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Flow cytometric analysis of MCF-7 cell line in its treatment with leaves extract of *Eugenia uniflora* L

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Abstract

The use of natural product in treatment of cancer has attracted more attention since the existing treatments have not yet given any satisfactory result. Dewandaru (*Eugenia uniflora.*L), one of promising medicinal plants in Indonesia, was reported to have suppression activity on DNA polymerase of EBV (Eipstein-Bar Virus). This study aimed to reveal the cytotoxicity of *E. uniflora* L leaves extract and examine more deeply whether or not this activity will trigger the apoptosis process of human breast cancer MCF-7.

The extraction process was conducted using petroleum ether, dichloromethane and methanol. Each extract was subjected for MTT assay for cytotoxicity analysis. The prospective compounds were then separated using vacuum column chromatography and preparative thin layer chromatography. MTT method was used to performed cytotoxicity test of each separated fraction on MCF-7 cells. The selected compound which show the most potential activity was analyze to its IC_{50} value. MCF-7 cells which treated with this toxic compound was analyze for its cell cycle using flow cytometry assay and propidium iodide staining.

The results showed that the IC_{50} of the toxic compound tested on MCF-7 cells were 10 µg/mL. Flow cytometry analysis showed that this compound has capability in inducing apoptosis. Cell cycle arrest was observed in MCF-7 cells in which cell accumulation occurred in G1 phase.

Key words: *Eugenia uniflora* L, MCF-7, flow cytometry

INTRODUCTION

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to disregulate balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host (Ruddon, 2007). Cancer replaced heart disease as the leading cause of death among men and women aged younger than 85 years in 1999 in the United States. The 3 most commonly diagnosed types of cancer among women in 2011 are breast, lung and bronchus, and colorectal, accounting for about 53% of estimated cancer cases in women. Breast cancer alone is expected to account for 30% (230,480) of all new cancer cases among women (Siegel, 2011)

The ultimate goal of any cancer drug discovery process is discovering and developing effective and non-toxic therapies (Collota, 2008). In this case,cancer treatment using natural products has attracted more attention as the existing treatments did not provide satisfactory results. One of the potential plants is Dewandaru (*Eugenia uniflora* L), also known as the Surinam Cherry

E. uniflora leaves extracts have been showed pronounced anti-inflammatory action (Schapoval, *et al.*, 1994), considerable contractile activity, with a resulting effect on intestinal transit (Gbolade, *et al.*, 1996), endothelium-dependent vasorelaxant effects (Wazlawik *et al.*, 1997) and hypotensive effects (Consolini, *et al.*, 1999; Consolini & Sarubbio, 2002), and inhibit the increase of plasma glucose and triglyceride levels (Arai *et al.*, 1999). Some compounds present in *E. uniflora* leaves extracts have also been shown to inhibit the Epstein– Barr virus, known to be closely associated with nasopharyngeal carcinoma (Lee, *et al.*, 2000), and have antimicrobial (Adebajo, *et al.*, 1989; Holetz *et al.*, 2002) and antifungal activity (Lima, *et al.*, 1993; Souza *et al.*, 2002). However, research on its anticancer activity has not been reported. Therefore, the objective of this research is to study the potential application of *E. uniflora* as anticancer agent, isolate the toxic compound and study its impact on cytotoxicity and apoptosis on MCF-7 breast cancer cells.

MATERIALS AND METHODS

Plant and extracts

Fresh leaves of *E. uniflora* were collected from Tawangmangu, Indonesia and was identified at Medicinal Plant and Traditional Medicine Research and Development Office, Tawangmangu. Their powder was macerated three times using petroleum ether for 24 hours. After filtration the resulting extracts were combined and evaporated to dryness. The residue from petroleum ether was macerated three times using dichloromethane for 24 hours. After filtration the resulting extracts were combined and evaporated to dryness. The residue from dichloromethane was macerated using methanol for 24 hours. Supernatant was evaporated to dryness. Each extracts was tested for cytotoxicity test on MCF-7 cell line.

Fractionation

The toxic extract was partitioned using petroleum ether, petroleum ether/chloroform {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, chloroform, chloroform/methanol {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, and methanol. The chemical composition of each fraction was monitored on thin layer chromatography. The sub fractions which show similar spots on TLC analysis, were then combined. Each fraction was subjected for cytotoxicity test on MCF-7 cell line.

The toxic fraction were partitioned with petroleum ether, petroleum ether/chloroform {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, chloroform, and methanol. The fractions that showed similarity on TLC were combined. Each fraction was subjected for its cytotoxicity on MCF-7 cell line.

Cytotoxicity assay using MTT method

Cell Culture

Human breast cancer cell lines MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) in an incubator with humidified air with 5% CO_2 at 37°C.

Cell viability assay

The viability of the cells was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) (Sigma) assay which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Cells were plated onto 96-well plates ($2x10^3$ cells/well) (Iwaki). After 24 h incubation, cells were treated with each extract/fraction with various concentrations for 48 h. Then, MTT solution was added in each well and cells were incubated for 4 h at 37°C and then incubated with 100 µl of soluble

solution at 37°C overnight. The quantity of formazan product was measured by using a spectrophotometric microtiter plate reader (Bio-Rad) at 595 nm wavelength.

Flow cytometry analysis

Apoptosis detection and analysis of cell cycle distribution were performed by flow cytometry. Briefly, cells were incubated for 24 h in a medium without FBS to synchronize the cell cycle. Cells were then treated for 24 and 48 h in the medium containing 10% FBS with isolated compounds solution . Cells were harvested by trypsinization, washed twice with PBS, incubated with 0.125% Triton X-100, and stained with propidium iodide (PI) in PBS containing 0.2 mg/mL RNase A. Stained cells were analyzed using a FACS calibur. For each sample, cells were counted until the count reached 5 x 10^5cells. The percentages of cells in the subG1, G1, S, and G2/M phases were determined using the CELLQUEST software.

RESULTS AND DISCUSSION

Extraction and Cytotoxicity Assay

Maceration of powdered leaf of Dewandaru was conducted using petroleum ether, dichloromethane and methanol. The four extracts was then tested on T47D breast cancer cells using concentration series as follows: 500, 250, 125, 62,5 dan 31,25 µg/mL. Each treatment was observed after 48 hours incubation. Figure 1 showed a curved of cell viability versus various concentration extract which is used on the treatment. The results indicate that dichloromethane extracts has the smallest IC_{50} value of 115 µg/ml, whereas the IC_{50} for petroleum ether extract and methanol extract was 160 µg /ml and 150 µg/mL respectively.

There were no significant differences among IC_{50} values of petroleum ether extract, dichloromethane extract and methanol extract. This is due to distribution of toxic compounds in each extract. Extraction process by maceration technique enables the toxic compounds can't extracted perfectly. Dichloromethane extract was then chosen for fractionation because of its smallest IC_{50} value.

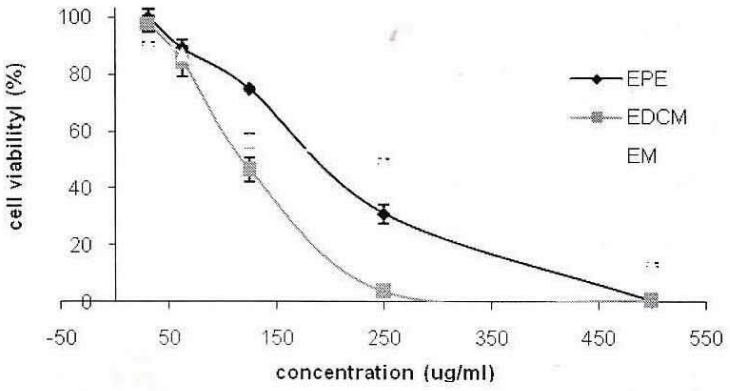

Figure 1. MCF-7 cell viability caused by treatment of petroleum ether extract (EPE), dichloromethane extract (EDCM) and methanol extract (EM) leaves of E. uniflora with MTT method

The portion of dichloromethane extract that showed the strongest cytotoxicity activity was partitioned by vacuum liquid chromatography (VLC). The fraction that showed similarity on TLC were combined to give four fractions. Each fractions were tested for cytotoxicity test. Two fraction had cytotoxicity activity (fraction II and III) and the other fraction not toxic.

The most toxic fraction (fraction II) were further were partitioned by VLC using gradient elution. The chemical composition of each fraction was monitored on thin layer chromatography. The fractions that showed similarity on TLC were combined and evaporated given two fraction. Cytotoxicity activity of F2V2 fraction against MCF-7 cells stronger than F2V1 fraction.

F2V2 fraction were further separated by preparatif thin layer chromatography. The TLC spot were scraped into two portion, upper and lower part and diluted in mixture chloroform/methanol 4:1. After filtration each fraction were evaporated and tested for cytotoxic activity. It was found that the lower part of PTLC had cytotoxic activity. The toxic compounds were purified by preparatif TLC given two portion upper and lower part. After 48 treatment of each portion, it was found that the upper part of preparative TLC had cytotoxic activity. Toxic compounds activity of *E. uniflora* against MCF-7 cells showed a linear correlation between the concentration of the test with cell viability as shown in Figure 2.

Cytotoxic activity of toxic compounds E uniflora against MCF-7 cells showed a linear correlation between the concentration of the test with cell viability as shown in Figure 2.

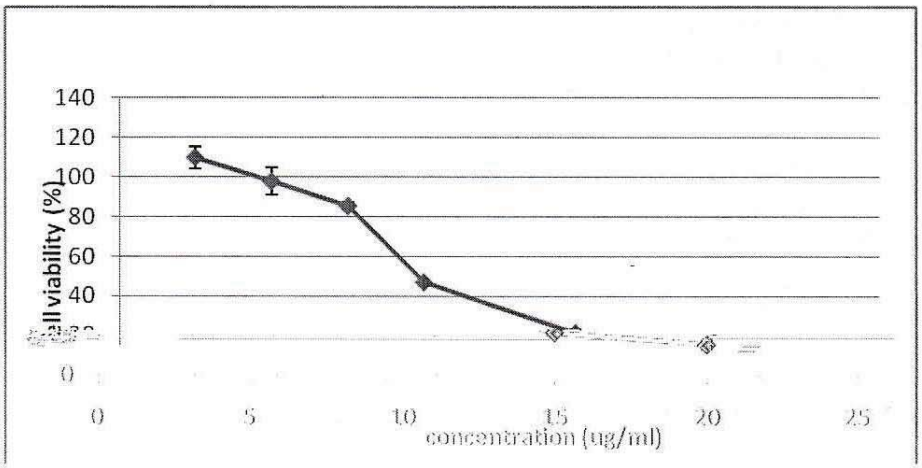

Figure 2. Treatment effects of toxic compounds extracted from *E. uniflora* against MCF-7 cell lines. The cell viability was determined by MTT method.

The calculation showed that IC_{50} of toxic compounds extracted from *E. uniflora* tested on MCF-7 cells is 10 µg/mL. There were morphological changes in MCF-7 cells due to the treatment of doxorubicin and toxic compounds extracted from *E. uniflora*. The cells look Tues looked round, flat and floats (Figure 3). Sel tampak berbentuk bulat, pipih dan mengapung (gambar 3).

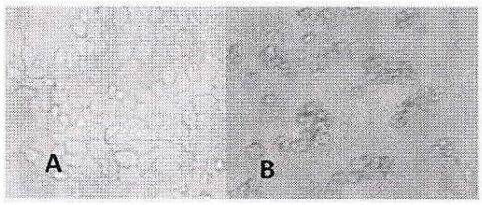

Figure 3. MCF-7 cell morphology (A) without treatment (B) treatment with toxic compounds isolated from *E.uniflora* 15 µg / mL

Flow cytometry analysis

We analyzed cell cycle distributions using flow cytometry to investigate the effects of toxic compounds extracted from *Eugenia uniflora* L. The distribution of MCF-7 cells in each phase after treatment can be observed in tables and figures below.

Table. 1. The distribution of MCF-7 cells in each phase of cell cycle after treatment using extract of *E. uniflora*. The cell cycle distribution was observed using flow cytometry method

Treatment	Concentration (μg/ml)	Incubation (hour)	Persentase jumlah sel (%)				
			Sub G1	G1	S	G2/M	Polyploidi
Control		*48*	*1.18*	*45,96*	*23.41*	*26,91*	*3,18*
Toxic compound	*8*	*24*	*0,98*	*76,05*	*12,08*	*8,33*	*2,90*
	8	*48*	*16,25*	*59,40*	*8,06*	*15,04*	*1,58*
	6,5	*24*	*1,00*	*53,63*	*21,86*	*21,42*	*2,60*
	6,5	*48*	*10,16*	*69,47*	*10,95*	*14,82*	*2,62*

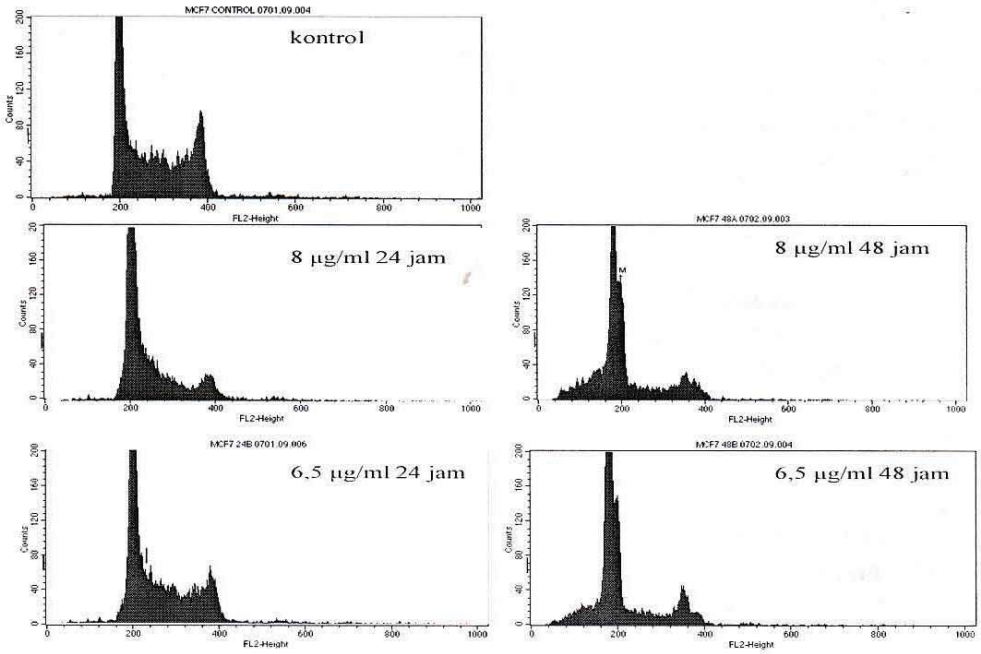

Figure 4. Flow cytometry of MCF-7 cell cancer. Cell were treated with 6.5 and 8 μg/mL of toxic compounds extracted from *E. uniflora*. Incubation process was conducted for 24 and 48 hours.

MCF-7 cell lines were treated with concentrations of toxic compounds of 8 μg/mL and 6.5 μg/mL. Incubation process was conducted for 24 and 48 hours. Both concentration were shown to induce the apoptosis process after 48-hour incubation. It was indicated by the increase of the percentage of cells in sub G1 phase. Inhibition of cell cycle which occurs in

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G1 phase can reduce the percentage of cell population in S phase. As a result, the cells that enter the phase of G2 / M also reduced. According to the data above, it can be inferred that toxic compounds from *E. uniflora* arrested cell cycle at G1 phase and lead to apoptosis.

In cell cycle analysis using flow cytometry, it can be known that toxic compounds of *E. uniflora* lead to apoptosis. To support these data, the cell was also observed for apoptosis using double staining method. Here we used fluorescent compounds that can bind to DNA / RNA, ethidium bromide-acridine orange (AO-EB), which was subjected against MCF-7 cells. Apoptotic morphology of MCF-7 cells on double staining test results can be examined in Figure 5.

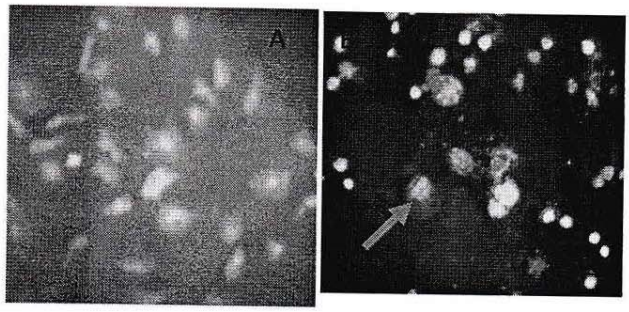

Figure 5. Apoptosis Induction of toxic compounds in the MCF-7 cells with double staining method (ethidium bromide-acridine orange). (A) MCF-7 cells without treatment, (B) MCF-7 cells in treatment with toxic compounds of 9 ug / mL.

The results showed that all apoptosis in control cells exhibit a green fluorescent. It means that there was no cell death. Treatment of MCF-7 using toxic compounds causing some cells be fluorescent which show the loss of cell membrane permeability and indicate the occurrence of apoptosis. Further observations indicate the presence of an enlarged cell nuclei size and the occurrence of multi nucleus. Some cells begin to divide the cell nucleus and form the apoptotic bodies. On one hand, acridine orange can penetrate the membrane of normal cells, binds to the DNA / RNA and cause a bright green fluorescence. On the other hand, ethidium bromide is more easily to penetrate into the decreased permeability of cell membranes and cause orange fluorescence. This method showed the presence of live and dead cells, and cells which undergo apoptosis. One of the characteristics of apoptotic cells is the fragmentation of the cell nucleus that followed by the fragmentation of cells into apoptotic bodies.

CONCLUSIONS

The IC_{50} values of toxic compounds from *E. uniflora* L tested on MCF-7 cells were 10 µg/ml, Flow cytometry analysis showed that the toxic compounds are capable of inducing apoptosis. Cell cycle arrest was observed in MCF-7 cells in which cell accumulation occurred in G1 phase.

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