Apoptosis Induction of SKOV-3 Ovarian Cancer Cells from Pacing Rhizome (Costus speciosus) Through the Modulation of BAX and P53 Genes Expression

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Abstract

Costus speciosus or Pacing has been investigated to have cytotoxic effect on several human cancer cells. Current was conducted to determine the cytotoxic activity of ethanol extract of the Pacing rhizome (EP) on SKOV-3 ovarian cancer cells, to investigate EP’s effect on BAX and p53 expression, and predict the inhibition activity on intrinsic pathway. EP were extracted using maceration method with 70% ethanol. The cytotoxic effect was performed using MTT Assay with various concentrations (375 µg/mL, 250 µg/mL, 62.5 µg/mL, 31.25 µg/mL, and 7.8125 µg/mL). The ability of EP to induce apoptosis in SKOV-3 cells was measured using flow cytometry. The BAX and p53 gene expressions on SKOV-3 cells were detected with RT-qPCR after treatment of EP with concentrations of ¼ IC₅₀, ½ IC₅₀, and IC₅₀. The ability of diosgenin to interact with BAX and p53 can be seen from the interaction with the MCL-1 protein and was predicted with molecular docking. The results showed that IC₅₀ of cytotoxic activity was 69.143 µg/mL. EP could induce apoptosis on SKOV-3 cells and can induce BAX and p53 protein expressions. The docking results show that diosgenin has the potential to act as an antagonist for the MCL-1 protein, it can increase apoptosis.

Keywords: apoptotic, Costus speciosus extract, SKOV-3, p53, BAX.

INTRODUCTION

Ovarian cancer mortality ranks first among gynecological cancers (Kuroki & Guntupalli, 2020). Most new cases can be diagnosed at advanced stages (III and IV) because symptoms are rarely found in the early stages, making ovarian cancer often referred to as a silent killer (Nersesian, et al., 2019; Pokhriyal, et al., 2019). Every year, as many as 314,000 women are diagnosed with ovarian cancer, and 207,000 women die from this disease. Ovarian cancer sufferers have the lowest chance of
survival of any other cancer in women, with 5-year survival rates ranging from 36-46% in developing countries (WHO, 2020).

Neoadjuvant chemotherapy is done as a first step to shrink the tumor so that it can facilitate surgery in the next step. Platinum chemotherapy is usually effective for most ovarian cancers, but among them there is platinum resistance, and survival is barely improved (Binju, et al., 2019; Liu, et al., 2017). As many as 80% of patients who receive standard treatment can be cured, but another 70% relapse within 6–12 months after completing chemotherapy with more aggressive tumors (Binju, et al., 2019; Huang, et al., 2020). Multidrug resistance and non-specific or dose-related cellular toxicity are the main causes of cancer chemotherapy’s limited use (Harsono, 2020). Apoptosis can be caused by chemotherapy by raising the levels of BAX and p53 proteins, which are proapoptotic proteins and transcription factors (Nurmaulawati, 2021).

Apoptosis is cell death that occurs regularly to maintain homeostatic balance (Singh, et al., 2019). The groups of proteins B-cell lymphoma-2 (BCL-2) and p53 play an important role in regulating apoptosis. BAX is a proapoptotic protein from the BCL-2 group that can increase tumor sensitivity to treatment, while p53 is a tumor suppressor gene (Liu, et al., 2019; Manne, et al., 2021). An imbalance between these proteins can cause apoptosis and abnormal cell growth.

In a previous study, we found that the pacing plant (Costus speciosus) can be an alternative treatment for ovarian cancer. Some of the chemicals that are found in the rhizomes and roots are sitosterol-β-D-glucoside, dioscin, dioscin prosapogenin A and B, gracilin, and quinine (Nafisah, et al., 2022). Diosgenin is a steroid saponin whose pharmacological effects mostly refer to anticancer activity in vitro and in pre-clinical animal models. The main way diosgenin works is by changing several cells signaling pathways that are involved in controlling the cell cycle, differentiating cells, and cell death (Sethi, et al., 2018). Research on C. speciosus with a high diosgenin content as an anticancer is still minimal. Therefore, this research was carried out with the aim of studying the induction of apoptosis in the ethanolic extract of pacing rhizomes through modulation of BAX and p53 gene expression.

Targeting Apoptotic for Anticancer Therapy

There are various strategies by cancer cells to escape apoptotic mechanisms, numerous mutations are identified in both the extrinsic and intrinsic pathways (Liu, et al., 2017). The intrinsic pathway is regulated by the BCL-2 family of proteins. BCL-2 family are grouped into three subsets include proapoptotic effector (BAK and BAX), antiapoptotic BCL-2 protein (BCL-2, BCL-W, MCL-1), and BH3-only protein.

The elevation of BH3-only proteins can initiate the activation of BAX/BAK, where BAX is controlled by p53. p53 has essential functions in overseeing the cell cycle, apoptosis, and maintaining genomic stability, earning its reputation as the “guardian of the genome” (Wang, et al., 2023). Upon activation, BAX and BAK undergo oligomerization, inducing mitochondrial outer membrane permeabilization (MOMP) (Dadsena, et al., 2021).

MCL-1 shows significant promise as a target for treating tumors. MCL-1 inhibitors specifically attach to MCL-1, releasing pro-apoptotic proteins, BAX/BAK, triggering the initiation of apoptosis. Selective inhibitors of MCL-1 could emerge as a novel category of anti-cancer medications (Wang, et al., 2021).

MATERIALS AND METHODS

Preparation and Extraction of Pacing Rhizomes (EP)

Pacing rhizomes were found on Jalan Kabupaten, Kranggahan I, Trihanggo, Gamping District, Sleman Regency, Special Region of Yogyakarta, Latitude 7°44’38.9” S, Longitude 110°21’04.4” E. The pacing rhizomes that were obtained were identified and authenticated by the
Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, with a plant determination letter number of 20.21.7/UNI/FFA.2/BF/PT/2023.

Fresh pacing rhizomes are washed thoroughly and dried in the oven for one day. The dried rhizomes are ground using a blender until they become powder. Then the simplicia powder was extracted with solvent 70% ethanol using the maceration method for 5×24 h. The filtrate is taken and then evaporated over a water bath until a thick extract is obtained. The result showed that the maceration method produced the yield value of 12.45%.

**Phytochemical Characterization by Thin Layer Chromatography-Densitometry**

TLC-Densitometry was carried out according to Foudah, et al. (2019), with modifications. 3 µL of the diosgenin standard solution was added, and 6 µL of the EP sample solution was placed on a TLC plate (Silicagel GF254 5×10 cm). The TLC plate was eluted with an acetone-hexane mobile phase (2:8 v/v). The elucidation components are colorless and difficult to see directly, thus a spray reagent of 50% sulfuric acid in methanol (v/v) was used to produce a visible color change. Then, the plate heated at a temperature of 100°C for 10 minutes. The TLC plate was read at λ 439 nm (Soegiardjo and Koensoemardiyah, 2005). Qualitative analysis was carried out by comparing the Rf of EP spots with the diosgenin standard. Quantification was carried out using the single calibration method for the area of the spot.

**In Vitro Testing of Pacing Rhizome Extract (EP) on SKOV-3 Cells**

_in vitro_ testing with SKOV-3 cells has received ethical approval from the Ethics Commission of the Faculty of Medicine, Public Health, and Nursing at UGM with number KE/FK/1261/EC/2023.

**Cytotoxicity Test with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay**

SKOV-3 cells with a concentration of 1×10^4 cells/well were planted in 96-well plates and incubated at 37°C and 5% CO₂ for 24 h. Cells were treated with EP at 10 series of different concentrations, then incubated for 24 h. Next, the media containing the EP was discarded. Add a 0.5 mg/mL MTT solution to each well and incubate for 4 h. After 4 h, the medium was discarded, then DMSO (200 µL/well) was added, and the plate was left overnight. The plate was shaken on a shaker for 10 minutes, and then the absorbance was measured using an ELISA reader, the Bio-Rad IMark Microplate Absorbance Reader, at λ 595 nm. Treatment absorbance data was converted into percent viability and used to calculate the IC<sub>50</sub> (Meiyanto, 2013).

**Apoptosis Induction Test with Flow Cytometry**

To test the induction of apoptosis, SKOV-3 cells were treated with pacing extract at a concentration of ½ IC<sub>50</sub> (EP1), ¼ IC<sub>50</sub> (EP2), and 1/8 IC<sub>50</sub> (EP3), and control cells (KS). Control cells and EP-treated cells were fixed in cold 70% ethanol and filtered through 300 mesh nylon, then left overnight at 4°C. Then, cells were washed with PBS and stained with DNA (50 µg/mL PI, 50 µg/mL RNase-A, and 0.1% Triton X-100). Cells were washed with trypsin and centrifuged (1000 rpm, 5 min). Cell pellets were washed once in cold PBS and twice in 1X binding buffer by centrifugation (1000 rpm, 5 min) at each washing step. The cell pellet was resuspended in PI and Annexin V-APC solution (BD Pharmingen™), then incubated for 15 minutes in the dark and at room temperature. The samples were then analyzed at various stages of cell apoptosis (Niar, et al., 2014).
Gene Expression Analysis (Quantitative Real-Time Polymerase Chain Reaction)

RT-qPCR was used to look at the expression of the Bax gene (which controls apoptosis) and the p53 gene (which stops tumors from growing) (Abou-Elhamd, et al., 2021). Total RNA was extracted with Favorgen. cDNA was synthesized with the SMOBIO marker, and then PCR was carried out using a Light-Cycler 480 with SYBR Green PCR master mix. PCR conditions were 95°C (5 min); 45 cycles of 95°C (30 s), 55°C (30 s), and 72°C (15 s); and a final extension of 72°C (5 minutes). The housekeeping gene GADPH standardized the relative amount of duplicated mRNA expression. The PCR results were calculated using the $2^{-\Delta\Delta CT}$ comparative method. In-situ testing of diosgenin compounds on MCL-1 ligands.

Molecular Docking

Compounds were analyzed using the MOE 2010.10 application with a license from the UGM Faculty of Pharmacy. Data were used in the form of protein structures obtained from the PDB database (https://www.rcsb.org/) with the PDB code 4HW3 and the SMILES database for diosgenin obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Finding out the energy value (kCal/mol) between the target receptor and diosgenin was used to look at the docking results (Wardani, et al., 2021).

Analysis Method

Data are presented as means with SD. Groups were compared by one-way ANOVA. Statistical analyses were performed using GraphPad Prism software, version 8.00 (GraphPad Software, La Jolla, CA). The value of statistical significance for differences is assumed to be $p<0.05$.

RESULTS

Pacing Rhizome Extract Profile (EP)

Pacing rhizomes were harvested independently in the morning at 09.00 WIB on the riverbank around the UGM Academic Hospital area, Yogyakarta, in July 2023. The Pacing rhizome contains various bioactive compounds that are abundant in the morning before

![Figure 1. EP Phytochemical Profile. (a) Spot EP; (b) Diosgenin standard spot.](image1)

![Figure 2. Graph of viability of SKOV-3 cells treated with EP doses of 375 µg/mL, 250 µg/mL, 62.5 µg/mL, 31.25 µg/mL, and 7.8125 µg/mL.](image2)
photosynthesis starts. These bioactive compounds play an important role in the biological activities of the Pacing rhizome, including antibacterial, anti-inflammatory, and antioxidant activities.

The extraction of 111.08 grams of dried simplicia pacing rhizomes using the maceration method produced 13.83 grams of thick extract with a yield percentage of 12.45%.

Phytochemical Profile of Pacing Rhizome Extract (EP) TLC-Densitometry

The EP spots resulting from the TLC plate elution in spot (a) appeared at the same Rf as the diosgenin standard comparison in spot (b), which is presented in Figure 1. Quantification was carried out by the densitometric method, and the mass fraction of the diosgenin compound in EP was 2.17 µg with a single calibration.

Cell Viability by Cytotoxicity Analysis of SKOV-3 Cell Pacing Extract

The experiment was repeated three times, each sample in triplicate. Mean±SD is presented.

The results of this research showed that the IC$_{50}$ value of the ethanol extract of rhizome pacing (EP) in ovarian epithelial cells (SKOV-3 cells) was 69.143 µg/mL. Another group of cells, called VERO cells, showed that a methanol extract of pacing rhizomes had an IC$_{50}$ value of 94.78 µg/mL (Baskar, et al., 2012). This shows that this dose of EP is more cytotoxic to cancer cells and less toxic to normal cells.

Induction of Apoptosis by Flow Cytometry of Pacing Rhizome Extract (EP) in SKOV-3 Cells

To test the induction of apoptosis, SKOV-3 cells were treated with pacing extract at concentrations of 8.625 µg/mL (EP1), 17.25 µg/mL (EP2), and 34.5 µg/mL (EP3), and control cells (KS). In Figure 3, the lowest left quadrant represents living cells (Q4), the upper left quadrant represents cells undergoing necrosis (Q1), the lowest right quadrant represents pre-apoptotic cells (Q3), and the upper right quadrant represents late apoptotic cells (Q2).
The changes in the increase in apoptosis events that can be seen in Figure 4 in the treatment of SKOV-3 cells with EP2, and EP3 significantly ($P<0.05$) can increase the percentage of apoptosis events compared to KS.

Gene Expression Analysis (Quantitative Real-Time Polymerase Chain Reaction) BAX and p53 in Pacing Rhizome Extract (EP)

The values and expression of BAX and p53 genes in SKOV-3 cell samples were obtained through relative quantification using the Livak formula. Results of calculating the expression levels of the BAX gene and the p53 gene in ovarian cancer cells towards the GAPDH gene. The expression levels of the BAX and p53 genes were obtained using the $2^{-\Delta\Delta Ct}$ comparative method.

Molecular Docking

In this study, MCL-1 was chosen as the target protein. MCL-1 (myeloid cell leukemia 1) is an anti-apoptotic protein from the BCL-2 family that is essential in inhibiting the apoptotic pathway through inhibiting BAX and BAK activation (Patel & Karch, 2020). Increased amounts of MCL-1 also contribute to tumorigenesis in various situations (Liao, 2021). Therefore, diosgenin is used as a candidate inhibitory compound on MCL-1 so that BAX can be expressed to carry out apoptosis. In this study, the MCL-1 protein with the PDB code 4HW3 was used, which was obtained via the website https://www.rcsb.org/. The selection of the GDP code is based on a good RMSD value, namely RMSD<2.

Re-docking the native ligand (19G) at the MCL-1 binding site served as validation of the docking method. The root mean square deviation (RMSD) value obtained was 0.6166, which means that this method is considered accurate because the copy ligand is similar to the original ligand in the in vitro experiments that have been carried out. The docking results between the MCL-1 protein and 19G produced a binding energy of -11.5015 kcal/mol. Visualization of the interaction between 19G and MCL-1 shows binding to the amino acids Phe270, Arg263, and Met250 via an aryl hydrogen-carbon bond.

Shown in Figure 6. (a) Diosgenin can bind to the MCL-1 protein with a binding energy of -11.8265 kcal/mol. This value is lower than the 19G binding energy in the MCL-1 protein.

Mechanism of Apoptosis Induction of Pacing Extract in SKOV-3 Cancer Cells

Shown in Figure 7. Mechanism of BAX and p53 Modulating Apoptosis of SKOV-3 Cancer Cells.

DISCUSSION

Pacing plant (*Costus speciosus*), containing diosgenin known for its pharmacological effects, particularly in exhibiting anticancer activity, presents itself as a potential alternative treatment...
for ovarian cancer. Extracted via the maceration method, the pacing rhizome, rich in diverse bioactive compounds, yielded a substantial 13.83 grams of thick extract, showcasing a yield percentage of 12.45%. Utilizing TLC densitometry, the phytochemical profile of the pacing rhizome extract was elucidated. Notably, the ethanol extract of rhizome pacing (EP) demonstrated an IC\(_{50}\) value of 69.143 µg/mL in ovarian epithelial cells (SKOV-3 cells), highlighting its heightened cytotoxicity towards cancer cells while exhibiting lower toxicity towards normal cells. The induction of apoptosis in SKOV-3 cells was examined via flow cytometry.

Treatment involved the application of pacing extract at three concentrations: 8.625 µg/mL (EP1), 17.25 µg/mL (EP2), and 34.5 µg/mL (EP3), alongside control cells (KS). In Figure 3, the lowest left quadrant represents living cells (Q4), the upper left quadrant represents cells undergoing necrosis (Q1), the lowest right quadrant represents pre-apoptotic cells (Q3), and the upper right quadrant represents late apoptotic cells (Q2). Apoptosis shows how the phospholipid phosphatidyserine (PS) moves from the inner layer to the outer layer of the plasma membrane so that phagocytes can find it in the early stages of apoptosis. V-FITC attachment allowed for the identification of early apoptosis by detecting PS extraction to the outer layer. PI is a red fluorescent dye that binds nucleic acids and is impermeable to living cells and early apoptotic cells. The changes in the increase in apoptosis events that can be seen in Figure 4 in the treatment of SKOV-3 cells with EP2, and EP3 significantly (\(P<0.05\)) can increase the percentage of apoptosis events compared to KS. In their exploration of *C. speciosus* anti-cancer potential, El-Far, *et al.* (2016) identified its ability to trigger programmed cell death (apoptosis) via a direct pathway involving upregulation of DR-4 and endoproteases, including caspase-3. This pro-apoptotic effect was then successfully validated against two established human cancer cell lines, like MCF-7 breast adenocarcinoma and HepG2 liver carcinoma.
One of the crucial anticancer compounds in *C. speciosus*, diosgenin, may be the reason for EP’s ability to induce apoptosis. The study by El-Far, *et al.* (2016) found that *C. speciosus* is involved in apoptosis because it raises the levels of DR-4 and endo-proteases (like caspase-3), and this activity has been studied against human breast adenocarcinoma with the MCF-7 cell line and human liver cancer (HepG2 cell line).

The values and expression of BAX and p53 genes in SKOV-3 cell samples were obtained through relative quantification using the Livak formula. Results of calculating the expression levels of the BAX gene and the p53 gene in ovarian cancer cells towards the GAPDH gene. The expression levels of the BAX and p53 genes were obtained using the $2^{(-\Delta\Delta Ct)}$ comparative method. As shown in Figure 5 (a), there was a statistically significant difference in the expression levels of the p53 gene between the exposed group (EP4) and the control group. The increase in BAX gene expression can be observed in Figure 5. (b) After treatment of SKOV-3 cells with EP2 and EP4, there was a significant difference ($P<0.05$) in the level of BAX gene expression between the exposed group and the control group. Increased expression of p53 and BAX in the SKOV-3 ovarian cancer cell line that was given pacing extract compared to those that were not given pacing extract. These results are in accordance with doxorubicin as an anticancer,
which can significantly increase the relative expression of the BAX gene in breast cancer cells that experience multidrug resistance (Cao, et al., 2021).

Molecular docking is a computational simulation to predict the binding between a drug or ligand and a receptor or protein by pairing a ligand (a small molecule) on the active site of the receptor. Molecular docking is widely used in the process of discovering and developing new drugs with the aim of finding better activity (Pratama, et al., 2017). Shown in Figure 6. (a), Diosgenin can bind to the MCL-1 protein with a binding energy of -11.8265 kcal/mol. This value is lower than the 19G binding energy in the MCL-1 protein. This indicates that diosgenin can bind more stably to the MCL-1 protein compared to 19G as a native ligand. Diosgenin is also able to bind to one of the key residues that correspond to the native ligand 19G. Thus, diosgenin has the potential to be used as an inhibitor of the MCL-1 protein.

The upregulation of the BAX gene in SKOV-3 cells makes the outer mitochondrial membrane permeable (MOMP) and releases factors that cause cell death, which starts the apoptosis process. Increased p53 gene expression can induce apoptosis in cancer cells through DNA damage, thereby preventing the proliferation of damaged cells (Aubrey, et al., 2017). The upregulated p53 gene can regulate the expression and activity of
BAX, further promoting apoptosis, as presented in Figure 7. *In silico* analysis using molecular docking of the diosgenin compound present in the extract against the MCL-1 ligand supports these findings. This protein is an anti-apoptotic protein that is a member of the BCL-2 family and is essential in inhibiting the apoptosis pathway through inhibiting BAX activation. The binding energy of diosgenin to the MCL-1 protein is -11.8265 kCal/mol. This means that diosgenin can bind more stably to the MCL-1 protein so that BAX can still work as a pro-apoptotic protein.

**CONCLUSIONS**

Pacing (*Costus speciosus*) rhizome extract was proven to have activity as an apoptosis indicator in SKOV-3 ovarian cancer cells with an IC$_{50}$ value of 69.143 µg/mL obtained from the MTT Assay cytotoxicity test, and the results of induction of apoptosis by flow cytometry showed the ability to induce apoptosis at a concentration of 34.5 µg/mL (EP3). The RT-qPCR test showed that pacing rhizome extract at a concentration of 69 µg/mL (EP4) could raise the levels of the p53 gene and the BAX gene, which are both involved in cell death. Molecular docking results show that diosgenin can bind MCL-1 protein with a binding energy of -11.8265 kcal/mol and can bind to key residues according to the native ligand.

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