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Determination of Apoptosis Level of Hela Cells Treated with Disobinol Compound from *Chisocheton Macrophyllus* Plant

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Abstract

Disobinol is a limonoid compound from the seeds of the Chisocheton macrophyllus plant that has been reported to have anticancer activity against cervical cancer cell lines. Cervical cancer is a type of cancer caused by infection with Human papillomavirus (HPV) types 16 and 18, leading to the transformation of normal cervical epithelial cells into cancerous cells. Previous studies show that Disobinol has a cytotoxic effect on HeLa cells with the IC_{50} value 52,92 µg/mL (24 hours' incubation). This study aims to determine the level of apoptosis in HeLa cells treated with Disobinol and observe the DNA fragmentation in HeLa cells undergoing apoptosis. The HeLa cells were treated with Disobinol at concentrations of 26,5 µg/mL (1/2 IC_{50}), 52,3 µg/mL (IC_{50}), and 104,6 µg/mL (2x IC_{50}) and incubated for 24 hours. The level of apoptosis was analyzed by using flow cytometry, and the DNA fragmentation pattern was analyzed by agarose gel electrophoresis. The results showed that Disobinol induces early apoptosis in HeLa cells, although the difference in the percentage of cell death is not very significant, which were 37.05%, 37.7%, and 41.60%, respectively. The DNA fragmentation pattern in HeLa cells treated with various concentrations of Disobinol was also observed on agarose gel. Therefore, Disobinol has the potential to be used as a chemotherapeutic drug or as a lead compound for the treatment of cervical cancer.

Keywords: *HeLa cell line, Disobinol, flow cytometry, agarose gel electrophoresis*

Introduction

Cancer is a disease characterized by the uncontrolled proliferation of transformed cells, which undergo evolution through natural selection, accompanied by the invasion of surrounding tissues and metastasis to distant parts of the body [1]. Globally, cancer is the second leading cause of death after cardiovascular diseases. In 2022, there were 20 million newly diagnosed cases and 9.7 million deaths attributed to cancer. Among all cancer types, cervical cancer ranks as the eighth leading cause of cancer-related mortality worldwide [2].

Several therapeutic approaches commonly employed to treat cancer include surgery, radiation therapy[3], chemotherapy[4],[5], and monoclonal antibody therapy[6]. Although numerous chemotherapeutic agents have been developed and applied, their efficacy remains limited due to adverse effects on healthy tissues, leading to significant health complications. Traditional approaches such as chemotherapy and radiation therapy impose considerable physical and psychological burdens on patients[7]. Therefore, the development of more specific and targeted chemotherapeutic agents remains a critical priority.

Cervical cancer is a primary malignant tumor that arises from the transformation of normal cervical epithelial cells into cancerous cells. The disease is predominantly caused by infection

with oncogenic human papillomavirus (HPV) genotypes 16 and 18[8]. HeLa cells, a widely used cervical cancer cell model, are characterized by their immortality, semi-adherent growth properties, and rapid, uncontrolled proliferation both in vivo and in vitro[9].

The Meliaceae family, native to tropical and subtropical regions, is recognized for its extensive diversity of secondary metabolites, including alkaloids, coumarins, flavonoids, terpenes, and limonoids[10]. These compounds have been reported to exhibit various biological activities, such as anticancer, antibacterial, antifungal, antimalarial, and antiviral[11],[12],[13]. Among them, the limonoids azadirachtin and nimbolide have shown notable anticancer properties, particularly by inhibiting cervical cancer cell proliferation through molecular mechanisms involving the induction of apoptosis and cell cycle arrest [14],[15]. Given the limitations of current therapeutic modalities and the significant burden they impose on patients, the exploration of natural products as alternative or complementary anticancer agents has gained increasing interest. In this context, members of the Meliaceae family, known for their rich repository of bioactive secondary metabolites, represent a promising source of novel therapeutic candidates against cervical cancer [10].

The genus Chisocheton, the second largest within the Meliaceae family, comprises approximately 50 species distributed across several East Asian countries. This genus is a rich source of various compounds, including limonoids, tirucallanes, dammaranes, lupanes, oleananes, steroids, sesquiterpenes, anthraquinones, alkaloids, coumarins, and simple phenolic compounds, many of which exhibit notable biological activities [16]. Several limonoids isolated from Chisocheton species have been reported to possess anticancer activity[17],[18],19].

The search for anticancer drugs has focused on compounds that specifically target mechanisms inhibiting cancer cell growth and survival [5]. The primary goal is to suppress cell proliferation, disrupt the cell cycle, or induce apoptosis, a form of programmed cell death, in cancer cells. In cancer cells, there is often an upregulation of several proteins involved in signaling pathways that promote growth, such as Her2, Ras, and MAPK. These proteins play crucial roles in accelerating cell division and cancer metastasis. Conversely, proteins responsible for triggering apoptosis, such as Bcl-2 and p53, are frequently downregulated or dysfunctional in cancer cells. The imbalance between growth-promoting proteins and those regulating cell death is a key factor in the uncontrolled proliferation of cancer cells. Therefore, effective cancer therapies must include compounds that can both suppress overactive signaling pathways and reactivate apoptotic mechanisms, thereby halting tumor growth and preventing cancer cell dissemination to other tissues[20].

In a previous study by Gaffar et al. (2024)[21], it was reported that Disobinol exhibits anticancer activity against HeLa cells and is categorized as toxic, with IC_{50} values for incubation periods of 24, 48, and 72 hours at 52.92, 52.70, and 14.96 µg/ml, respectively, as determined by the MTT assay. Gene expression analysis via qPCR indicated that Disobinol has the potential to induce apoptosis through the extrinsic pathway and inhibit the cell cycle. Therefore, Disobinol may be considered as a candidate for chemotherapy or as an adjuvant agent for cervical cancer treatment. This study aims to assess the level of apoptosis and observe DNA fragmentation in HeLa cells treated with Disobinol, using flow cytometry and agarose gel electrophoresis methods.

Experimental

Equipment

The equipment used in this study includes a Flow Cytometer (BD Bioscience), Hemocytometer (BD Bioscience), CO_2 incubator (Thermo Scientific), Inverted Microscope (BD Bioscience), Mini-Sub Cell GT Agarose Gel Electrophoresis System (Biorad), Refrigerated Centrifuge (Eppendorf), 25 cm^2 cell culture flasks, 100 cm^2 cell culture petri dishes (NEST), 10 mL and 5 mL serological pipettes (NEST), and 15 mL and 50 mL centrifuge tubes (Biologix).

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General glassware, pipettes, and pipette tips commonly used in molecular biology laboratories were also utilized.

Material

The Disobinol used in this study is a pure compound with a purity of approximately 98%, obtained from Nurlelasari et al. (2017). HeLa cells were a laboratory collection from the Faculty of Pharmacy, Universitas Padjadjaran (Unpad). The following reagents were used: Annexin V-FITC and PI (Propidium Iodide) Apoptosis Kit (Biolegend), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin antibiotics, Trypsin-EDTA, Phosphate-Buffered Saline (PBS), and Trypan Blue (Gibco). Cisplatin, agarose, DMSO, DNA Marker, and TAE buffer (Tris-acetate-EDTA) were purchased from MilliporeSigma; GelRed (Biotium); and the innuPREP DNA Mini Kit 2.0 (Analytik-Jena).

Methods

HeLa Cell Revitalization

HeLa cell proliferation was performed aseptically in an Airstream laminar flow cabinet (ESCO) using sterilized equipment and media. The cells were thawed from liquid nitrogen and cultured in 25 cm^2 cell culture flasks containing 5 mL of DMEM supplemented with 10% FBS and Penicillin-Streptomycin (100 U/mL and 100 µg/mL), and incubated in a 5% CO2 incubator at 37°C until the culture reached approximately 80% confluence. The cells were then washed twice with 1 mL PBS. HeLa cells were harvested by adding 300 µL of trypsin-EDTA and incubated in the CO_2 incubator for 3 minutes (until cells detached from the flask surface). Cell viability and condition were monitored under an inverted microscope. Subsequently, 4 mL of DMEM was added to stop trypsin activity. The cell culture was centrifuged at 6000 rpm for 5 minutes, and the pellet was resuspended in 3 mL DMEM. The cell count was determined using a hemocytometer.

Treatment of HeLa Cell with Disobinol

Disobinol stock solution 1 mg/mL was made in 100 µL DMSO. The solution was vortexed for 5 minutes at maximum speed. Then, 900 µL of water was added, and the solution was vortexed for 10 minutes at maximum speed. The Disobinol stock solution was then diluted into three concentrations: 26.5 µg/mL (1/2 IC_{50}), 52.3 µg/mL (IC_{50}), and 104.6 µg/mL (2x IC_{50}), based on the IC_{50} value of Disobinol against HeLa cells after a 24-hour incubation[21]. A total of 1 mL of HeLa cell culture, approximately $5x10^5$ cells per well, was seeded into 6-well culture plates. Then, 1 mL of Disobinol at each concentration was added, along with a 12.0 µM Cisplatin control, a solvent control (culture medium and DMSO), and a negative control (HeLa cells without treatment). The cells were incubated for 24 hours at 37°C in a 5% CO_2 incubator.

Flow Cytometry Analysis

HeLa cells treated and incubated for 24 hours were removed from the incubator and observed using an inverted microscope. The medium was then aspirated, and the cells were washed with 2 mL PBS. Next, 150 µL of Trypsin-EDTA was added, and the cells were incubated for 3 minutes. The cell culture was observed under an inverted microscope to confirm that all HeLa cells had detached. Subsequently, 2 mL of fresh DMEM medium was added to inactivate the Trypsin-EDTA. The cell culture was then centrifuged for 5 minutes at 6000 rpm to collect the cells. The supernatant was discarded, and the pellet was washed with 1 mL PBS and centrifuged again for 5 minutes. The cell pellet was resuspended in 100 µL Annexin V binding buffer, followed by the addition of 5 µL Annexin V-FITC and 10 µL PI, and vortexed. The cells were then incubated in the dark for 15 minutes at room temperature. After incubation, 400 µL of Annexin V binding buffer was added to each microtube. The samples were analyzed using a BD C6 Accuri Flow Cytometer, and the flow cytometry data were processed using BD C6 Accuri software.

Determination of DNA Fragmentation in HeLa Cells

HeLa cells were revitalized, harvested, counted, and treated with Disobinol as described in the previous experiment until a cell pellet was

obtained. The cell pellet was washed with 200 µL PBS, and the cells were detached from the well surface using a scraper. The cells were then transferred to a microtube and centrifuged for 10 minutes at 7500 x g. The tube was placed on ice, and the supernatant was discarded. DNA isolation was performed following the innuPREP DNA Mini Kit 2.0 protocol (Analytik-Jena). To the pellet, 100 µL PBS was added, and the cells were resuspended by pipetting. Next, 200 µL of CBV lysis buffer and 20 µL of proteinase K were added and vortexed for 5 seconds. The mixture was incubated at 56°C for 30 minutes in a thermoblock for cell lysis. After incubation, 200 µL of SBS binding buffer was added and vortexed for 5 seconds. The sample was then transferred to a spin filter tube placed in a collection tube and centrifuged at 11,000 x g for 2 minutes. The filtrate in the collection tube was discarded, and the DNA bound to the spin filter was washed twice with 650 µL MS wash buffer, followed by centrifugation for 30 seconds at 11,000 rpm. Residual solution on the spin filter was removed by centrifugation at maximum speed for 2 minutes. The spin filter was then transferred to a new microtube, and 50 µL of elution buffer was added, incubated for 1 minute, and centrifuged for 1 minute at 11,000 x g. A total of 20 µL of the eluate was electrophoresed on a 1% agarose gel with 1x TAE buffer. Electrophoresis was performed at 100 volts for 45 minutes, and the gel was visualized under UV light.

Results and Disscusion

HeLa Cell Revitalization

Figure 1 illustrates the morphological characteristics of healthy HeLa cells as observed through inverted microscopy. The cells exhibit typical features of healthy HeLa cells, including a normal appearance and firm adherence to the surface of the culture flask. Notably, the cells display an angular, leaf-like shape, which is commonly observed in HeLa cell morphology. Additionally, the cells exhibit a patchy monolayer growth pattern, a distinctive feature that indicates normal cellular behavior and growth in a controlled environment. These observations confirm the overall health and viability of the HeLa cells in culture.

Apoptosis Levels in HeLa Cells Treated with Disobinol

Before flow cytometry analysis, HeLa cells were treated with Disobinol, incubated for 24 hours, and observed using an inverted microscope. Figure 2 shows the morphology of HeLa cells under each treatment. The negative control (Figure 2a) represents untreated cells, which exhibit a typical monolayer pattern with angular, leaf-like shaped HeLa cells and normal cell membranes. Figure 2b shows cells treated with the solvent DMSO. Compared to the negative control, a decrease in cell population and a morphological shift from angular to slightly round cells can be observed in the solvent control. This suggests that DMSO solvent affects HeLa cell growth.



Figure 1. Observation of HeLa cells using an inverted microscope, showing the morphology of cultured HeLa cells at 100x magnification.

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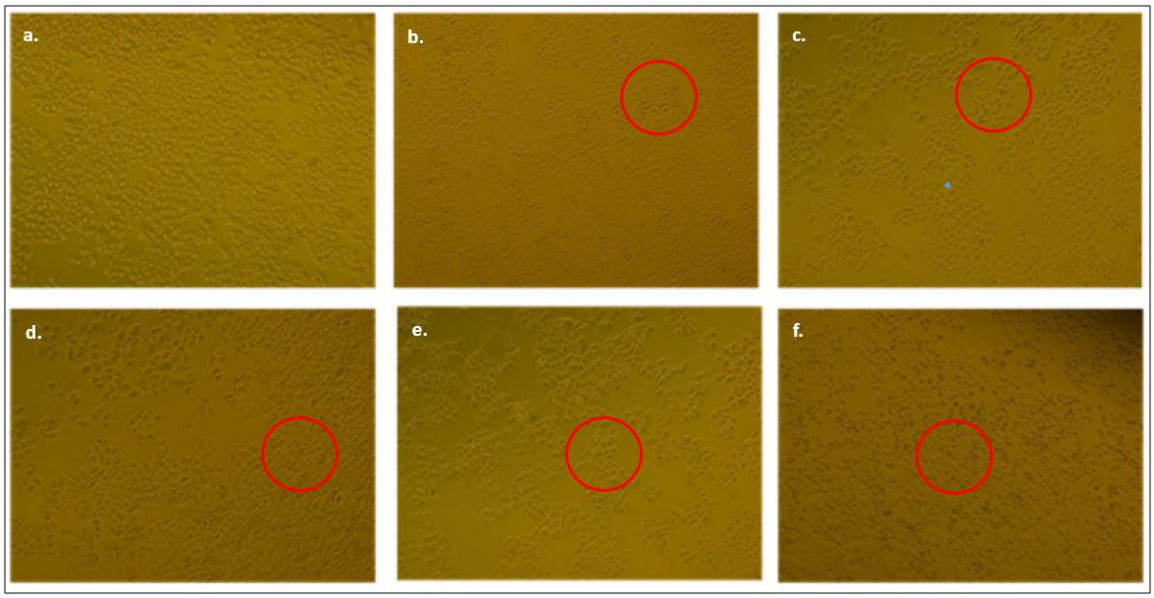


Figure 2. Inverted microscope analysis showing the morphology of HeLa cells: a) negative control, b) solvent control, c) ½ IC_{50} Disobinol, d) IC_{50} Disobinol, e) 2x IC_{50} Disobinol, and f) 12.0 µM Cisplatin, at 100x magnification.

Additionally, cells treated with Disobinol and Cisplatin also exhibit notable changes, including a decrease in cell population, shrinkage in cell size, reduction in cell volume, granulation, and altered cell shape. These changes indicate that both Disobinol and Cisplatin can inhibit HeLa cell proliferation. However, changes in cell population were not significantly different between the concentrations of ½ IC_{50}, IC_{50}, and 2x IC_{50} (Figure 2).

Flow cytometry analysis was performed to determine whether the cytotoxic effects of Disobinol are mediated through apoptosis. Cells were stained with Annexin V conjugated to FITC, which serves as a marker for the occurrence of apoptosis. Cells were also simultaneously stained with PI to assess the loss of membrane integrity. This dual staining procedure allows differentiation between early apoptotic cells (Annexin V-FITC positive, PI negative) and late apoptotic cells (Annexin V-FITC positive, PI positive).

Figure 3 presents the results of flow cytometry analysis, divided into four quadrants, each representing different cell states. Q2-UL in the upper left quadrant represents HeLa cells undergoing necrosis, Q2-LL in the lower left quadrant represents live HeLa cells, Q2-UR in the upper right quadrant represents HeLa cells undergoing late apoptosis, and Q2-LR in the lower right quadrant represents HeLa cells undergoing early apoptosis. In Figure 3, it is observed that all treatment groups, including the control cells, exhibit apoptosis, which could be attributed to culture conditions, environmental stress, or intrinsic factors. However, the percentage of apoptosis increased in each treatment group (Table 1), allowing for further analysis of Disobinol's effect on HeLa cells. Treatment of HeLa cells with Disobinol at concentrations of ½ IC_{50}, IC_{50}, and 2x IC_{50} led to an increase in the percentage of apoptosis, as observed by the shift in the population of live cells towards early and late apoptosis. However, the differences were not highly significant. The total percentage of apoptotic cells increased from 22.8% in the control cells to 39.1%, 40.0%, and 43.65% in cells treated with Disobinol after 24 hours **(Table 1)**.

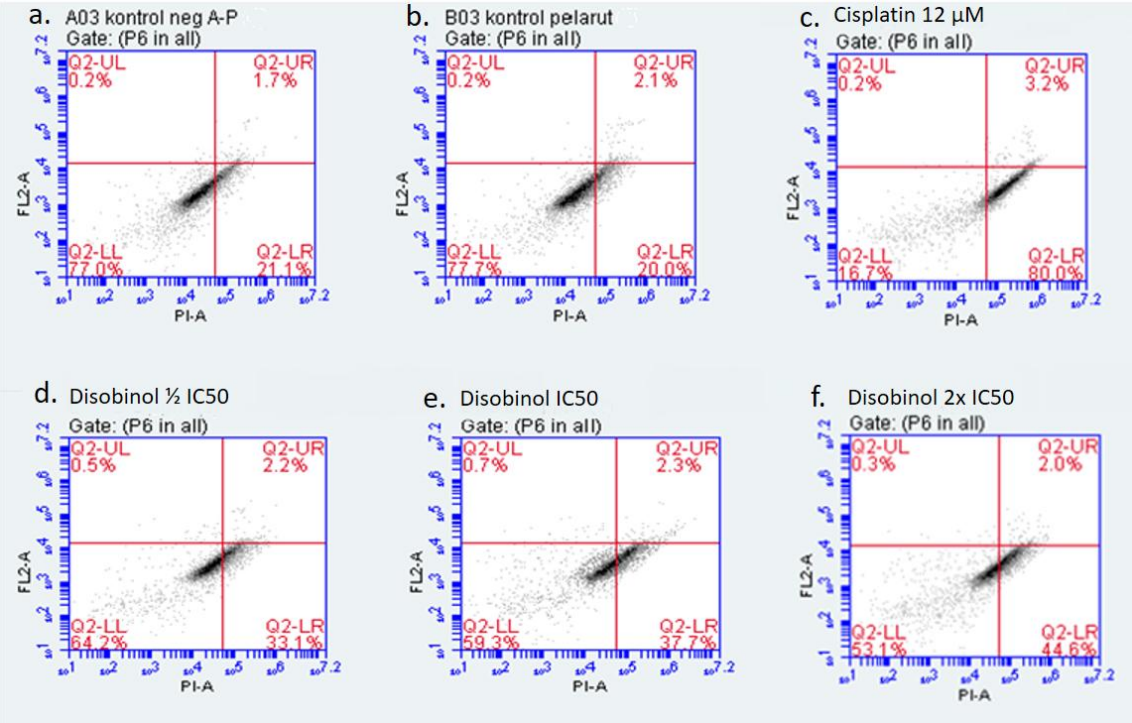


Figure 3. Flow cytometry analysis results of HeLa cells: a) control, b) solvent control, c) 12 µg/mL Cisplatin, d) ½ IC_{50} Disobinol, e) IC_{50} Disobinol, f) 2x IC_{50} Disobinol..

Table 1. Percentage of HeLa cell death based on flow cytometry analysis.

	Percentage of HeLa cell (%)			
	Living cell	Early Apoptosis	Late Apoptosis	Necrosis
Negative control	77,0	21,1	1,7	0,20
Solvent control	77,7	20,0	2,1	0,20
Cisplatin 12 µM	16,7	80,0	3,2	0,20
½ IC_{50} Disobinol	60,55	37,05	2,05	1,00
IC_{50} Disobinol	59,3	37,7	2,3	0,70
2x IC_{50} Disobinol	56	41,6	2,05	0,35

Previous studies have shown that Disobinol exhibits cytotoxicity against HeLa cells and induces apoptosis by increasing the expression levels of caspase 8 and 3, as observed through qPCR analysis [21]. The results of this flow cytometry analysis support these findings, showing a decrease in the percentage of live cells and an increase in the percentage of apoptotic cells in the Disobinol-treated group. Although the increase in apoptotic cells was not highly significant, it suggests that Disobinol has the potential to induce apoptosis.

The animal cell membrane is a complex structure composed of a phospholipid bilayer. The outer layer of the plasma membrane predominantly contains phosphatidylcholine (PC) and sphingomyelin (SM), while the inner layer is enriched with phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidyl ethanolamine (PE) [22]. In viable cells, plasma membrane integrity is preserved, preventing Annexin V-FITC from binding to PS, as PS remains located on the inner leaflet of the membrane [23]. During early apoptosis, membrane asymmetry is lost, resulting in the

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externalization of PS to the outer leaflet. The negatively charged PS disrupts phospholipid interactions, leading to membrane damage. Annexin V, a phospholipid-binding protein, binds to externalized PS with high affinity in a Ca^2-dependent manner [24]. FITC conjugated to Annexin V emits fluorescence, allowing for the visualization of PS exposed on the outer membrane surface [25]. In late apoptotic cells, PS externalization occurs alongside loss of plasma membrane integrity, permitting propidium iodide (PI) to penetrate the cell and intercalate with nucleic acids, which can be detected as PI fluorescence emission. In contrast, necrotic cells are only PI-positive due to membrane lysis, allowing PI to bind nucleic acids without prior PS externalization [23].

The results of the flow cytometry analysis presented in Table 1 demonstrate that the percentage of viable HeLa cells decreased with increasing concentrations of Disobinol; however, the reduction was not as pronounced compared to the effect observed with cisplatin treatment. Similarly, the percentage of HeLa cells undergoing late apoptosis was relatively low. This outcome may be attributed to the relatively short incubation time employed in this study, which was limited to 24 hours. In the previous study by Gaffar et al. [21], the lowest IC_{50} value for Disobinol was reported following 72 hours of incubation, reaching IC_{50} of 14.96 µg/mL. Therefore, it is likely that extending the incubation period to 72 hours would result in a higher proportion of HeLa cells undergoing late-stage apoptosis.

DNA Fragmentation of HeLa Cells Treated with Disobinol

The occurrence of apoptosis can also be detected through the observation of DNA fragmentation. HeLa cells treated with Disobinol were subjected to DNA isolation using the innuPREP DNA Micro Kit (Analytik-Jena), followed by characterization via agarose gel electrophoresis. As shown in Figure 3, the presence of DNA fragments indicates internucleosomal DNA fragmentation in HeLa cells treated with Disobinol and cisplatin (lanes 1–4). In contrast, in the solvent control (lane 5), a faint DNA fragmentation pattern was observed, while no fragmentation was detected in the negative control (lane 6). When compared, the intensity of the DNA fragmentation bands in cells treated with cisplatin appeared thicker than those treated with Disobinol. Although the differences in band intensity among lanes 1–4 were not highly significant, this observation is consistent with the flow cytometry results, which showed a relatively low percentage of late-stage apoptotic cells.

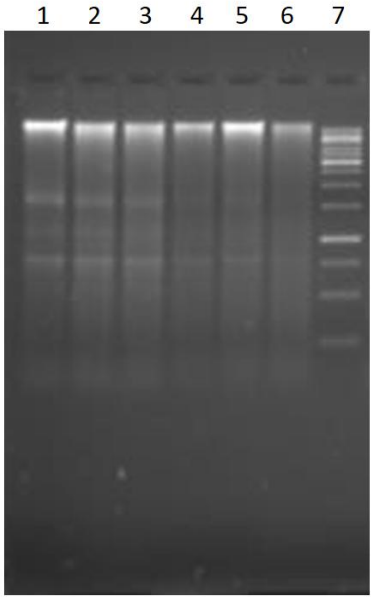


Figure 4. Agarose gel electrophoresis results of isolated DNA from HeLa cells after treatment: (1) 12 µM cisplatin; (2) Disobinol 2× IC_{50}; (3) Disobinol IC_{50}; (4) Disobinol ½ IC_{50}; (5) solvent control; (6) negative control; (7) DNA marker.

DNA fragmentation is the final process of apoptosis. During apoptosis, DNA cleavage typically occurs in two stages: first, cleavage at the chromatin loop domains (50–300 kb) generates DNA fragments with relatively high molecular mass; second, cleavage of the internucleosomal regions of DNA (in multiples of approximately 180 bp, the size of oligonucleosomes) produces DNA fragments with relatively low molecular mass [26].

The DNA fragmentation process involves two major nucleases, namely endonuclease G (endoG) and DNA fragmentation factor (DFF). DFF is composed of two subunits: caspase-activated DNase (CAD) and the inhibitor of CAD (ICAD) [27]. Initially, ICAD functions as a chaperone, assisting in the proper folding of CAD. In proliferating cells, CAD remains inactive as it forms a heterodimeric complex with ICAD. Various apoptotic signals, such as death factors, DNA damage, or genotoxic agents, trigger the activation of the caspase cascade. Caspase-3, located downstream in the cascade, cleaves ICAD at two sites, thereby abolishing its inhibitory activity. The release of CAD from ICAD allows CAD to degrade chromosomal DNA, leading to DNA fragmentation [27,28].

Previous research shows that the gene expression analysis using the qPCR in HeLa cells treated with Disobinol demonstrated significant alterations in the expression levels of Bax, Caspase-8, Caspase-3, and Cyclin D1 genes. Additionally, changes in the expression of Caspase-9 (Cas-9) and NF-κB genes were also observed; however, these changes were only statistically significant at the higher concentration of Disobinol, corresponding to 2× IC_{50}. In contrast, no significant changes were detected in the expression of the p53 transcription factor or the anti-apoptotic gene Bcl-2 [21].

Cisplatin is a chemotherapeutic agent widely used for the treatment of various types of human cancers, including ovarian, cervical, neck, ovarian, and lung cancers [29]. Previous studies have reported that 18 μM cisplatin effectively inhibits 50% of HeLa cell proliferation [30]. In the present study, treatment with 12 μM cisplatin for 24 hours resulted in approximately 80% of HeLa cells undergoing early-stage apoptosis. Cisplatin is a coordination compound consisting of a central platinum ion surrounded by four ligands, namely two amine groups and two chloride ions. The amine ligands form strong bonds with platinum, while the chloride ligands act as leaving groups, allowing the platinum ion to bind to DNA bases [31]. Research findings suggest that cisplatin inhibits HeLa cell proliferation through multiple mechanisms. Cisplatin induces DNA damage, leading to the activation of p53 and triggering downstream effects, including apoptosis; activates the death receptor TRAIL; enhances the expression of the pro-apoptotic protein Bax; and upregulates the expression of Caspase-3, Caspase-8, and Caspase-9, thereby amplifying death signaling through the caspase cascade [29].

Previous studies have demonstrated that other limonoid compounds, such as nimbin, nimbidin, and nimbolide, possess anti-metastatic and anti-invasive activities. These compounds have been shown to activate tumor suppressor genes and inhibit signaling pathways, including VEGF, PI3K/Akt, and NF-κB pathways. Nimbolide induces apoptosis in HeLa cells by upregulating the expression levels of Bax, Bid, and p53, while downregulating the expression of anti-apoptotic genes such as Bcl-2 and Bcl-xL [14]. Furthermore, epoxyazadiradione has been reported to induce intrinsic apoptosis in HeLa cells by increasing the expression levels of pro-apoptotic genes and members of the Bcl-2 family, while simultaneously reducing the expression of anti-apoptotic genes. This compound also promotes the activation of caspase-8, the release of cytochrome c into the cytoplasm, and downregulates the nuclear translocation of NF-κB from the cytosol [32].

This study suggest that Disobinol may exert potential anticancer effects on HeLa cells by inducing apoptosis, though the proportion of apoptotic cells was not as high as that induced by Cisplatin. This observation raises the possibility of further exploring Disobinol as a promising candidate either as a novel

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chemotherapy agent or as a supplementary treatment for cervical cancer. The data showed a notable increase in the percentage of apoptotic cells in response to Disobinol treatment, indicating that it can activate apoptotic pathways within cancer cells. However, the observed increase in apoptotic cells was not markedly significant when compared to Cisplatin, implying that further investigations are necessary to elucidate the precise mechanisms through which Disobinol triggers apoptosis. Additionally, enhancing the potency of Disobinol and improving its efficacy as an anticancer therapeutic may require more extensive studies to optimize its therapeutic potential.

Conclusions

Disobinol has the potential to induce apoptosis in HeLa cells, as evidenced by the increased percentage of cells undergoing early apoptosis and DNA fragmentation in HeLa cells treated with Disobinol at higher concentrations. Therefore, Disobinol shows promise as a chemotherapy drug or lead compound for the treatment of cervical cancer. Further research is needed using higher concentrations, as well as toxicity studies of Disobinol on normal cells.

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Declaration of Generative AI and AI-Assisted Technologies

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

Author Contributions

E.H.: Methodology and data collection. D.H.A.: Investigation and formal analysis. R.M.: Visualization, and writing —original draft preparation. T.H.: Methodology and validation.N.P.: Data collection and validation. R.A.: Formal analysis and writing —review and editing. N.N.: Data curation and visualization. S.G.: Conceptualization, supervision, and corresponding author responsibilities.

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Declaration of Conflicting Interests

The authors declare no conflict of interest.

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