin.

3.3. Measurement of Apoptosis Level by ELISA

Measurement of apoptosis level was detected using enzyme-linked immunosorbent assay (ELISA) in HeLa cell lines treated with different concentrations of novel ligand L_2b (5, 10, 25, 50, 100 µM) and 15 µM of cisplatin. Cisplatin was used as positive control and dose 0 µM as a negative control to compare the results of ligand L_2b.

A significant apoptotic effect was recorded for all L_2b ligand doses compared to the negative control (at p < 0.05 value). In another hand, comparing the effect of ligand L_2b on HeLa cells with cisplatin, data have shown that doses 50 and 100 µM of novel ligand L_2b

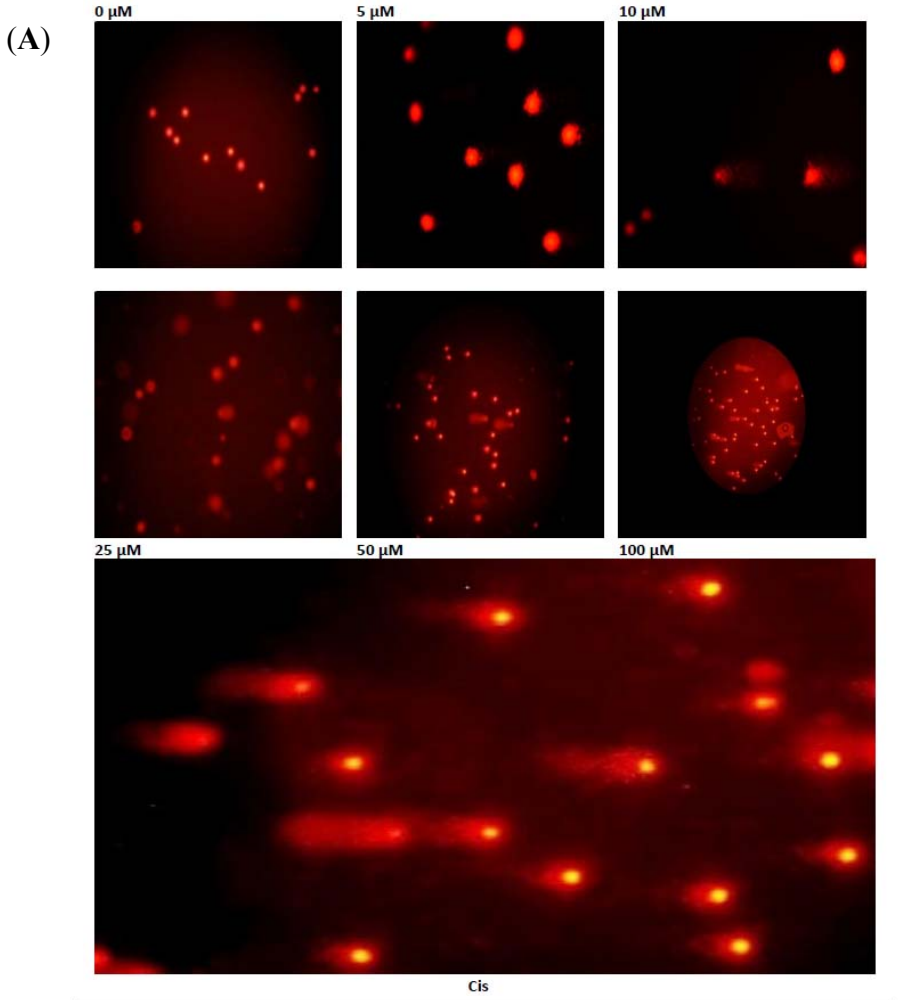


Fig. (3A and B). DNA damaging levels of HeLa cells in various doses of ligand L_2b and Cisplatin. (**a**) $p < 0.05$ compared with 0 µM negative control group, (**b**) $p < 0.05$ compared with Cisplatin positive control group. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

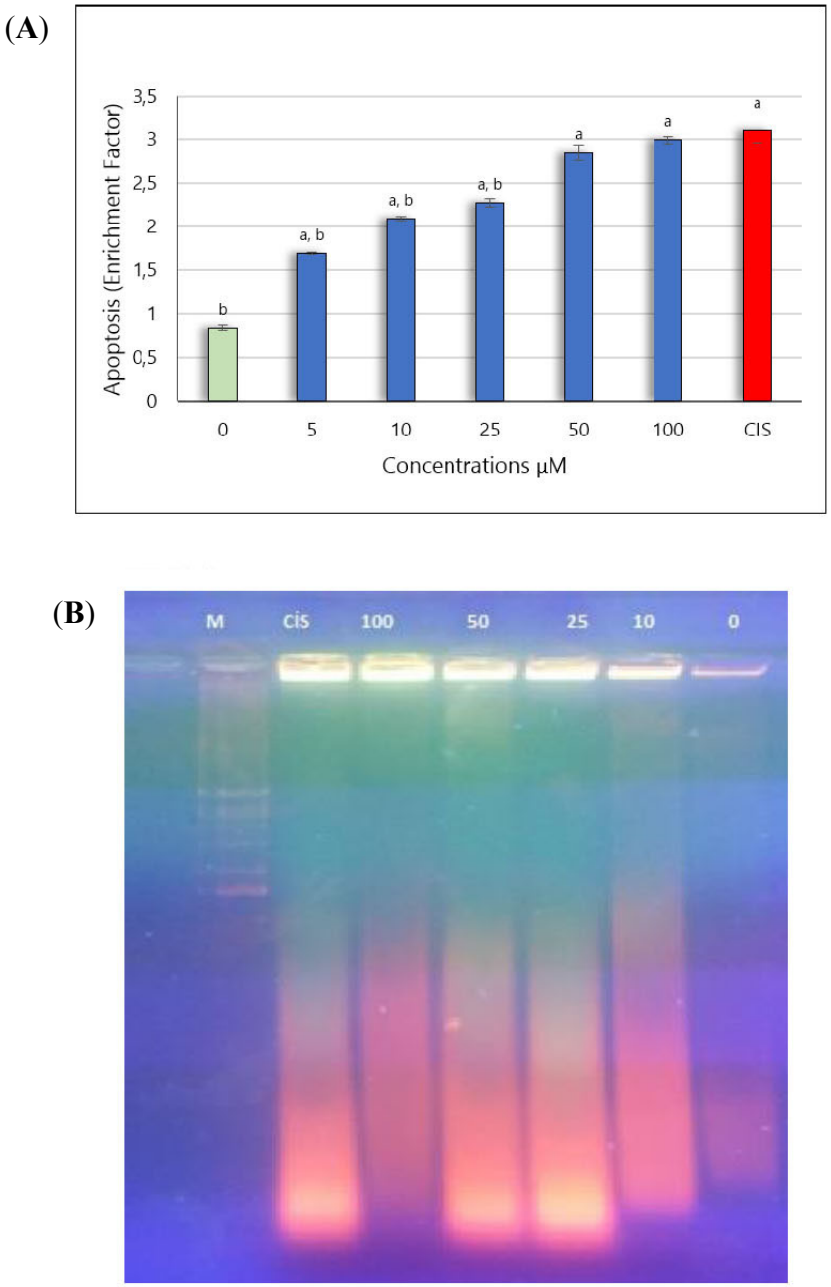


Fig. (4A). Apoptotic effect of various doses ligand L_2b and Cisplatin on HeLa cells. Data represent mean ± SD. (**a**) p- value compared with 0 µM control group; (**b**) p-value compared with Cisplatin group. **(4B)**. DNA laddering of HeLa cells treated with L_2b and cisplatin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

have the same effect of cisplatin on HeLa cells in this assay. Fig. (**4A**) shows the apoptotic level of HeLa cells.

3.4. DNA Fragmentation in HeLa Cells

DNA laddering can be observed when fragments resulting from apoptotic DNA are visualized by gel electrophoresis. Various concentrations (0-100 µM) of L_2b were applied to demonstrate DNA damage to HeLa cells. The ladder formation emerged as an indicator of DNA fragmentation which began to increase to the extension at a dose of 10 µM. We assume that the fragmentation at 25 and 50 µM increases depending on the reading result (Fig. **4B**).

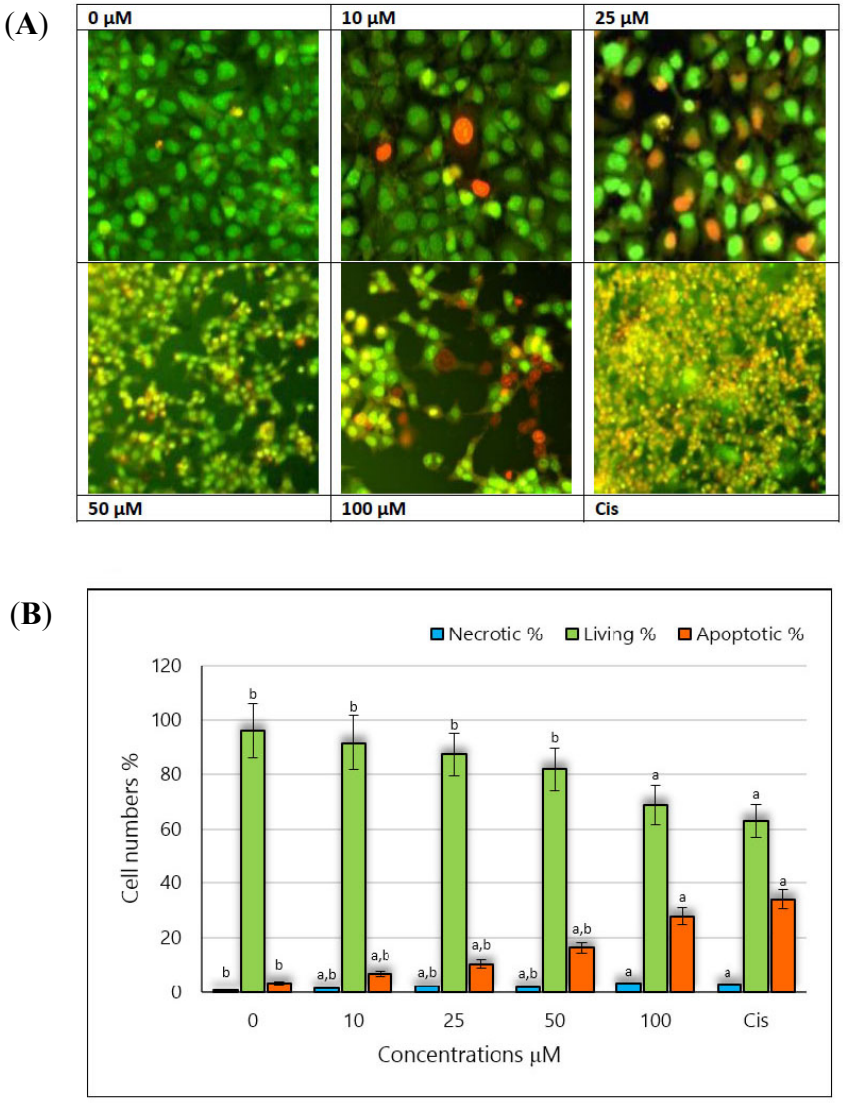


Fig. (5A and B). The extent of apoptosis, necrosis, and living cells treated with L_2b and Cisplatin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.5. Measurement of Apoptosis, Necrosis, and Living Cells by AO/EB

Results of AO/EB detection, due to its nucleic acid selective metachromatic properties, are commonly used in fluorescent microscopic examination of cell living status. This test result demonstrates the potential of ligand L_2b to induce the death of the HeLa cell line through the cell death program of apoptosis. Cells that began to undergo apoptosis at the 10 µM dose increased to 6.88% at 10 µM; the apoptosis rate was 3.37% compared to the 0 doses.

The proportion of dead cells in the HeLa cell line increased as the amount of ligand L_2b doses increased, with apoptosis rates increasing to 25 µM, 10.48%, 50 µM, 16.39%, and 27.88% at 100 µM.

The AO/EB test results, which show the necrosis rate, formed a result chart in which low and stable necrotic cell rates of the newly synthesized salicylaldimine derivative were observed at all doses.

That is one of the great points of effect and activity of ligand L_2b on the Hela cell line, due to the best drug agent way to decrease and kill cancer cells is an apoptotic way and not necrosis that depends on the change happening on the eukaryotic body. Fig. (**5A** and **B**) has shown the rates of apoptosis, necrosis, and living cells applied with ligand L_2b and compared with 0 doses as

a negative control and 15 µM dose of cisplatin as a positive control.

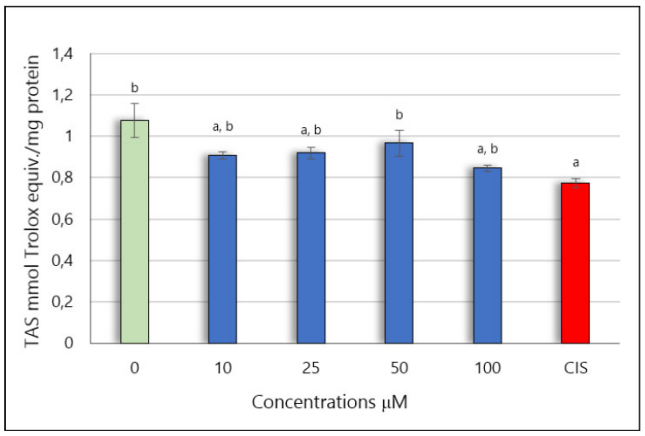


Fig. (6A). TAS values of HeLa cells treated with various doses of L_2b and Cisplatin. The results are presented as the mean ± SD, $p < 0.05$. (**a**) $p < 0.05$ compared with 0 µM negative control group, (**b**) $p < 0.05$ compared with Cisplatin positive group. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

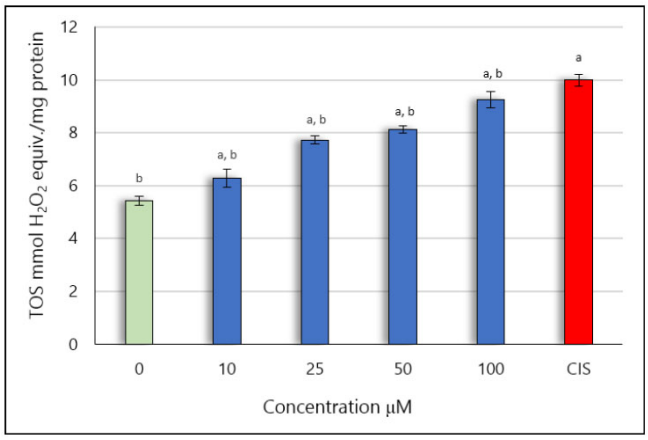


Fig. (6B). TOS values of HeLa cells treated by various doses of L_2b and Cisplatin. (**a**) $p < 0.05$ compared with 0 negative control group, (**b**) $p < 0.05$ compared with Cisplatin positive control group.

3.6. Total Antioxidant Status (TAS)

TAS level has been measured in the HeLa cancer cell line treated with various concentrations (10, 25, 50,100 µM) of novel synthesis ligand L_2b and 15 µM Cisplatin. These results show that the TAS level in the HeLa cell line decreases with increasing L_2b concentration, and the findings obtained at a dose of 100 µM have effects close to the effect of cisplatin (anti-cancer agent drug), which is used as a positive control. All concentrations of L_2b were significant at $p < 0.05$ compared with the negative control (0 µM) except for dose 10 µM. Fig. (**6A**) showed diminishing TAS levels in HeLa cells in the presence of ligand L_2b and cisplatin.

3.7. Total Oxidation Status (TOS)

TOS levels have been measured in HeLa cell lines treated with different concentrations (10, 25, 50, 100 µM) of the novel ligand L_2b and a 15 µM dose of cisplatin. In this context, a similarly increased TOS value was observed in the HeLa cell line in correlation with increasing doses of L_2b ligand. All doses of L_2b were significant at p-value < 0.05 when compared with the negative 0 µM and positive15 µM of cisplatin control groups. Fig. (**6B**) appeared the TOS levels when treated with various doses of L_2b compared with a 15 µM dose of cisplatin.

3.8. Oxidative Stress Index (OSI)

OSI values are an oxidative stress index obtained by dividing TOS by TAS. Based on the results of TAS and TOS, we calculated the OSI level of the HeLa cell line, which showed significant p-value at all various concentrations of the novel synthesis ligand L_2b tested in this study to demonstrate efficacy as an anticancer agent.

Fig. (**6C**) describes the oxidative stress index measured in the L_2b-treated HeLa cell line compared to 15 µM cisplatin.

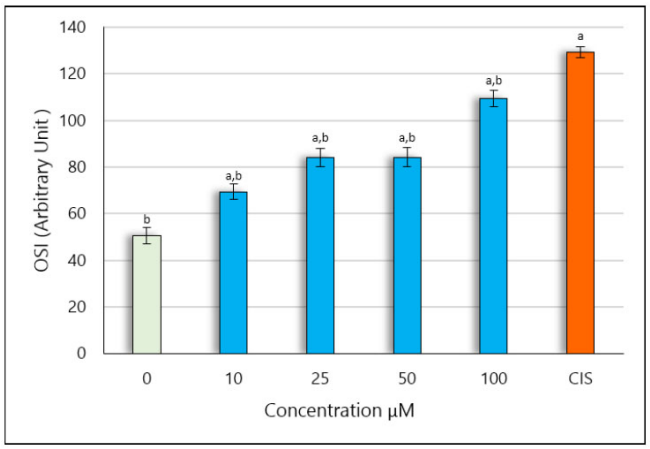


Fig. (6C). OSI values of HeLa cells treated with various doses of L_2b and Cisplatin. (**a**) $p < 0.05$ compared with 0 negative control group, (**b**) $p < 0.05$ compared with Cisplatin positive control group. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.9. Measurement of FRAP Assay

We used the FRAP assay as a safe method to determine the ferric-reducing ability of HeLa cells treated

with different concentrations (10, 25, 50, and 100 µM) of L_2b ligand and 15 µM Cisplatin. In this experiment, we show that all doses of L_2b reduce the FRAP value ($p < 0.05$) in the HeLa cell line compared to the negative control. The decreasing FRAP ratios showed that all L_2b concentrations had the same efficacy as cisplatin. Below (Fig. **7**) appears the reading of the FRAP test for HeLa cell lines treated with various concentrations of L_2b and 15 µM of cisplatin.

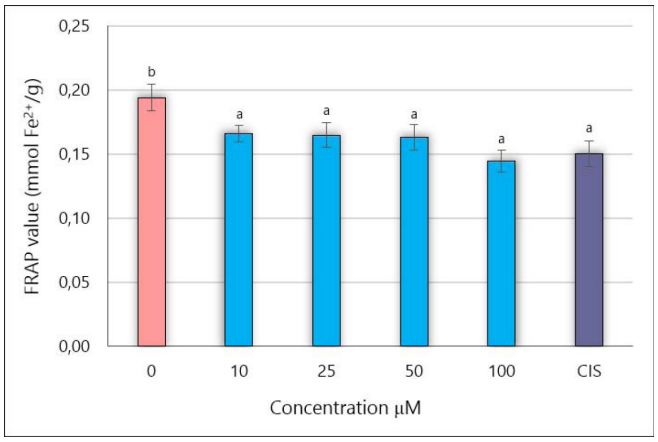


Fig. (7). FRAP values of HeLa cells treated with different doses of L_2b and Cisplatin. (**a**) $p < 0.05$ compared with 0 µM negative control group, (**b**) $p < 0.05$ compared with Cisplatin positive control group. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.10. Measurement of Total Thiol

Within our results, different concentrations of L_2b ligand (10, 25, 50, and 100 µM) were found to be effective at varying rates in HeLa cervical cell lines. The first dose, 10 µM L_2b, began to act by reducing the total thiol of cervical cancer HeLa cell lines. Nevertheless, in cervical HeLa cell lines, L_2b at a dose of 100 µM showed greater efficacy when compared to the negative control and equally potent when compared to the positive control. We predicted that increased ligand doses produced a resultant chart that reduced antioxidants in HeLa cervical cancer cell lines (Fig. **8**).

3.11. Measurement of Reduced Glutathione (GSH)

Glutathione, a notable intracellular non-protein thiol, protects against free radical damage. The first dose (10 µM) of L_2b ligand to the last dose (100 µM) administration significantly decreased the amount of GSH in cervical cancer HeLa cell lines. The 50 µM dose of L_2b was the active dose in HeLa cell line cancer, but a 100 µM dose of L_2b was more effective than others compared to negative control and positive control (Fig. **9**).

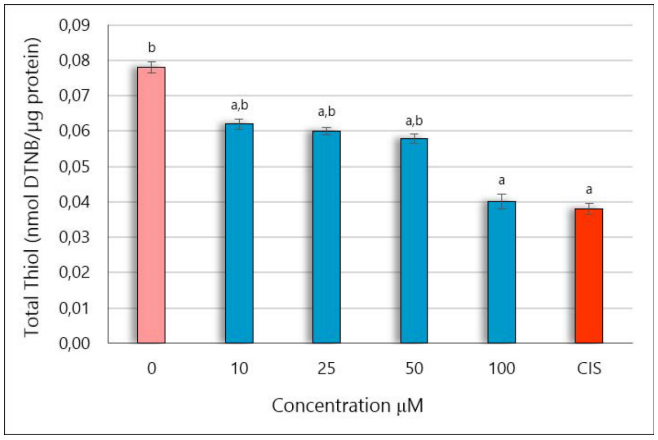


Fig. (8). Total thiol values of HeLa cells treated with doses of L_2b and Cisplatin. (**a**) $p < 0.05$ compared with 0 µM negative control group, (**b**) $p < 0.05$ compared with Cisplatin positive control group. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

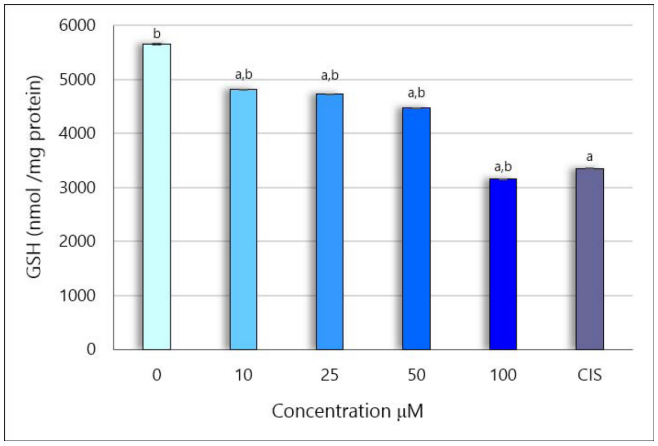


Fig. (9). GSH values of HeLa cells treated with doses of L_2b and Cisplatin. (**a**) $p < 0.05$ compared with 0 µM negative control group, (**b**) $p < 0.05$ compared with Cisplatin positive control group. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

Cervical cancer is the famous second tumor after breast cancer that causes death in females worldwide [26, 27]. Chemo medications are one of the selections for cancer therapy in the world, but the resistance of some of this medicine and a high side effect on human health after taking them. Researchers in the ongoing work of discovery of medication agents that are effective for treating cancer and with little effect on healthy cells. In our study, novel ligand L_2b, a derivative of reduced salicylaldimine explored apoptosis cured in human cervical HeLa cell lines and fewer side effects on sanitary cells.

To the best of our knowledge, there are no available data regarding the anticancer effect of salicylaldimine derivatives. Ligand L_2b is a novel synthesis; we first used it in this work to prove the antiproliferation effect on cervical cancer cell lines. In this study, we showed that besides having a cytotoxic and genotoxic effect, it also damages DNA and induces cancer cells to cell death apoptosis.

In our assay, we calculated IC_{50} values of ligand L_2b on several tumoral and nontumoral cell lines (HeLa, DU-145, PC3, DLD-1, HT-29, ECC, and PNT1-A). Based on these values indicating the higher cytotoxic effect of L_2b on HeLa cell lines, we selected HeLa cells for further experimentation to check the efficacy of the new ligand L_2b as an anti-cancer on cervical cancer cells. According to the results of a previously published article using different chemicals, similar to the findings of the current study, the IC_{50} values, which indicate the dose that kills half of the cell count, measured in the cervical cancer HeLa cell line support the MTT results of L_2b [28].

MTT assay showed that all doses of L_2b in cervical cancer HeLa cell lines reduced cell viability due to increased apoptosis. When evaluated in the light of cytotoxicity data, all subgroup doses were significant considering the $p < 0.05$ value compared to the negative control (0 μM) (Fig. **2A**). In HeLa cell lines, where the cytotoxic effect is closely related between antiproliferation and apoptosis, analyses with different treatments are encountered [29-31].

We also screened the cytotoxic effect of L_2b on a normal PNT1-A cell line and compared it with the anticancer drug Cisplatin. These results clarified the increase of apoptotic levels in PNT1-A cell lines but recorded the effect less than Cisplatin. The findings mentioned above mean that the novel ligand L_2b has fewer side effects than anticancer chemotherapy (Cisplatin) currently used to treat cancer. Synthesizing a new chemical that can eliminate cancer cells without any side effects is one of the great points and the aims of new studies to discover antiproliferation work on cancer cells with less effect on normal cells. (Fig. **2B**) expressed the cytotoxicity effects of L_2b and Cisplatin by different doses applied on PNT1-A cell lines.

We have been studying the results of genotoxicity detected by comet assay and DNA fragmentation using DNA ladder assay. It has been shown that human cervical cancer HeLa cells are significantly affected by different doses of novel synthesis ligand L_2b; for instance, doses of L_2b that work equally effectively with cisplatin are 25 and 50 μM. In the 100 μM, ligand L_2b was recorded more among those affected by HeLa cells than Cisplatin (Fig. **3A** and **3B**). All application groups exhibited a significant $p < 0.05$ value compared with the negative control group as untreated with L_2b. DNA damage at all L_2b concentrations that treated HeLa cell lines induced apoptosis and cell death. Further confirmed by ELISA, Fig. (**4A**) showed DNA damage caused the uprising of apoptotic rate on HeLa cell lines. In the literature, it has been reported that the measured DNA damage rate of HeLa cell lines treated with AgNPs extracted from *Podophyllum hexandrum* recorded increased DNA damage in HeLa cells treated with various doses of AgNPs [29].

In our experiment, the ELISA assay determines the cell death-inducing apoptosis rate of HeLa cell lines treated with various doses of ligand L_2b. These results have shown a significant effect in all dose manner p-value < 0.05. The rising dose of L_2b caused an increase in the apoptotic levels in HeLa cell lines, and doses 50 and 100 μM of L_2b correspond to the effect of cisplatin. This test was also confirmed by the cytotoxicity results of DNA damage from L_2b, causing cell death *via* an apoptotic pathway.

Additionally, the induction of apoptosis following DNA damage may be the primary cytotoxic mechanism of the chemotherapeutic agent or other DNA-binding anticancer drugs [32]. In the present study, we have demonstrated that DNA damage increases in treated cervical cancer cell lines by L_2b. However, it has been viewed that ligand L_2b can induce peroxidation in nuclear membrane lipids along with the rising break strand of DNA. The expected genotoxic behavior of L_2b can be explained by their high levels of pro-oxidant production or pro-oxidant activities, leading to elevated H_2O_2 in cancer cells [33]. H_2O_2 can kill any cervical cancer cells and abort their development at any stage, resulting in DNA damage by the hydroxyl radical generated *via* the Fenton reaction since the main aim of our research was to obtain a chemical agent that elevates or induces apoptosis. Our results demonstrated that ligand L_2b had shown higher apoptotic activity on HeLa cancer cells (Fig. **5A** and **B**).

Oxidative stress arises from an imbalance between pro-oxidant reactive species and antioxidant molecules, both endogenous and exogenous. It has been associated with many non-communicable diseases, such as cancer and aging [34]. As oxidative stress plays an important role in disease status, numerous commercial test kits for oxidative stress assessment have become available. Clinical studies require the use

of these test kits in large numbers as these are used in experiments with cell cultures.

TAS and TOS are the additive measurement parameters of oxidative stress that enable the measurement of all antioxidants and oxidant parameters by being evaluated together. The TOS/TAS ratio, in other words, the OSI, is an indicator of the oxidative stress degree and shows the anti-oxidation and oxidation redox balance.

The results of TAS analysis of HeLa cell lines treated with various concentrations of L_2b ligand revealed that TAS level decreased with increasing L_2b concentration. The effects of all L_2b doses were significant compared to the untreated negative control group, except for the 5 μM dose.

TOS levels have been investigated in HeLa cell lines treated with the same doses used in the TAS assay. The test results showed that all dose-dependent administration of L_2b significantly increased TOS correlatedly. OSI values we obtained from TAS and TOS results and showed the balance between them. The data of OSI increased by uprising concentration of L_2b. Finally, we understand that the oxidant level is higher than the antioxidant in L_2b-treated HeLa cell lines.

Our study investigated ferric-reducing antioxidant power by FRAP assay in HeLa cell lines that healed with different doses of L_2b. The data of this experiment showed dropped FRAP values with rising doses of L_2b. All results recorded statistically significant $p< 0.05$ values compared with negative control. All doses of the ligand L_2b tested in this study had the same effect as Cisplatin, resulting in a reduction in FRAP.

It has been noted that thiol, and GSH assays decreased the total thiol and GSH values of HeLa cells with rising applying concentrations of L_2b. Laboratory data showed a reduction of antioxidants in HeLa cells affected by L_2b.

Cancer therapies such as radiotherapy and chemotherapy use ROS overproduction to prevent the proliferation of cancer cells [35]; ROS elevation has also been observed after the administration of the novel synthesis ligand L_2b. Down-regulation of antioxidant defense systems is responsible for ROS elevation, as seen in the markedly reduced levels of TAS and FRAP in HeLa cancer cell lines, followed by increased TOS and OSI. In addition, the induction of chronic oxidative damage on biomolecules such as DNA has also emerged [36, 37]. ROS triggers the apoptotic cascade, which is assumed to be the implementer of apoptosis [38]. Many studies have implicated intracellular ROS in the signal transduction pathways that lead to apoptosis [39, 40]. In the present study, TAS, TOS, OSI, FRAP, Thiol, and GSH tests were used to calculate the production of ROS at 24 h culture setup; in this context, we observed a statistically significant increase in ROS rate during ligand L_2b treatment. These data suggest that the ligand L_2b can induce cell death in HeLa cells through a ROS-mediated apoptotic process, as implied by the pro-oxidant effect in HeLa cells. The increased ROS rates might be the reason for the go-up apoptosis morphological alteration in the ligand L_2b applied cells.

AO/EB staining was performed to check for cell death, apoptosis, and necrosis and to account for viable cells. In our study, apoptosis, necrosis, and live cell detection were conducted in HeLa cell lines treated with various doses of L_2b, handling fluorescent microscopy. The outcome of AO/EB has shown an increasing number of apoptotic with stable necrosis levels by the raised concentration of L_2b. The dose of 100 μM obtained a higher effect than the cisplatin used as a positive control group. In the paper, the authors reported that their study on the apoptotic pathway induced by different chemical hexapeptide treatments in the HeLa cell line reduced the viable cell rate, similar to our result [41].

The alteration of morphology of the cells during cell death and apoptosis, such as chromatin condensation, and a reduction in cell volume, fragmentation of DNA, or multiples thereof in such system apoptosis bodies are formed [42]. However, the necrosis cell death program, which causes inflammation and infection, is not preferred in designing any drug to control and kill cancer cells due to its effect on the human body. Depending on the morphological and physiological changes that occur in the human body induction of cell death, apoptosis has beena considerable mechanism for anticancer agent discovery. In this context, studies on the development of agents that inhibit abnormal cancer cell growth and abolish it *via* the apoptosis pathway have accelerated [8, 9, 43-47].

CONCLUSION

In conclusion, the present *in vitro* study strictly confirms the capacity of the novel ligand L_2b in cytotoxic, genotoxic, and regulating the proliferation of human cervical cancer HeLa cell lines mainly via apoptosis emerging through increasing ROS production in cancer cells. Less effect of L_2b on normal PNT1-A cells were observed compared with its highly effective on HeLa cancer cells. Therefore, the derivative compound

of L_2b might be used as an antiproliferative agent for cancer therapy. Since our data was based on *in vitro* experiments, further *in vivo* studies are required to consolidate the genotoxic and antiproliferative properties of L_2b and need to identify the pathways that induce apoptosis and signaling involved.

LIST OF ABBREVIATIONS

HPV	= Human papillomavirus
ROS	= Reactive oxygen species
TNF	= Tumor necrosis factor
L_2b	= Novel synthesis salicylaldimine derivatives ligand
HeLa	= Human cervical carcinoma cell line
PNT1-A	= Normal prostate cell line
MP	= Per microplate
AO/EB	= Acridine orange/ethidium bromide
TAS	= Total antioxidant status
TOS	= Total oxidation status
MTT	= Cell viability assay
IC_{50}	= Half maximal inhibitory dose
HP	= Hydrogen peroxide
FRAP	= Ferric reducing antioxidant power
TPTZ	= 2,4,6-tripyridyl-S-triazine
UV/VIS	= Ultraviolet-visible
GSH	= Reduced glutathione
DU-145 and PC3	= Prostate cancer cell lines
DLD-1 and HT-29	= Colon cancer cell lines
ECC	= Endometrial cancer cell line
SCGE	= Single-cell gel electrophoresis
ELISA	= Enzyme-linked immunosorbent assay
OSI	= Oxidative Stress Index

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The approval was provided by the ethical committee of Van Yuzuncu Yil University, Türkiye. The ethics approval number: 03.06.2016/13.

HUMAN AND ANIMAL RIGHTS

No animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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