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Cell Cycle Inhibition and Apoptotic Induction of *Vernonia amygdalina* Del. Leaves Extract on MCF-7 Cell Line

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Abstract

AIM: The research aimed to determine the cytotoxic activity, cell cycle inhibition, and apoptosis induction of the ethyl acetate extract of the African leaves *Vernonia amygdalina Del*. on the MCF-7 cancer cells.

METHODS: The extraction of *Vernonia amygdalina Del*. leaves was done using the maceration method whereas the cytotoxic was performed using MTT assay. After that, the cell cycle testing and apoptosis induction were conducted using flow cytometry assay.

RESULTS: The IC_{50} values of n-hexane, ethyl acetate, and ethanol extract of *Vernonia amygdalina Del*. on the MCF-7 cancer cells were 206.211 ± 0.99, 50,365 ± 0.07, and 967.033 ± 2.68 µg/mL, respectively. The percentage of the cycle cell results in the G_0-G_1 phase in the cell control with 72.08% decreased in the treatment with ethyl acetate extract 1/2 IC_{50} with 62.58% and 1/5 IC_{50} with 44.72%. For the S and G2-M phase, the highest percentage was found in the ethyl acetate extract 1/5 IC_{50} treatment with 47.27% and 9.50% which were higher than the control cells with 23.26% and 5.90%.

CONCLUSION: Based on the results, the *Vernonia amygdalina Del*. extract provides chemopreventive agent as anti-cancer. Our future study will assess the mechanism of ethyl acetate fraction in inhibiting angiogenesis and metastatic in breast cancer.

Introduction

Cancer is an abnormal cell growth that can attack normal body tissues and affect the body function. There are some factors that can affect breast cancer risk, such as long-term hormone use, dietary factors, reproductive factors and first pregnancy in an old age, lack of physical activity, hormone replacement therapy in chronic patients, and congenital genetic factors associated with breast cancer, such as gene mutations and radiation during the development of breast cancer [1].

The entry and development of cells through the cell cycle is controlled by changes in the level and activity of a group of proteins named cyclin. The cell cycle consists of several phases, namely the phase of Gap 1 (G1), S (Synthesis), Gap 2 (G2), and M (Mitosis) [2]. The duration of the cycle varies. In normal human cells, the cycle takes 20-24 hours in which the G1 phase takes 8-10 hours, the S phase takes 6-8 hours, the G2 phase takes 5 hours, and the M phase takes 1 hour. The generation time for the cell culture is generally the same as normal cells [3]

Cells will also experience controlled death as a physiological response of the cell to eliminate cells that are not needed by the body which is known as apoptosis. Apoptosis also has a role in monitoring changes in cancer cells and becomes the first line of defense to fight mutations by cleaning abnormal DNA cells which can be malignant. Thus, apoptosis is part of the immune system which controls normal cell populations in the body [2].

In general, cancer treatment still depends on chemotherapy derived from synthetic chemicals. However, chemical compounds might cause multi-

drug resistance effects. Besides that, anti-cancer with synthetic chemical compounds will affect not only the target cells but also the normal cells around them [4].

Vernonia amygdalina Del. which is the family of Asteraceae come from West Africa. Several studies found some chemical components, such as sesquiterpene lactones flavonoids, steroidal, fatty acids and saponins [5], [6], [7], [8], [9], [10], [11] and indicated some of the pharmacological activities, such as anti-obesity, anti-tumor, anti-malaria, anti-inflammation, and other activities [12], [13], [14], [15]. The aim of this study was to evaluate cytotoxic activities of n-hexane, ethyl acetate, and ethanol extract of *Vernonia amygdalina* Del. leaves.

In this study, the cytotoxic test was conducted to determine IC_{50} values that showed the concentration values that resulted in 50% inhibition of cell proliferation and reflected the drug toxicity to MCF-7 cells [16]. The test was done to analyse the cell growth and find the cell cycle phase in the research which experience growth in the G2 phase. In the G2 phase, DNA which is damaged due to exposure to a substance or carcinogenic compound will be repaired. If the damaged DNA cannot be repaired, there would be apoptosis in the cells. The cell cycle and apoptosis test were performed using flow cytometry method.

Material and Methods

Fresh leaves of *Vernonia amygdalina Delile* were collected from the garden of medicinal plants at the Faculty of Pharmacy, Universitas Sumatera Utara, Jl. Tridharma, North Sumatera Province, Indonesia. *Vernonia amygdalina Delile* was identified by the Herbarium Bogoriense, Bogor Botanical Garden, Bogor, Indonesia. The chemical used were Sorafenib, DMSO (Sigma), [3-(4,5-dimethyl thiazole-2-yl) -2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), and Annexin V (Biolegend). Propidium Iodide kit (Biolegend).

The process of making *Vernonia amygdalina Delile* leaves extract was done using graded maceration method. Five hundred gr of simplicia was weighed and put into a container. In the first maceration, the simplicia was soaked with 5 L of n-hexane for six hours, stirred occasionally, and left out for 18 hours. After that, the filtrate was separated from the residue. The residue was then dried in an oven at 50°C. The dried residue was re-macerated with 5 L of ethyl acetate solvent, soaked it for 6 hours while stirred occasionally, and left out for 18 hours. The filtrate was then separated from the residue. After dried, the residue was macerated again with 96% ethanol solvent with the same procedure. Each solvent was soaked 3 times. The macerate from the three solvents was evaporated with a rotary evaporator to obtain a thick extract. This study used ethyl acetate as a solvent due to its characteristic as a semi-polar solvent which can pull out polar and nonpolar compounds [17].

The cells were treated with n-hexane, ethyl acetate, ethanol extract, and sorafenib. 4T1 cells were planted in a 96-well microplate so that density of 1 x 10^4 cells/mL was obtained, and the cells were incubated for 24 hours to obtain good growth. After 24 hours, the medium was replaced with a new one. After that, the test solution was added with various series of concentrations using DSMO cosolvent and incubated at 37°C in a 5% CO2 incubator for 24 hours. At the end of the incubation, the media and test solution were removed, and the cells were washed with PBS. Then, 100 µL of culture media and 10µL MTT 5 mg/mL were added into each well. To observe its viability, the cells were incubated again for 4-6 hours in a 5% CO2 incubator at 37°C. The MTT reaction was stopped with a reagent stopper (10% SDS in 0.1 N HCl), and the plate was wrapped in aluminium foil so that it was not translucent at room temperature and left out for one night. Living cells would react with MTT and form a purple color. The test results were read by ELISA reader at a wavelength of 595 nm [17], [18], [19].

The MCF-7 cells (5 x 10^5cells/mL) were plated in 6-well plate and incubated for 24 hours. The cells were treated with ethyl acetate extract of *Vernonia amygdalina Del*. and sorafenib and then incubated for 24 hours. After 24 hours, the media was moved into the conical tube, and 0.025% trypsin was added into the plate. After washed twice with PBS and collected into the conical, the media was centrifuged at 2500 rpm for 5 minutes. The supernatant was then discarded, but 70% of cold ethanol was added into the sediment for 2 hours for cell fixation. After added with PBS and centrifuged at 3000 rpm for 3 minutes, the supernatant was removed, PI kit containing 40 µg/g/µmL PI and RNAse 100 mL was added into the sediment, and it was resuspended. Next, the mixture was incubated at 37°C for 30 minutes. The sample was analyzed using FAC Scan Flow cytometer. Based on its DNA content, the percentage of the cell accumulation in the cell cycle (G_1, S, and G_2/M) were calculated using modfit lt.3.0 [20].

The MCF-7 cells (5 x 10^5cells/mL) were plated in 6-well plate, incubated for 24 hours, treated with EEAL and sorafenib, and incubated for 24 hours. After 24 hours, the media was put into the conical tube, and 0.025% trypsin was added into the well. After washed with PBS, collected into the conical, and centrifuged at 2500 rpm for 5 minutes, the supernatant was discarded. Subsequently, PBS was added into the sediment, and the suspension was centrifuged 300 rpm for 3 minutes. The supernatant was then moved. Next, Annexin V kit was added into the microtube, resuspended, and incubated at 37°C for 30 minutes. The sample was then analyzed using FAC Scan Flow

cytometer [20].

The cytotoxic test data was analyzed using SPSS 22 software with probit analysis.

Results

Inhibition Concentration 50% (IC_{50})

Table 1. Table of Cytotoxicity Assay

Treatment	IC_{50} value (µg/mL)
n-Hexane Africa	206.211 ± 0.99
Ethyl Acetate Africa	50.365 ± 0.068
Ethanol Africa	967.033 ± 2.68

Among several test solvents, the cytotoxic test results which showed a good value of IC_{50} µg/mL for MCF-7 cells was ethyl acetate extract. The IC_{50} value obtained on the MCF-7 cells was 50.365 µg/mL in which ethyl acetate extract was able to inhibit 50% of the cell growth tested at this concentration.

Analysis of the cell cycle inhibition by flow cytometry method was done by giving ethyl acetate extract 1/2 IC_{50} and 1/5 IC_{50}. The result for the G0-G1 phase in the cell control was 71.08%. However, the percentage decreased in the treatment with ethyl acetate extract 1/2 IC_{50} with 62.58% and 1/5 IC_{50} with 44.72%. For the S and G2-M phases, the highest percentage was found in the treatment with ethyl acetate extract 1/5 IC_{50} with 47.27% and 9.50% respectively in which both percentages were higher than the control cells with 23.26% and 5.90%. Increased inhibition in the S phase indicates that the extraction prevented cell DNA synthesis whereas increased inhibition in the G2-M phase indicates that there was a repair of damaged DNA.

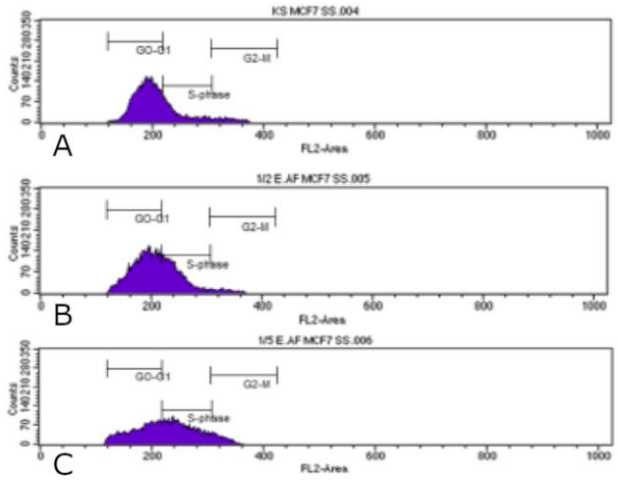

Figure 1: The cell cycle on the EEAF analysis on MCF-7 line; A) Cell control; B) 1/2 IC_{50}; and C) 1/5 IC_{50}

The percentage of apoptosis induction of the African leaf's ethyl acetate extract 1/2 IC_{50}, 1/5 IC_{50} and cell control in the initial apoptosis were 1.86%, 4.39%, and 1.764%, in the late apoptosis / early necrosis were 6.12%, 5.69%, and 0.62%, and in the final necrosis were 90.56%, 54.36%, and 7.49%, respectively.

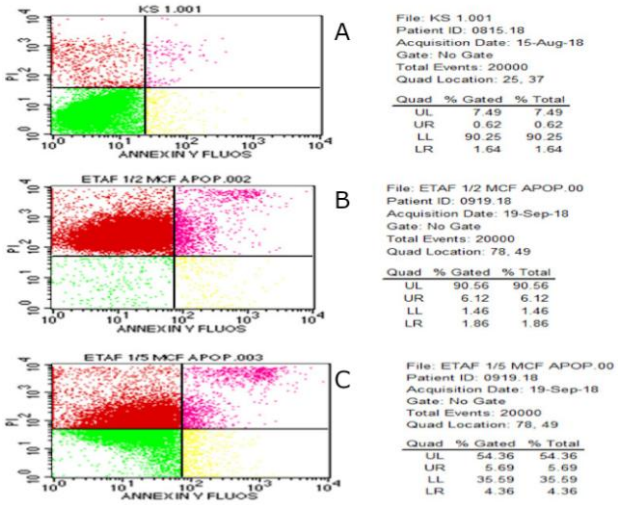

Figure 2: Apoptosis analysis of EEAF on MCF-7 cell lines: A) cell control; B) 1/2 IC_{50}; and C) 1/5 IC_{50}

Discussion

An extract is stated to have potential if it has an IC_{50} value of less than 100 µg/mL [21]. The greater number of living cells indicates the more active cells perform metabolism so that the amount of formazan crystals formed also accumulates and causes purple intensity to increase on the plate. Dead cells cannot be colored by MTT salts, so they do not form purple as in living cells. As a result, dead cells do not form purple formazan, but the color remains yellow like a medium [22]. If the damaged DNA can be repaired, the cell cycle can occur in which the cell enters the G0-G1 phase after passing M phase [21].

The inhibition of the G0-G1 cycle allows apoptosis to occur. Hence, there was no activation of CDK4 and CDK6 which resulted in the inhibition of phosphorylation of pRb (retinoblastoma protein). Non-phosphorylated Rb binds to the E2F transcription factor binding to DNA and inhibits gene transcription whose products are required for the cell cycle in the S phase. Thus, the cells are retained in the G1 phase, or G1 arrest occurs [3].

Flow cytometry method was used to determine apoptosis. This method is a method used for calculating the number of living cells, cell necrosis, and apoptosis within a short period of time. In this test, Annexin V was used to bind phosphatidylserine found in the cell plasma membrane during fluorescence apoptosis [21].

The potential of African leaves ethyl acetate

extract in promoting cell apoptosis may be caused by steroid/triterpenoid compounds through several mechanisms including inhibition of DNA topoisomerase I/II activities, signal path modulation, decreased Bcl-2 and Bcl-XL gene expression, increased Bax and Bak gene expression, and endonuclease activation [23].

Based on the results, it is found that EEAF had immensely good effect; thus, it is potentially used in breast cancer therapy through the cell cycle inhibition and apoptosis-induced activity.

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