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Ethyl Acetate Fraction of *Moringa oleifera* Leaves Induces Cell Cycle Arrest on T47D Breast Cancer Cell via G0/G1 through Cyclin D1 Expression

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Abstract

Cancer has one of the highest mortality rates worldwide. Exploration of a natural product containing anticancer agent provides a promising line for research on cancer. One of plant that used in traditional medicine for cancer treatment is *Moringa oleifera*. The previous study has reported that the *n*-hexane fraction of *M. oleifera* leaves induce apoptosis and cell cycle arrest in T47D cells. Based on the preliminary result, ethyl acetate fraction of *M. oleifera* (EMO) leaves showed medium cytotoxic activity on T47D cells with IC_{50} values of 243.58 µg/mL and induced apoptosis cell death. This study was carried out to determine the anticancer activity of EMO against T47D breast cancer cell line by observing cell cycle progression and Cyclin D1 expression level. Analysis of the cell cycle was performed using flow cytometry and cyclin D1 expression was analyzed using the immunocytochemistry method. The result shows that EMO induced cell cycle arrest in G0/G1 phases. Immunocytochemistry assay showed that the EMO decreased expression of cyclin D1. These results indicate that EMO potentially containing compounds that induce cell cycle arrest and can be developed as a candidate for an anticancer drug. Further investigation is needed to know the responsible compound for the anticancer activity of EMO.

Keywords: Breast cancer, cell cycle, cyclin D1, *Moringa oleifera*, T47D cell

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1. INTRODUCTION

Breast cancer is the most common invasive cancer in women and the second leading cause of cancer death in women after lung cancer. It was estimated that 2.1 million cases every year, and also cause the greatest number of cancer-related deaths among women. In 2018, it is estimated that 627,000 women died from breast cancer–that is approximately 15% of all cancer deaths among women (WHO, 2018). It will afflict an estimated 9.1 million women in poorer countries over the following decade. Of the 5 million women expected to die from breast cancer in the next decade, 70 percent will live in low and middle-income countries (Babaee *et al.*, 2016; Malki *et al.,* 2017). There is evidence for a genetic contribution to the risk of developing breast cancer, as well as an association with modern affluence (diet and alcohol consumption). Besides, the influence of reproductive factors supports a hormonal role within the etiology of the disease (Coles *et al.*, 1992; Sun *et al.*, 2017).

Moringa oleifera is an edible plant native to Northern Indian subcontinents, however recently the plants are widely cultivated and become naturalized in many countries like Pakistan, Afghanistan, Shri Lanka, Bangladesh, East, and West Africa, throughout West Indies, from Mexico to Peru, Paraguay and Bazil[6,7,8]. *M. oleifera* is also called with various names such as horseradish tree, drumstick tree, and locally named 'kelor'. It belongs to the family of Moringaceae and has been used in traditional medicine for centuries. The traditional uses of *M. oleifera* including the treatment of bacterial, fungal,

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viral and parasitic issues, along with asthma, circulatory, headaches, anemia, bronchitis, cholera, digestive and inflammatory disorders, malaria, typhoid fever, arthritis, hypertension, and diabetes (Guevara *et al.*, 1999; Fahey, 2005; Madi *et L.*, 2016; Welch & Tietje, 2017).

Almost every part of the *M. oleifera* plant from the leaves to the fruit, bark, and seeds can be used to treat a diverse array of ailments, but the leaves are the most widely cultivated due to its phytochemical composition and their associated medicinal properties (Panda *et al.*, 2013; Mehta *et al.*, 2003; Cheenpracha *et al.*, 2010; Mahajan *et al.*, 2007; Gupta *et al.*, 2010; Mishra *et al.*, 2011). The moringa tree is highly nutritious since it is a significant source of fats, proteins, vitamins, carotenoids, polyphenol, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins, saponins and oxalates and phytates (Leone *et al*., 2015).

Several studies on anticancer activity of *M. oleifera* leaves extract has been published (Jung, 2014; Al-Asmari *et al.*, 2015; Jung *et al.*, 2015; Leone *et al.*, 2015). *M. oleifera* aqueous crude leaf extracts have expressed anticancer effects in both A549 lung cancer cells and SNO oesophageal cancer cells (Tiloke *et al.*, 2013) as well as KB tumor cells (Sreelatha *et al.*, 2011) in a ROS-dependent manner. Gaffar *et al*. (2019) show that the *n*-hexane fraction of *M. oleifera* has properties to induces apoptosis and cell cycle arrest on T47D cells. Our previous study indicates that ethyl acetate fraction of *M. oleifera* (EMO) leaves showed medium cytotoxic activity on T47D cells with IC_{50} values of 243.58 µg/mL by either inducing apoptosis (Apriani *et al.*, 2019). Gothai *et al.* (2017) have reported that phenolic compound in ethyl acetate fraction of *M. oleifera* leaf was found to be relatively high. Moreover, Rashid *et al.* (2003) stated that flavonoids are primarily derivatives of phenolic compounds. Thus, we assume that flavonoid compounds like quercetin, kaempferol, catechin, epicatechin, rutin are bioactive compounds that could possibly exist in EMO.

The development of many anticancer drugs is directed to apoptosis-inducing process (Xu *et al*., 2009) and cell cycle regulation (Saphiro & Harper, 1999). In our previous research, we observed the anticancer activity of EMO through inducing the apoptosis process and reduce the Bcl-2 expression level. This study aims to investigate the mechanism of anticancer activity of EMO against T47D breast cancer cell line through observing cell cycle progression and Cyclin D1 expression level.

2. MATERIAL AND METHODS

Plant Material

M. oleifera L. leaves were taken from Mangunreja, Tasikmalaya, Indonesia on February 2018, during the rainy season. The samples were authenticated and deposited in Biology Department herbarium (Ref. No. MOL0027), Universitas Padjadjaran, Indonesia.

Chemicals

Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS), 0.25% trypsin-EDTA solution, and all antibiotics were purchased from Gibco Invitrogen, Life Technologies, USA. Propidium iodide (PI) were purchased from BD Bioscience, New Jersey, USA. Anti-cyclin D1 antibody, biotinylated secondary antibody, streptavidin-HRP, and Mayer-hematoxylin reagent were purchased from Bio-care, California, USA. Hydrogen peroxide was obtained from Millipore Sigma, Burlington, USA). DAB (3, 3 diamino-benzidine) was purchased from Alfa Aesar, Ward Hill, USA. Other chemicals and reagents were of cell culture grade and were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA.

Extraction

M. oleifera leaves were dried with an oven at 30°C. Dry powder of *M. oleifera* leaves was extracted with ethanol 96% for 3x24 hours. The filtrate was concentrated using a rotary evaporator at 50°C. *M. oleifera* ethanol extract was then partitioned with *n*-hexane and ethyl acetate by liquid-liquid extraction. Ethyl acetate fraction was evaporated by using a rotary evaporator to obtain ethyl acetate fraction of *M. oleifera* leaves (EMO).

Cell Culture

Human breast cancer T47D culture cells were obtained from the collection of the Paracytology Laboratorium, Universitas Gadjah Mada, Yogyakarta. The cells were grown in a medium culture of RPMI-1640 (Roswell Park Memorial Institute-1640) from

Gibco which containing 10% Fetal Bovine Serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA), and was incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air. Trypsin-EDTA 0.025% (Gibco, USA) solution was used to separate cells on the flask.

Cell Cycle Analysis

FACS analysis was carried out to investigate cell cycle distribution. T47D cells were grown in 6-well plate (10^6 cells/well) and treated with 150, 200, 250, and 300 µg/mL of EMO, respectively. After 24 hours of treatment, cells were trypsinized and centrifuged at 2000 rpm for 3 minutes. Trypsinized adherent cells were collected and detected by adding 25 µL propidium iodide, 2.5 µL RNAse, 0.5 µL Triton-X, then incubated at room temperature for 10 minutes. The cell suspension was transferred into a flow cytometer (BD FACS-Calibur, USA).

Immunocytochemistry

T47D cells were grown with a density of $5x10^4$ cells/coverslip in 24-well plate and incubated for 24 hours. Cells were treated for 24 hours and then fixed with cold methanol for 10 minutes and washed twice using PBS and sterile water. Cells were blocked in hydrogen peroxide (Millipore Sigma, Burlington, USA) blocking solution for 10 minutes at room temperature. Then, washed again with PBS, and incubated with pre-diluted blocking serum for 10 minutes at room temperature. Next, the cells were incubated with primary cyclin D1 antibody (Biocare Medical, California, USA) for 1 hour at room temperature. After three time-washing with PBS, cells were incubated with biotinylated universal secondary antibody for 20 minutes, followed by streptavidin-biotin complex (Biocare Medical, California, USA) for 10 minutes. Substrate solution chromogen 3, 3 diamino-benzidine (DAB) (Alfa Aesar, Ward Hill, USA) was exposed for 5 minutes and washed with distilled water. Cells were counterstained with Mayer-Hematoxilin reagent for 3 minutes. Then, cells were fixed with ethanol and xylol. Between each immunostaining step, cells were washed shortly in PBS. The cyclin D1 expression on the cells was observed by a light microscope (Olympus Life Science, Shinjuku, Tokyo, Japan).

3. RESULTS AND DISCUSSION

Cytotoxic activity was used to evaluate the potential of EMO cytotoxicity on T47D cells. Furthermore, IC_{50} value was acquired as a parameter of EMO concentration to inhibit 50% T47D cell growth. According to Apriani *et al*. (2019), EMO giving an IC_{50} value of 135.321 µg/mL. Based on the IC_{50} value, EMO expressed medium activity as an anticancer because according to Kamuhabwa *et al*. (2000), an extract is considered active if it has an IC_{50} value less than 100 µg/mL, but it can be still developed as an anticancer because an extract is considered inactive if the IC_{50} value more than 500 µg/mL (Machana *et al.*, 2011).

However, this research did not analyze the effect of EMO to normal cell as a control, there was so many research show that *M. oleifera* extract has minor cytotoxicity for normal cells, indicates that normal cells are more resistant to the extract (Jung et al., 2014). Also, Khor *et al.* (2018) report that the leaf extract of *M. oleifera* has relatively low toxicity against normal cells, and very low toxicity was detected when the extract was administered orally during in vivo testing on mice and rats. These results indicate the leaf extract of *M. oleifera* is relatively safe and possibly become a chemotherapy agent.

EMO Induce Cell Cycle Arrest: According to the cytotoxic activity mentioned above, cell cycle distribution was determined by flow cytometry. Figure 1 shows the distribution of T47D cells treated with various concentrations of EMO for 24 hours incubation. In this case, we used untreated cell T47D as a control cell to compare the distribution of T47D cells accumulation in the cell cycle. T47D cells treated with 150, 200, 250, and 300 µg/mL of EMO were accumulated in the G0/G1 phase, which is from 47.64% in untreated cells to 70.24%, 58.27%, 48.36%, and 55.77%, respectively, in treated cells. Doxorubicin has been reported to induce cell accumulation in the G0/G1 phase in T47D cells, which is 50.64% (Gaffar *et al.*, 2019). This study has proven that T47D cells treated with EMO caused cell cycle arrest in the G0/G1 phase. This result indicates that one of the mechanisms of EMO may act as an anticancer agent is inhibition of cell cycle progression.

Generally, the proliferation of cells is regulated by a spread of extracellular growth factors that control the progression of cells

through the restriction point in late G1. Within the absence of growth factors, cells unable to pass the restriction point and become quiescent, frequently getting into the resting state called G0. They will reenter the cell cycle in response to growth factors implies that the extracellular signaling pathways stimulated downstream of growth factor receptors ultimately act to control parts of the cell cycle (Cooper & Hausman, 2007). The accumulation of cells within the G0/G1 phase observed in this study indicates that T47D cells did not get any stimulus from extracellular growth factor signaling so that the cells could not enter the S phase to continuing cell cycle progression.

Expression of Cyclin D1 was decreased on T47D cell treated with EMO: The cell cycle progression in the G0/G1 phase might be caused by some proteins that play a role in cell cycle checkpoints. Cyclin D1 is an important regulator protein of G1 to S phase progression. Therefore, the expression of cyclin D1 was also investigated using the immunocytochemistry method. As shown in Figure 2, the result of immunocytochemistry assay showed that cyclin D1 level decreased significantly confirmed by an intensive purple color in the nucleus after being treated with 150, 200, 250 and 300 μg/mL EMO, while the control cell did not show intensive purple color in the nucleus, indicates that the control cell expressed a high level of cyclin D1.

Table 1. Cell cycle distribution of T47D cells in the G0/G1, S, and G2/M phases

Treatment	Concentration (μg/mL)	Cell cycle phase (%)		
		G0/G1	S	G2/M
Control	-	47.64	19.94	32.12
EMO	150	70.24	7.17	22.60
EMO	200	58.27	12.74	25.99
EMO	250	48.36	17.60	28.80
EMO	300	55.77	16.52	23.57

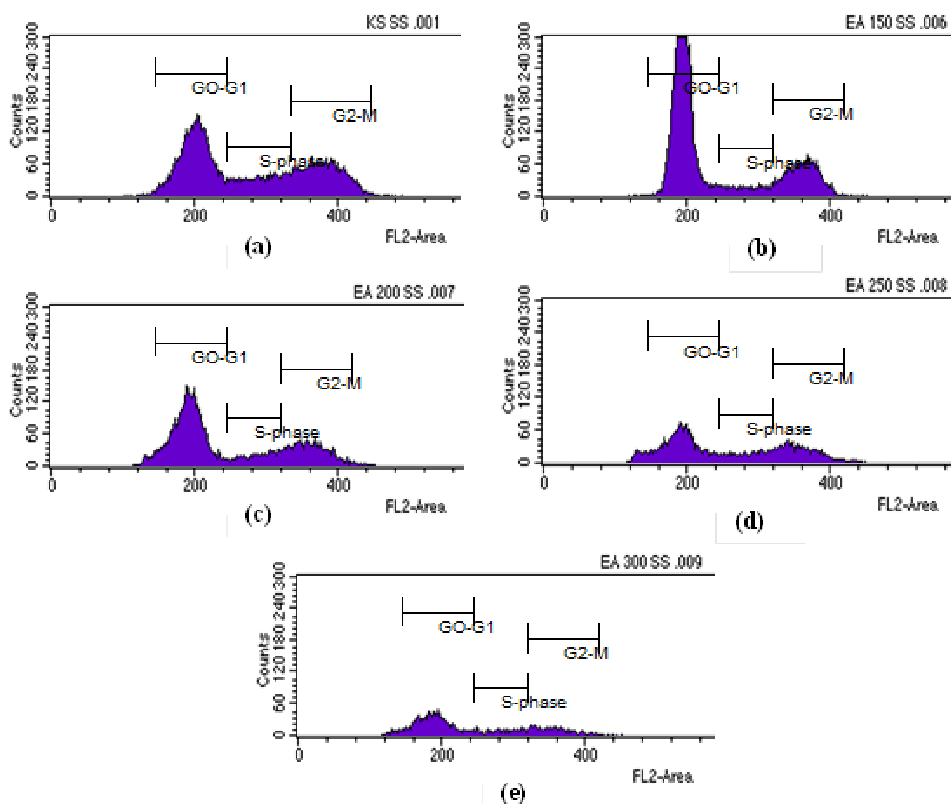

Figure 1. EMO caused cell cycle arrest on T47D cells. Cell treated with EMO (a) 0 μg/ml (control); (b) 150 μg/ml; (c) 200 μg/ml; (d) 250 μg/ml; (e) 300 μg/ml. Cell cycle distribution was calculated by flow cytometry.

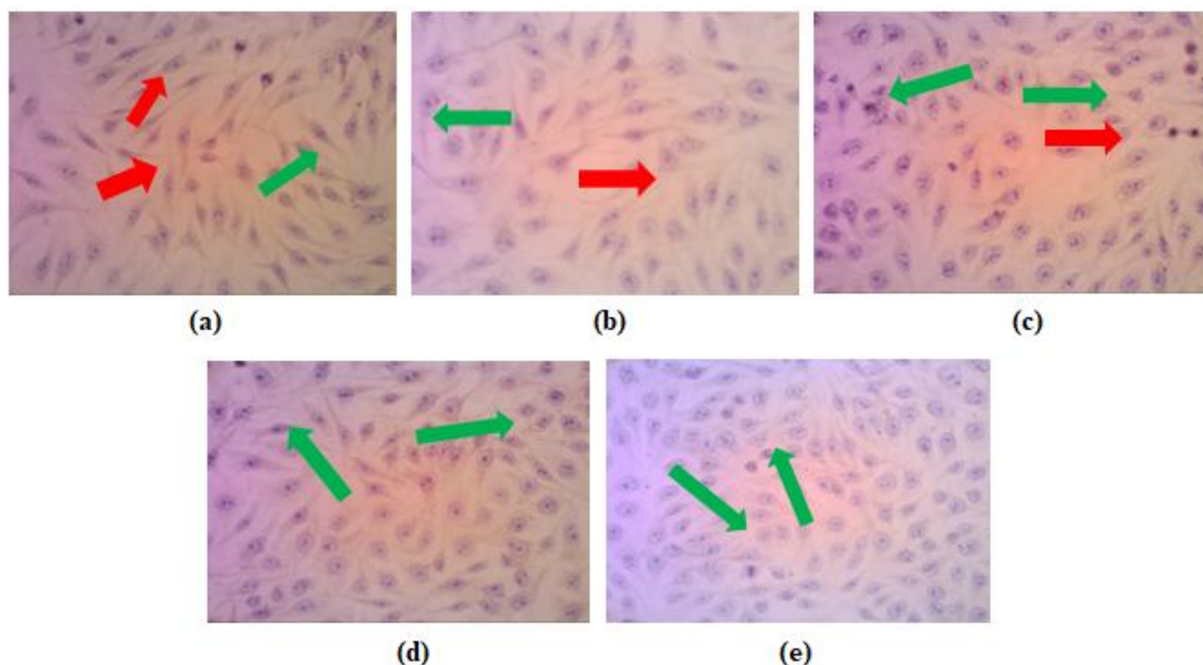

Figure 2. Determination of cyclin D1 expression on EMO treated T47D cells using immunocytochemistry method. (a) control cells, (b) EMO 150 µg/mL, (c) EMO 200 µg/mL, (d) EMO 250 µg/mL, (e) EMO 300 µg/mL. (Red arrows: cells expressing cyclin D1; green arrows: no Cyclin D1 observed)

Cyclin D1 is synthesized in a response to growth factor stimulation through the Ras/Raf/ERK signaling pathway (Gaffar *et al.*, 2019). Together with its binding partner's cyclin-dependent kinase 4 and 6 (CDK4 and CDK6), cyclin D1 forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (RB) (Alao, 2007) and cells are able to reach the restriction point on the cell cycle. Contrarily, if the presence of growth factors is not available in G1, the cyclin D1 level will decrease and could not form complexes with CDK 4 and CDK 6. In this study, the cyclin D1 level was decreased, cells cannot pass the restriction point and then enter a quiescent stage of the cell cycle known as G0 within which they will stay for long periods while not proliferating.

4. CONCLUSION

EMO causes accumulation of T47D cell in the G0/G1 phase compared to control cells, also causes a decrease of cyclin D1 level compared to control cells. These findings indicate that EMO has anticancer activity through the cell cycle arrest on T47D cells and decrease the cyclin D1 expression. Further investigation is needed to know the responsible compound as a candidate for an anticancer drug. Observation of its selectivity as part of the safety aspect is also needed.

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