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# Dysobinol Extracted from *Chisocheton Macrophyllus* Triggers Proliferation Inhibition, Potential Apoptosis, and Cell Cycle Arrest of He La Cancer Cell Lines

**Shabarni Gaffar,[1,2] Ersanda Hafiz,[1] Hesti Lina Wiraswati,[3] Safri Ishmayana,[1] Nurlelasari Nurlelasari[1,4]**
[1]Departement of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia
[2]Graduate School, Universitas Padjadjaran, Indonesia
[3]Department of Biomedical Sciences, Faculty of Medicine Universitas Padjadjaran
[4]Research Center of Natural Product, Universitas Padjadjaran, Indonesia

**Abstract**

Dysobinol is a new limonoid from *C. macrophyllus* seeds reported to have an anticancer activity. This study aimed to determine the cytotoxic activity of Dysobinol against HeLa cancer cell lines and evaluate its mechanism of action by determining the expression level of several carcinogenesis genes related to apoptosis and cell cycle. In this experimental study, the cytotoxic activity was determined using the MTS assay and gene expression by real-time reverse transcriptase PCR. The result shows that Dysobinol has an anticancer activity in a dose and time-dependent manner against HeLa cells and was categorized as toxic with $IC_{50}$ values of 52.92, 52.70, and 14.96 µg/ml for 24, 48, and 72 hours, respectively. Dysobinol significantly increased the expression of Bax, Cas-8, and Cas-3 and decreased the expression of Cyc D1 at both doses ($IC_{50}$ and 2x $IC_{50}$) but only high doses (2x $IC_{50}$) could affect Cas9 and NF-κB expressions, indicating that Dysobinol can induce apoptosis via the extrinsic pathway and inhibits the cell cycle through the Cyc D1 regulator. Dysobinol has the potential to be developed as a chemotherapy drug or an adjuvant agent for cervical cancer treatment.

**Keywords:** Apoptosis, cell cycle arrest, cytotoxic activity, dysobinol, HeLa cell line

## Introduction

Meliaceae family plants in tropical and subtropical areas contain secondary metabolites, namely terpenoids, limonoids, alkaloids, flavonoids, and phenols, which have biological activities, such as anticancer, antibacterial, antifungal, antimalarial, and antiviral.[1,2] The Chisocheton genus is the second largest genus of the Meliaceae family which is known to contain limonoid, tirukalan, resin, lupan, oleanan, steroid, sesquiterpene, anthraquinone, alkaloid, coumarin, and simple phenolic compounds. It has been used traditionally to treat several diseases, such as stomach pain, back pain, fever, rheumatism, and malaria.[3] Recently, secondary metabolites have been extracted from the Chisocheton genus with biological activity, including anticancer, dysobinol, disobinin, nimonol, and 7α-hydroxyneotrisilenone.[4]

Limonoids are triterpenoid derivative compounds that are oxygenated and lose four-terminal carbon atoms in the side chain to form a furan ring.[5] These compounds display cytotoxic activity,[6,7] antiplasmodial,[8] antimycobacterial,[7] antiviral,[9] insecticidal,[10] antifungal,[11] anti-inflammatory properties,[12] induce apoptosis,[13] and inhibit lipid droplet accumulation.[14] Dysobinol (Figure 1) is a new limonoid compound that belongs to the group with intact A, B, C, and D rings and α and β unsaturated ketones in ring A.[4] The presence of α, β unsaturated ketones in ring A has an essential role in its anticancer activity.[3]

The search for anticancer drugs today is mainly directed at finding natural compounds that can specifically inhibit the cell cycle or induce apoptosis in cancer cells. Several proteins in the signaling pathway are overexpressed in cancer cells, such as the Her2, Ras, and MAPK proteins, while apoptotic proteins are suppressed so cancer cells grow uncontrolled.[15]

Finding natural compounds for cancer cell treatment is important because its have a lower risk of toxicity and side effects compared to

**Corresponding Author:**
Shabarni Gaffar,
Departement of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jalan Ir. Soekarno Km 21, Jatinangor, Sumedang, West Java, Indonesia
Email: shabarni.gaffar@unpad.ac.id

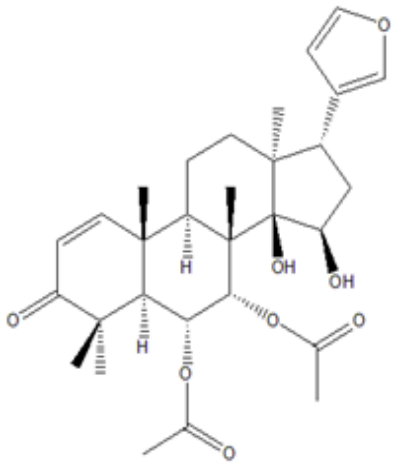


**Figure 1 Structure of Dysobinol from seed of C. *macrophyllus*[4]**

synthetic compounds. Additionally, natural compounds are often more accessible and affordable than synthetic drugs, especially in developing countries where access to modern cancer treatments may be limited. Current cancer treatments such as chemotherapy, radiation therapy, and targeted therapy often have significant side effects and may not be effective for all types of cancer. Chemotherapy, targets both cancer cells and healthy cells, which can lead to various side effects such as nausea, vomiting, hair loss, and fatigue. Radiation therapy can cause damage to healthy tissue and organs, leading to long-term complications. Targeted therapy drugs can be effective but are often very expensive and may not be accessible to all patients. Therefore, finding natural compounds that can specifically target cancer cells while leaving healthy cells unharmed would be a major breakthrough in cancer treatment.[13]

The incidence of cervical cancer in Indonesia is high, and 50% of those affected die.[16] HeLa cells are cervical cancer cells infected with HPV-18 and contain a wild-type p53 protein, but p53 expression is low. The tumor suppressor p53 is a transcription factor that regulates about 500 gene targets in controlling various cellular processes such as cell cycle arrest, cell aging, DNA repair, metabolic adaptation, and apoptosis,[17,18] This study determined the cytotoxic activity of Dysobinol against HeLa cells, and the expression of genes related to apoptosis and the cell cycle that play a role in carcinogenesis, such as p53, Bcl-2, Bax, Cas-9, Cas-8, Cas-3, Cyc D1, and NF-κB.

Apoptosis is also influenced by changes in the shape of the mitochondrion and the availability of the Bcl-2 protein family. Bax and Bcl-2 belong to the Bcl-2 protein family that regulates the permeability of the mitochondrial membrane, resulting in the release of cytochrome-C. The release of cytochrome-C will activate caspase-9, initiates of the intrinsic apoptosis pathway.[19]

Apoptosis is also closely related to the cell cycle that is linked to cell cycle regulators protein, one of which is cyclin D1. Cyclin D1 is a proto-oncogene that is often overexpressed in cancer patients. Excessive expression of cyclin D1 results in hyperphosphorylation of the famous transcriptional regulator, retinoblastoma protein (RB or pRB), which play an important role in the cell cycle.[20] One mechanism that causes high levels of cyclin D1 is the signalling of the transcription factor NF-κB. NF-κB activates the *cyclin D1* gene and thus plays a role in regulating the cell cycle. NF-κB is a transcription factor that controls inflammation, immunity, cell proliferation, differentiation, and cell survival.[21]

The objective of this study is to determine the cytotoxic activity of Dysobinol against HeLa cancer cell lines and evaluate its mechanism of action by determining the expression level of some carcinogenesis genes related to apoptosis and the cell cycle. This study will provide information of the potency of Dysobinol for chemotherapy or as an adjuvant agent for the treatment of cervical cancer, especially for patients who do not respond to current treatments or who are unable to access them.

## Methods

The Dysobinol used in this study was a pure compound, and its structure was determined spectroscopically by Nurlelasari et al. (2017). HeLa cell lines were from the culture collection of Central Laboratory Universitas Padjadajaran. RPMI-1640, fetal bovine serum (FBS), 0.25% Trypsin-EDTA solution, DMSO, chloroform, isopropyl alcohol, 75% ethanol, DEPC-treated water, and all antibiotics were purchased from Sigma Aldrich. The MTS assay kit (CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay was from Promega, TRIsure™ was from Meridian-Bioscience, and SensiFAST™ SYBR® No-ROX One-Step Kit from Bioline.

Cell viability was assessed using the colorimetric MTS assay. HeLa cells were grown until 80% confluent in complete medium (RPMI supplemented with 10% PBS, penicillin, and streptomycin), harvested and counted, then

**Table 1 Primers Sequences Used in This Study**

| Genes | Forward Primer | Reverse Primer |
|---|---|---|
| p53 | 5'-CCT CAG CAT CTT ATC CGA G -3' | 5'-TGG ATG GTG GTA CAG TCA G -3' |
| Bax | 5'-GCG AGT GTC TCA AGC GCA TC -3' | 5'-CCA GTT GAA GTT GCC GTC AG - 3' |
| Bcl-2 | 5'-CAG GCC GGC GAC GAC TTC TC -3' | 5'-TCC CGG TTG ACG CTC TCC AC -3' |
| CycD1 | 5-'AGG CGG AGG AGA ACA AAC AC-3' | 5'-GTG AGG CGG TAG TAG GAC AG -3' |
| Cas-3 | 5'- GTC GAT GCA GCA AAC CTC AG -3' | 5'-TTC TAC AAC GAT CCC CTC TG - 3' |
| Cas-8 | 5'- CAT CCA GTC ACT TTG CCA GA -3' | 5'- GCA TCT GTT TCC CCA TGT TT -3' |
| Cas-9 | 5'-CCT CAC CCT GCC TTA TCT TG -3' | 5'-TCC ACG GCA TTC ATC TGT CC -3' |
| $NF_KB$ | 5'-CAA GGC AGC AAA TAG ACG AG -3' | 5'-GGG CAT TTT GTT GAG AGT TAG -3' |
| B-act | 5'-GAT CAT TGC TCC TCC TGA GC -3' | 5'-TAG AAG CAT TTG CGG TGG AC -3' |

diluted with complete culture medium and transferred into 96-well plates with 1.7 x 104 cells/well and incubated for 24 hours. The media was replaced, and cells were incubated for 24 hours with increasing concentrations of Dysobinol (1.95; 3.91; 7.8; 15.63; 31.25; 62.5; and 125 µg/mL) in DMSO (final concentration <0.2%). Doxorubicin (0.75–48 µg/mL) was used as a positive control, medium and DMSO 0.1% as negative control. All experiment was done in three replicated. All samples were incubated for 24, 48, and 72 hours at 37°C in a 5% CO2 incubator (NuAire, Plymouth, MN, USA). After incubation, 20 µL MTS reagent was added into each well and incubated for 4 hours at 37°C. Then, 10% SDS was added to stop the MTS reduction reaction and the absorbance was measured at 490 nm using a 96-well plate reader (NanoQuant, TECAN). The % of viable cells was calculated based on equation: $(A_{sample}-A_{medium}/A_{DMSO}-A_{medium})$ x100%. By plotting the % of viable cells (y) vs concentration (x), the $IC_{50}$ value will be obtained at the 50% of viable cell.

HeLa cells were grown in 12-well plates (1.7 x $10^4$ cells/well) and incubated for 24 hours or until 80% confluent. The concentration of Dysobinol used in this study was the $IC_{50}$ and 2x $IC_{50}$ values of the 24-hour incubation. The total RNA was extracted using a TRI-sure™ reagent (Meridian-Bioline) according to the manufacturer's protocol and the concentration was measured using a Multimode reader (Nano Quant, Tecan).

Quantitative PCR was performed using the SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline) and 5 ng/µL of RNA template per reaction according to the manufacturer's instructions on an AriaMx *Real-Time* qRT-PCR (Agilent). The cycling conditions used were two-step cycling, the first cycle was 1 cycle at 45ºC for 10 minutes (reverse transcription) and the second step was 1 cycle at 95ºC for 2 minutes, and 40 cycles at 95ºC for 5 seconds, 60ºC for 20 seconds. The primers were designed based on the gene sequences available in NCBI (Table 1). Genes expression was quantified using the $2^{-\Delta\Delta CT}$ method and the housekeeping gene β-actin.[22]

The data presented are means of three independent experiments. The qPCR results were analyzed by Minitab version 17 using ANOVA analysis with a 95% confidence level. The difference was considered statistically significant at the p-value <0.05. Tukey Post hoc test was used to compare the means of every treatment to the standards of every other treatment.

## Results

The percentage of viable cells with the administration of Dysobinol decreased with increasing Dysobinol concentrations and the $IC_{50}$ value decreased with the longer incubation time. The $IC_{50}$ of Dysobinol against HeLa cells were: 52.92; 52.70 and 14.96 µg/ml with incubation times of 24, 48, and 72 hours, respectively (Figure 2). Observation of the cells under a light microscope shows that Dysobinol altered cell morphology. It can be seen that high Dysobinol caused more cells undergoes morphological alteration, indicated by small brown rounded form cells looked cloudy and floated compared to control cells which were elongated and attached to a petri dish. Cell turbidity also decreased with increasing dysobinol concentration (data was not shown). There was a decrease in the percentages of living HeLa cells along with the increasing concentration of Dysobinol and the length of the incubation time.

Based on the National Cancer Institute, a

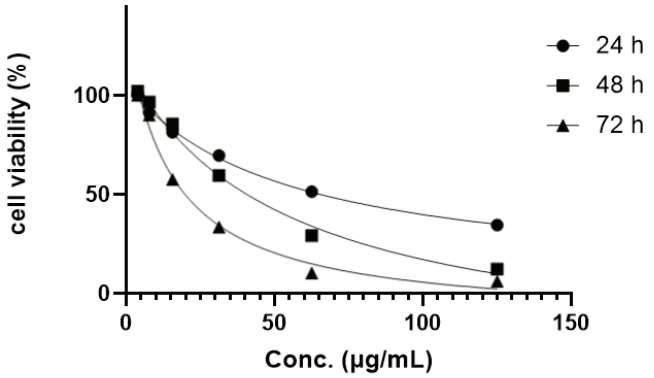


**Figure 2 HeLa Cell Viability Curve That Treated with Dysobinol at Different Incubation Time. The cell viability of HeLa cells treated with Doxorubicin. Data represent the mean ± SD of the three independent experiments**

powerful cytotoxic effect was characterized by $IC_{50}$ <21 µg/ml, moderate cytotoxic effect by $IC_{50}$ of 21-200 µg/ml, and weak cytotoxic effect by $IC_{50}$ of 201-500 µg/ml; meanwhile, IC>500 µg/ml are considered non-toxic. Based on its $IC_{50}$ value, the cytotoxicity of Dysobinol was considered moderate. Meanwhile, $IC_{50}$ of doxorubicin, a well-known chemotherapy agent to the HeLa cells is 2.13 µg/mL.

The qPCR results are depicted in Figure 3. Based on one-way ANOVA followed by the Tukey Post hoc test, there was a significant change in the expression of Bax (3.12x and 3.28x), Cas-8 (3.30x and 2.95x), Cas-3 (4.31 and 3.09x), and Cyc D1 (-0.46x and -0.41x) in Dysobinol-treated HeLa cells. Furthermore, the change in Cas-9 and $NF_KB$ gene expression only reached significance at the higher dose of 2x $IC_{50}$ of Dysobinol. However, there were no significant changes in the expression of the transcription factor p53 and the anti-apoptosis gene Bcl-2. In the qPCR, doxorubicin is not used as a positive control, because its mechanism is well-known and cannot be compared to Dysobinol.

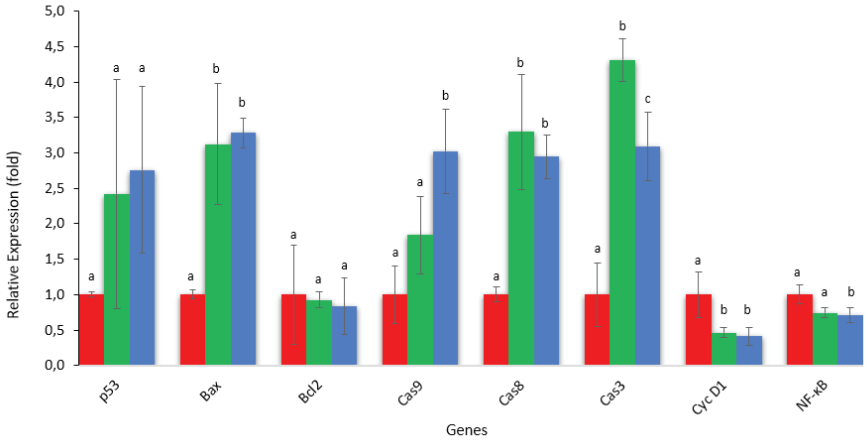


**Figure 3 Changes in the Expression of p53, Bax, Bcl-2, Cas-9, Cas-8, Cas-3, Cyc D1, and $NF_KB$ Genes After Administration of Dysobinol to HeLa cells for 24 Hours**

Red = Control Cells (without treatment), Green = Dysobinol $IC_{50}$, Blue = Dysobinol 2x $IC_{50}$. Gene expression was normalized to ß-actin. Data are presented as the mean ± SD and representative of three experiments. Bars that do not share a letter are significantly different for each gene based on one-way ANOVA, followed by the Tukey post hoc test with a significance level of = 0.05.

## Discussion

Dysobinol has a similar structure to disobinin derived from *C. siamensis* which is toxic to NCI-H187, KB, and MCF-7 cancer cell lines with an $IC_{50}$ of 1.67, 3.17, and 2.15 µg/ml, respectively.[9] However, the $IC_{50}$ of Dysobinol against the HeLa cell line was not as high as Disobinin. Disobinin has two ester groups (acetyl group) on the B-ring, a double bond on the D-ring, and a methylene group (CH2) at the C-16,[23] whereas Dysobinol has no double bond on the D-ring and two hydroxyl groups on the D-ring at the C-14 and C-15 positions. The existence of these two hydroxyls may reduce its anticancer activity and the presence of double bonds in disobinin compounds plays an important role in anticancer activity.[23]

There was a significant increase in the expression of Caspase-8 (extrinsic apoptosis markers) and the executor protein Cas-3 (both extrinsic and intrinsic apoptosis markers). Although the expression at 2x $IC_{50}$ concentration decreased in both genes, this did not reach significance but suggests that Dysobinol has the potential to trigger apoptosis via the extrinsic pathway. Cas-8 is activated due to the presence of death signals, TNF, and FAS, which are recognized by death receptors. This binding causes a change in shape and oligomerization of the receptors. The adapter protein recognizes changes in active receptors so that it is directed to procaspase 8 aggregation for Cas-8 activation. In some cells, Cas-8 can directly cut Cas-3 as the executor caspase.[24]

Dysobinol also significantly decreases the expression of Cyc D1, a cell cycle regulator gene in HeLa cells, and thus has the potential to inhibit the cell cycle by reducing Cyc D1 expression. However, this result should be confirmed by western blot analysis. Higher Cyc D1 expression is found in several human cancers such as breast cancer and cervical cancer.[25] Overexpression of cyclin D1 results in dysregulated CDK activity, rapid cell growth under conditions of restricted mitogenic signaling, bypassing key cellular checkpoints, and ultimately, neoplastic growth.[25] One of the cancer therapies targets Cyc D1 by inhibiting the formation of the Cyclin-CDK complex which phosphorylates the target protein such as retinoblastoma (RB) protein that plays an important role in the cell cycle. The decrease in Cyc D1 expression will trigger a checkpoint in the G1 phase due to the unavailability of several proteins required for DNA replication and the cell cycle. Therefore, inhibition of the cell cycle in the G1 phase will allow DNA repair, thereby maintaining the integrity of chromosomes and improving the survival of damaged cells.[19]

Dysobinol had no significant effect on increasing p53 expression and was unable to normalize the function of p53 which is the target of HPV virus E6 protein degradation. However, several studies have shown that apoptosis induction and cell cycle inhibition can still occur without p53, one of which is with sufficient Bax protein concentration through extrinsic pathways, and lowering Cyclin expression. Cyclin B1 and CDK1 are independent of p53.[27]

Dysobinol only had a significant effect on Bax expression. Bax and Bcl-2 are members of the Bcl-2 protein family that plays an important role in the regulation of the intrinsic pathway of apoptosis along with the release of procaspase-9 from the mitochondria. The translocation of Bax and the cleavage of Caspase 3 by Caspase-9 initiates the intrinsic pathway. Bax and Bak act as executor proteins for mitochondrial outer membrane permeability (MOMP),[19] therefore, the effect of increased Bax expression should be further investigated by flow cytometry. However, the small changes in Cas-9 expression and the insignificant changes in Bcl-2 expression indicate that Dysobinol does not trigger intrinsic pathway apoptosis.

NF-κB is also a family of transcription factors that have essential regulatory functions in inflammation, the immune response, cell proliferation, and apoptosis. The decrease in NF-κB expression was only significant at 2x IC50, indicating that Dysobinol has no significant effect on the expression of proteins that regulate the cell cycle and apoptosis through the NF-κB pathway, which is in line with the absence of a significant decrease in Bcl-2 expression.

In conclusion, Dysobinol has a dose- and time-dependent cytotoxic effect on HeLa cells and the potential to trigger apoptosis via the extrinsic pathway and inhibit the cell cycle, therefore it could be investigated as a potential drug for chemotherapy or as an adjuvant agent for the treatment of cervical cancer.

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