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n-Hexane fraction of *Moringa oleifera* Lam. leaves induces apoptosis and cell cycle arrest on T47D breast cancer cell line

[Fracción de n-hexano de hojas de *Moringa oleifera* Lam. inducen apoptosis y detención del ciclo celular en la línea celular de cáncer de mama T47D]

Shabarni Gaffar[1*], Riza Apriani[2], Tati Herlina[1]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia.
[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Garut, Garut, Jawa Barat, Indonesia.
*E-mail: shabarni.gaffar@unpad.ac.id

Abstract

Context: Moringa plant (*Moringa oleifera*) is one of the medicinal plants used as traditional medicine for the treatment of the variety of diseases including cancer. Recently, extensive research has been conducted on leaf extracts of *M. oleifera* to evaluate their potential cytotoxic effects. Several studies have reported anti proliferative activity of water and alcoholic extracts of leaf, bark and seed of *M. oleifera* on some cancer cell line, including HepG2 liver cancer cell, A549 lung cancer cell, Caco-2 colon cancer cell, MCF7 and MDA-MB-231 breast cancer cells.

Aims: To evaluate the cytotoxic effect of n-hexane fraction of *M. oleifera* (hMO) leaves on T47D breast cancer cells.

Methods: The *M. oleifera* leaves were extracted using ethanol, then fractionated with n-hexane. The cytotoxic activity was determined using MTT assay. Apoptosis and cell cycle arrest were analyzed using flow-cytometry and expression of Bcl-2 and cyclin D1 were analyzed using immunocytochemistry.

Results: Based on preliminary MTT assay, T47D treated with hMO demonstrated a medium cytotoxic effect and inhibited cell proliferation with an IC_{50} value of 235.58 µg/mL. Detection of apoptosis showed that hMO induced apoptosis mediated cell death in slow manner. In addition, hMO was also induce cell cycle arrest on G0-G1 and G2-M phase. Immunocytochemistry assay showed that the hMO decreased expression of anti-apoptosis protein, Bcl-2, and cell cycle regulator protein, cyclin D1, in concentration dependent manner.

Conclusions: hMO has properties to induce apoptosis and cell cycle arrest on T47D cells. hMO has a potential compound to be explored and developed as a chemo preventive agent for breast cancer. These results provide evidence for anticancer activity of *M. oleifera* leaves extract since it cause growth inhibition, induced apoptosis and cell cycle arrest.

Keywords: apoptosis; cell cycle arrest; hMO; T47D cell.

Resumen

Contexto: La planta de moringa (*Moringa oleifera*) es una de las plantas medicinales utilizadas como medicina tradicional para el tratamiento de variedad de enfermedades, incluido el cáncer. Recientemente, se ha realizado una extensa investigación sobre extractos de hojas de *M. oleifera* para evaluar sus efectos citotóxicos potenciales. Varios estudios han reportado actividad antiproliferativa de extractos acuosos y alcohólicos de hojas, cortezas y semillas de *M. oleifera* en alguna línea celular de cáncer, incluyendo hígado HepG2, pulmón A549, colon Caco-2 y mama MCF7, MDA y MB-231.

Objetivos: Evaluar el efecto citotóxico de la fracción de n-hexano de las hojas de *M. oleifera* (hMO) en las células de cáncer de mama T47D.

Métodos: Las hojas de *M. oleifera* se extrajeron con etanol, luego se fraccionaron con n-hexano. La actividad citotóxica se determinó utilizando el ensayo MTT. La apoptosis y la detención del ciclo celular se analizaron mediante citometría de flujo y la expresión de Bcl-2 y la ciclina D1 se analizaron mediante inmunocitoquímica.

Resultados: Basado en el ensayo MTT preliminar, las T47D tratadas con hMO demostraron un efecto citotóxico e inhibió la proliferación celular con un valor de CI_{50} de 235,58 µg/mL. La detección de la apoptosis mostró que la hMO indujo la muerte celular mediada por la apoptosis de manera lenta. Además, hMO también indujo la detención del ciclo celular en las fases G0-G1 y G2-M. El ensayo de inmunocitoquímica mostró que hMO disminuyó la expresión de la proteína anti-apoptosis, Bcl-2 y la proteína reguladora del ciclo celular, ciclina D1, de manera dependiente de la concentración.

Conclusiones: hMO tiene propiedades para inducir la apoptosis y la detención del ciclo celular en las células T47D. hMO tiene un compuesto potencial para ser explorado y desarrollado como un agente quimio preventivo para el cáncer de mama. Estos resultados proporcionan evidencia de la actividad anticancerígena del extracto de hojas de *M. oleifera*, ya que causa inhibición del crecimiento, apoptosis inducida y detención del ciclo celular.

Palabras Clave: apoptosis; célula T47D; detención del ciclo celular; hMO.

INTRODUCTION

Cancer is the second most fatal disease after heart attack. Breast cancer is one type of cancer that has high prevalence and the second most common cancer worldwide, which causes mortality (Bray et al., 2018). In Indonesia, breast cancer is the second most frequent one in women after cervical cancer (Setyowibowo et al, 2018). The higher rate of death caused by cancer diseases has led to a lot of research focused on the search for alternative therapies to prevent and treat cancer. Exploration of natural products containing anticancer agent was also widely carried out. Scientific evidence regarding the safety and effectiveness of treatment with natural products can strengthen its use as an alternative to modern medicine (Pal and Shukla, 2003; Enioutina et al., 2017). The use of herbal medicines for cancer has also increased. About 9-69% of patients who have been diagnosed with cancer take complementary and alternative herbal medicine (Gratus et al., 2009; Sun et al., 2016).

For centuries, all parts of *Moringa oleifera, Moringaceae,* (leaves, fruits, immature pods, and flowers) are combined into the traditional food for human consumption. Additionally, besides being edible, all parts of the moringa tree have long been employed for the treatment of many diseases such as antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, antitumor, diuretic, antihypertensive, cholesterol lowering, antioxidant, anti-diabetic, hepatoprotective, antibacterial and antifungal activities, and are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia, therefore it's called a *miracle tree* (Fuglie, 1999; Anwar et al., 2007; Paikra et al, 2017; Abubakar et al., 2017; Bhattacharya et al., 2018; Kou et al., 2018). The moringa tree is highly nutritious since it is a significant source of fats, proteins, beta-carotene, vitamin C, minerals, essential amino acids, antioxidants, and flavonoids, as well as isothiocyanates (Chumark et al., 2008; Mahmood et al., 2010; Kou et al 2018). For these reasons, some parts of this plant have drawn much attention and have been studied for its various biological activities,

including anti cardiovascular diseases (Khalafalla et al., 2010), antiviral (Murakami et al., 1998; Lilipun et al., 2003; Iqbal and Bhanger, 2006; Waiyaput et al., 2012), antioxidant (Kumar and Pari, 2003; Sultana et al., 2009; Kumar et al., 2012; Gupta et al., 2013), anti-microbial (Sultana et al., 2009), anti-inflammatory (Budda et al., 2011) and tumor suppressive effects in skin papillomagenesis, hepatocarcinoma cancer, colon cancer, and myeloma (Bharali et al., 2003; Brunelli et al., 2010). However, only a few studies have reported the anticancer activity of *M. oleifera* leaves on the molecular basis, most of reference had focused on the evaluation of their efficacy with respect to tumor suppressive activity, but not on the molecular basis of the tumor suppressive activity. Al-Asmari et al. (2015) showed that the ethanol extract of *M. oleifera* leaves and bark induced a significant level of apoptosis as well as cell cycle arrest at G2/M in MD-MB-231 breast cancer cells. In this study, *n*-hexane fraction of *M. oleifera* leaves was investigated for its potential to induce apoptosis and cell cycle arrest on T47D breast cancer cell line. T47D cell was choose because it different in many ways with MCF7 and MDA-MB-231. Briefly, T47D and MCF-7 cells are molecularly classified as Luminal A (ER+/PR+/HER2-), while MDA-MB-231 is a triple-negative (ER-/PR-/HER2-) cell line. MCF-7 cells are reportedly p53 wild-type, while T47D and MDA-MB-231 are p53 mutant (Yu et al., 2017). hMO considered to contain many non-polar bioactive molecules that have not been explore recently. The present study evaluated cytotoxic effect of hMO on T47D breast cancer cell.

MATERIAL AND METHODS

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. Dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent, annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, propidium iodide (PI) were purchased

from BD Bioscience, New Jersey, USA. Anti Bcl2 antibody, 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 diaminobenzidine) 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.

Plant material

M. oleifera plant was collected from Mangunreja, Tasikmalaya, Indonesia on February 2018. Geographically the latitude of the city was 7°19'10.4268'' S and longitude was 108°12'10.6992'' E on degree minutes second (DMS) unit. The samples were authenticated and deposited in Biology Department herbarium (Ref. No. MOL0027), Padjadjaran University, Indonesia.

Extraction

M. oleifera leaves were dried in an oven at 30°C. Dry simplisia was ground in a mill to obtain powder. Briefly, 500 g powder was macerated with 800 mL ethanol 96% for 3x24 hours. Filtrate was concentrated using rotary evaporator (Sigma Scientific Glass, Gujarat, India) at 50°C in atmospheric pressure. *M. oleifera* ethanol extract was then partitioned with n-hexane by liquid-liquid extraction. Furthermore, n-hexane fraction was evaporated by using rotary evaporator at 50°C in atmospheric pressure to yield semi solid hMO. Extracts thus obtained, were collected and stored at 4°C until further use.

Cells culture

Human breast cancer T47D cells was a culture collection of Parasitology Laboratory, Gadjah Mada University, Yogyakarta. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum.

In vitro cytotoxicity assay

Cell viability was assessed based on the reduction MTT to a purple formazan product by the activity of mitochondrial dehydrogenase enzymes of viable cells (Mosmann, 1983; van Meerloo et al., 2011). Briefly, T47D that were grown in 80% confluent were harvested and counted, then diluted with complete culture medium. The cells then were transferred into a 96-well plate with a total of 1×10^4 cells/well. After overnight growth, the cells were treated with increasing concentrations of hMO (1.95; 3.90; 7.8125; 15.625; 31.25; 62.5; 125; 250 and 500 μg/mL) with co-solvent 10% (v/v) DMSO in PBS and no color interference was observed. The final concentration of the solvent was always <0.2%. Doxorubicin (0.375 – 24 μg/mL) was used as positive control. All samples were incubated at 37°C in a 5% CO_2 incubator (NuAire, Plymouth, MN, USA) for 24 hours. After incubation, the medium was immediately replaced by 100 μL of MTT 0.5 mg/mL in RPMI medium. The plates were wrapped with aluminum foil and then incubated at 37°C for 4 hours until formazan was formed. Furthermore, stopped solution was added (SDS 10% in HCl 0.01N). The plate was then wrapped with paper and incubated in dark condition overnight. The absorbance was measured at 595 nm using a microplate reader (Bio-Rad, Hercules, California, USA).

Annexin V-FITC/PI analysis

Detection of apoptosis was conducted using the annexin V-FITC/PI apoptosis detection kit according to manufacturer's protocol (BD FACSVerse™ System, New Jersey, USA). T47D cells were seeded on a six-tissue culture well-plate at 5×10^5 cells per well. After 24 h incubation, the cells were treated with various concentration of hMO according to IC_{50} value obtained with the range of 50 μg/mL (150; 200; 250 and 300 μg/mL). After 24 hours incubation, the cells were removed using 0.25% trypsin solution and then spinned at 2000 rpm for 3 minutes, and then washed twice with

cold PBS. The cells were re-suspended in 500 mL of annexin V buffer and then treated with annexin V and propidium iodide (PI) for 10 minutes at room temperature and protected from light. The treated cells were subjected to a FAC-scan flow-cytometer (BD FACS-Calibur, USA) and followed by flowing software (version 2.5.1) and Excel MS Office 2013. Bivariant analysis of FITC-fluorescence (FL-1) and PI-fluorescence (FL-3) gave different cell populations, viable cells (annexin V negative, PI negative), early apoptotic cells (annexin V positive, PI negative), late apoptotic cells (annexin V positive, PI positive), necrotic cells (annexin V negative, PI negative).

Cell cycle analysis

Cell cycle was analyzed by quantitation of DNA content with propidium iodide staining (BD Biosciences, New Jersey, USA) by using flow cytometry. T47D cells were distributed into 6-well plate with the density of 10^6 cells/well and incubated for 24 hours. Furthermore, cells were treated with various concentration of hMO and incubation was continued for 24 hours. Finally, cells were treated with 150 µL trypsin-EDTA 0.25% and centrifuged (Eppendorf, Hamburg, Germany) at 2000 rpm for 3 minutes. Pellets were then washed twice with cold PBS. Cells were resuspended in PI solution and treated with 1 mL of RNAse-DNAse-free water for 10 minutes at 37°C. The DNA content was analyzed using flow cytometry and followed by flowing software (version 2.5.1) and Excel MS Office 2013.

Immunocytochemistry

Indirect immunocytochemistry (Renshaw, 2017) was done as follow. Briefly, 1 mL (~5 x 10⁴) T47D cells in RPMI medium were seeded on the cover slips placed on 24 well-plate and incubated for 24 hours (or until 80% confluent). The medium was replaced by 1 mL fresh RPMI medium containing various concentrations of hMO and then placed in a humidified incubator at 37°C within an atmosphere of 5% CO_2 and 95% air for 24 hours. Furthermore, the cells were harvested and washed with 500 µL PBS and fixed with 300 µL cold methanol for 10 minutes at 4°C. After that, the cells in

cover slips were placed on a respective slide. The cells were washed with PBS and distilled water, then 500 µL hydrogen peroxide (Millipore Sigma, Burlington, USA) was added as blocking solution and incubated for 10 minutes at room temperature. Then, washed again with PBS, and incubated with pre-diluted blocking serum for 10 minutes at room temperature. Furthermore, 50 µL of primary antibody anti-Bcl-2 and anti-cyclin D1 (1: 100) (Biocare medical, California, USA) were added respectively and incubated for 1 hour at room temperature. After three time-washing with PBS, 100 µL of biotinylated universal secondary antibody (Biocare Medical, California, USA) was added and incubated for 15-20 min, and then washed with PBS three times. The slides were incubated with streptavidin-HRP solution (Biocare Medical, California, USA) for 10 minutes, and then washed with PBS three times. The slides were incubated in the peroxidase substrate DAB (3, 3 diaminobenzidine) (Alfa Aesar, Ward Hill, USA) solution for 3-5 minutes and washed with distilled water. Cells were counterstained with Mayer-hematoxylin reagent (Biocare Medical, California, USA) for 3-4 minutes. After incubation, the coverslips were washed with distilled water and then immersed in absolute ethanol and in xylol. The protein expression was assessed under a light microscope Olympus BX43 (Olympus Life Science, Shinjuku, Tokyo, Japan). Cells that expressed protein will give a dark brown color in the cell membrane, while the cells with no expressed protein will give purple/blue color.

Statistical analysis

Data presented are means of three independent experiments and showed significant difference between the two means ($p \leq 0.05$. Statistical data were analyzed by t-test.

RESULTS AND DISCUSSION

Cytotoxic activity of hMO on T47D cells

Treatment of T47D cell with hMO (1.95, 3.90, 7.8, 15.6, 31.25, 62.5, 125, 250 and 500 µg/mL) decreased cells viability in a dose dependent manner with IC_{50} value of 235.56 µg/mL (Fig. 1). After the

treatment, cells were observed under light microscope. *M. oleifera* extract altered cells morphology. Increase of *M. oleifera* extract concentration caused more cells undergoing morphological alteration, which was indicated by cells becoming rounded form and detached from well (data was not showed). Meanwhile, doxorubicin has IC_{50} value of 1.82 µg/mL. Based on the IC_{50} value, hMO was considered medium active 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, in this case the cytotoxic activity of hMO cannot be compared with doxorubicin, because hMO might be contain many compounds that have synergic effect and doxorubicin is pure compound with high cytotoxicity that already used as commercial chemotherapy drug.

hMO induces apoptosis mediated cell death on T47D cells

In order to determine whether the hMO cytotoxic affect were mediated through apoptosis, the flow-cytometry analysis was performed. An increase in cellular staining with FITC-conjugated annexin V serves as an early marker for apoptosis. Cells were simultaneously stained with PI to investigate loss of cell membrane integrity. This double staining procedure distinguishes early stage apoptotic cells (annexin V positive) from late stage apoptotic cells (annexin V positive, PI positive). Treatment of T47D cells with 150, 200, 250 and 300 µg/mL of hMO was found to induce apoptotic cell death through the observation of shift in viable cell population from early to late stage of apoptosis, followed by secondary necrosis (Fig. 2). The total percentage of apoptotic cell was increase from 2.27% in control cell to 15.65, 33.17, 33.25 and 39.68% respectively, in hMO treated cells after 24 hours.
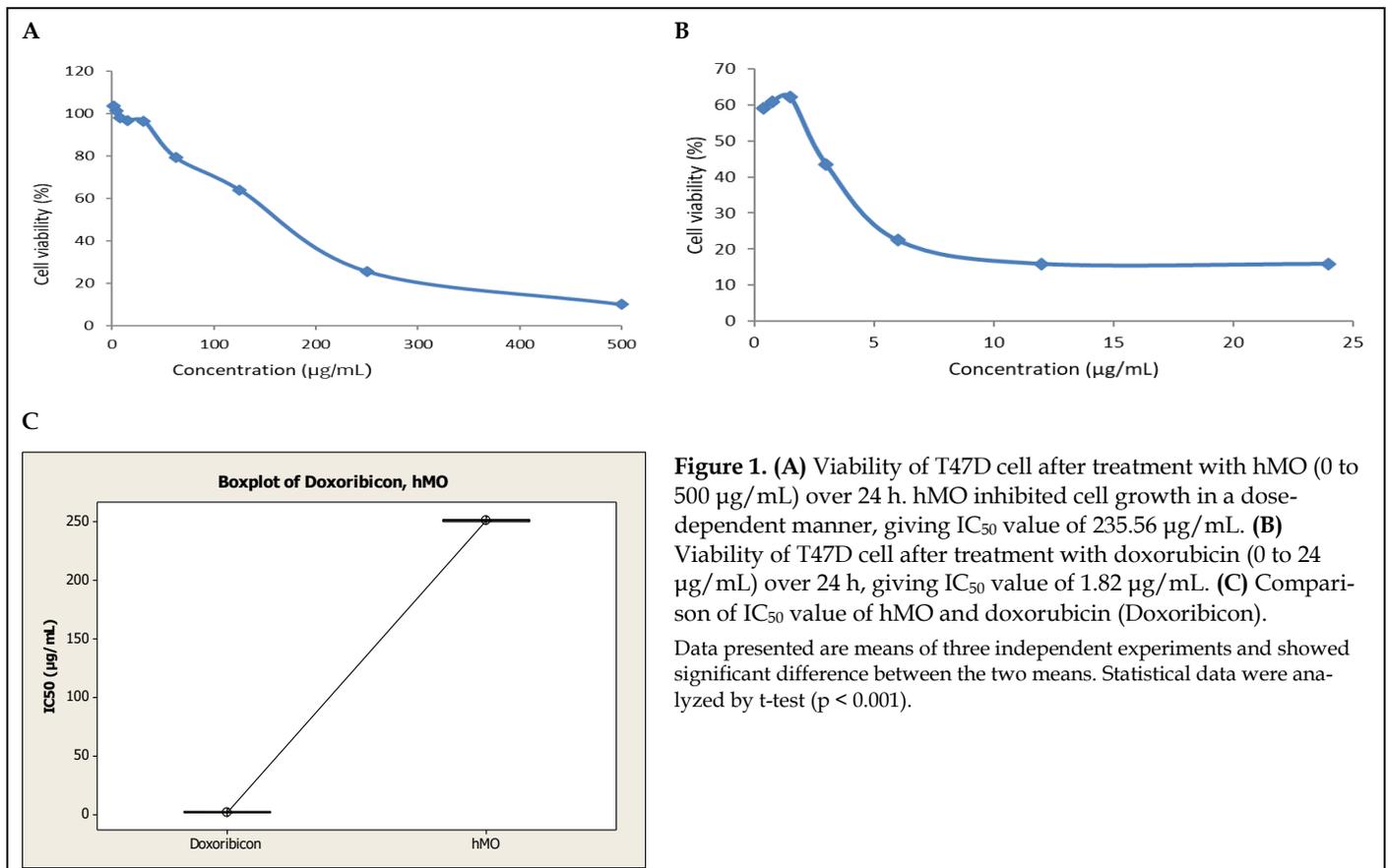

Figure 1. (A) Viability of T47D cell after treatment with hMO (0 to 500 µg/mL) over 24 h. hMO inhibited cell growth in a dose-dependent manner, giving IC_{50} value of 235.56 µg/mL. **(B)** Viability of T47D cell after treatment with doxorubicin (0 to 24 µg/mL) over 24 h, giving IC_{50} value of 1.82 µg/mL. **(C)** Comparison of IC_{50} value of hMO and doxorubicin (Doxoribicon).

Data presented are means of three independent experiments and showed significant difference between the two means. Statistical data were analyzed by t-test ($p < 0.001$).

Treatment of T47D cells with ½ IC_{50} of doxorubicin (0.9 µg/mL) induce apoptosis in 67.88% of cells. Doxorubicin is a common chemotherapy and potent apoptosis inductor. Utomo et al. (2018) showed that 201 nM doxorubicin induced 50% cells to performed apoptosis. Even though, the percentage of necrotic cells caused by hMO treatment were high (more than 50% in all variant concentration). This result needs to be confirmed because necrosis is not favor for cancer treatment due to it involved the sudden release of pro-inflammatory mediator.

To confirm the hMO induced apoptosis on T47D cell, the study of the effect of hMO on the expression of antiapoptotic protein, Bcl-2 was also performed by using immunocytochemistry method. Interestingly, the level of Bcl-2 in hMO treated cell was lower compare to control cells, indicated by more blue color cells that were seen under light microscope 100x (Fig. 3). This data suggest that might be hMO induced apoptosis mediated cell death through the intrinsic pathway.

T47D cell is one of breast cancer cells with the character of p53 mutation implicating in reducing or even loss of p53 ability to regulate cell cycle and apoptosis (Schafer, et al., 2000). Possible strategy to overcome p53 mutated breast cancer cells was by the using of apoptosis inductor. One of central function of p53 is repressing the expression of anti-apoptotic protein Bcl-2. Therefore, mutant p53 in T47D cell might be result in un-inhibited expression of Bcl-2. Immunocytochemistry result show that treatment of T47D cell with hMO decreased the expression of Bcl-2, so it is possible that hMO induce apoptosis via a p53-independent pathway.

The apoptosis is a programmed-cell death that has become the desired route for morphological and biochemical changes such as membrane blebbing, cellular shrinkage, chromatin condensation and activation of caspase and protease. Bcl-2 is one of anti-apoptotic protein that located on the outer membrane of mitochondria due to its role as anti-apoptosis protein by increasing the time-to-death and cell-to-cell variability (Skommer et al., 2010). Overexpression of Bcl-2 proteins, prevented the release of cytochrome c from mitochondria,

and was responsible for the survival of many types of cancer cells such as breast and prostate cancer (Fernández et al., 2002). NF-κB from downstream PI3K/Akt pathway is an important transcription factor for the transcription of anti-apoptotic proteins such as Bcl-2, IAP and Bcl-xL (Simstein et al., 2003). The decreased of Bcl-2 expression in this study might be due to the inhibition of the PI3K/Akt/NF-κB pathway. Berkovich et al. (2013) reported that *M. oleifera* leaf extract inhibit NF-κB transcription factors in Panc-1 cells. Apoptosis occurs probably involves an increase in proapoptotic protein expression such as Bax and Bak. The ratio between Bcl-2 and Bax regulated the induction of apoptosis by dimerizing with each other, thereby triggering the release of cytochrome c from the mitochondria (Ricci and Zong, 2006). Therefore, further research is also needed to determine the effect of giving the hMO to the expression of proapoptotic proteins.

hMO induce cell cycle arrest

The effects of hMO on cell cycle progression using flow-cytometry PI-based staining showed an increase in the cell's population in G0/G1 and G2/M phase. Fig. 4 shows the population of T47D cells treated with 150, 200, 250 and 300 µg/mL of hMO for 24 h incubation. T47D cells treated with 150 and 200 µg/mL of hMO were accumulated in G0/G1 phase, that is from 47.64% in normal cells to 63.86% and 57.05%, respectively, in treated cells. While T47D cells treated with 250 and 300 ug/mL were accumulated in G2/M phase, that is from 32.12% in normal cell to 45.89% and 45.34% respectively. Treatment with doxorubicin causes the cells to accumulate in G0/G1 phase. The accumulation of T47D cells in G0/G1 phase was due to doxorubicin can intercalates with DNA, therefore it directly affects transcription and replication. Doxorubicin can form complex tripartite with topoisomerase II and DNA. Topoisomerase has very important function in DNA replication and repair. Tripartite complex will inhibit DNA strand connection, it causes inhibition of the cell cycle stopped in G0 and G1 phase and also accelerate the apoptosis (Gewirtz, 1999; Minotti et al., 2004).
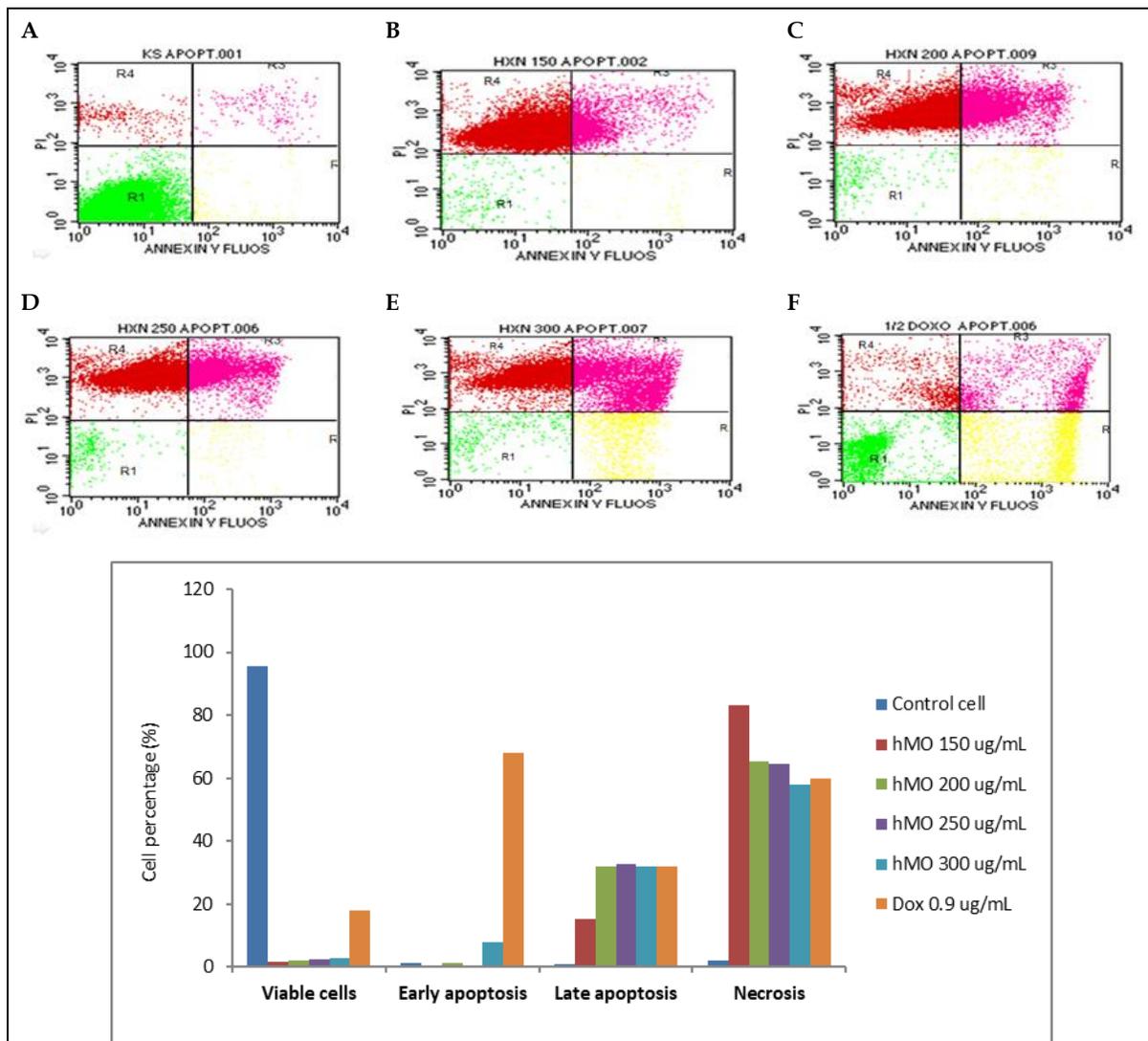
Figure 2. Detection of apoptosis using annexin V-FITC and PI dual staining of **(A)** control cell; **(B-E)** hMO treated cells; and **(F)** doxorubicin (Dox) treated cells.

The cells were seeded at 5×10^5 cells/well on six wells tissue culture plate, then treated with 150, 200, 250 and 300 µg/mL of hMO, respectively. The flow-cytometric profiles of cells: **(A)** control cell; **(B)** 150 µg/mL; **(C)** 200 µg/mL; **(D)** 250 µg/mL; **(E)** 300 µg/mL; and **(F)** Dox 0.9 µg/mL. Quadrans were designed as follow, **R1**: viable cells; **R2**: early apoptotic cells; **R3**: late apoptotic cells; **R4**: necrotic cells. Apoptosis percentage was calculated by flowing software (version 2.5.1).
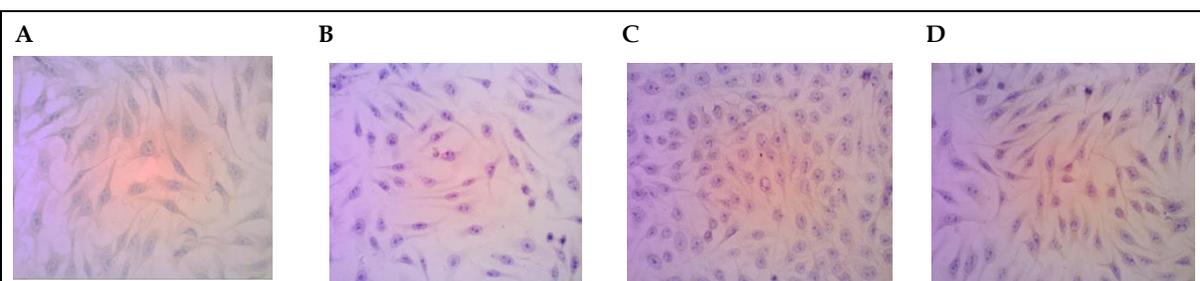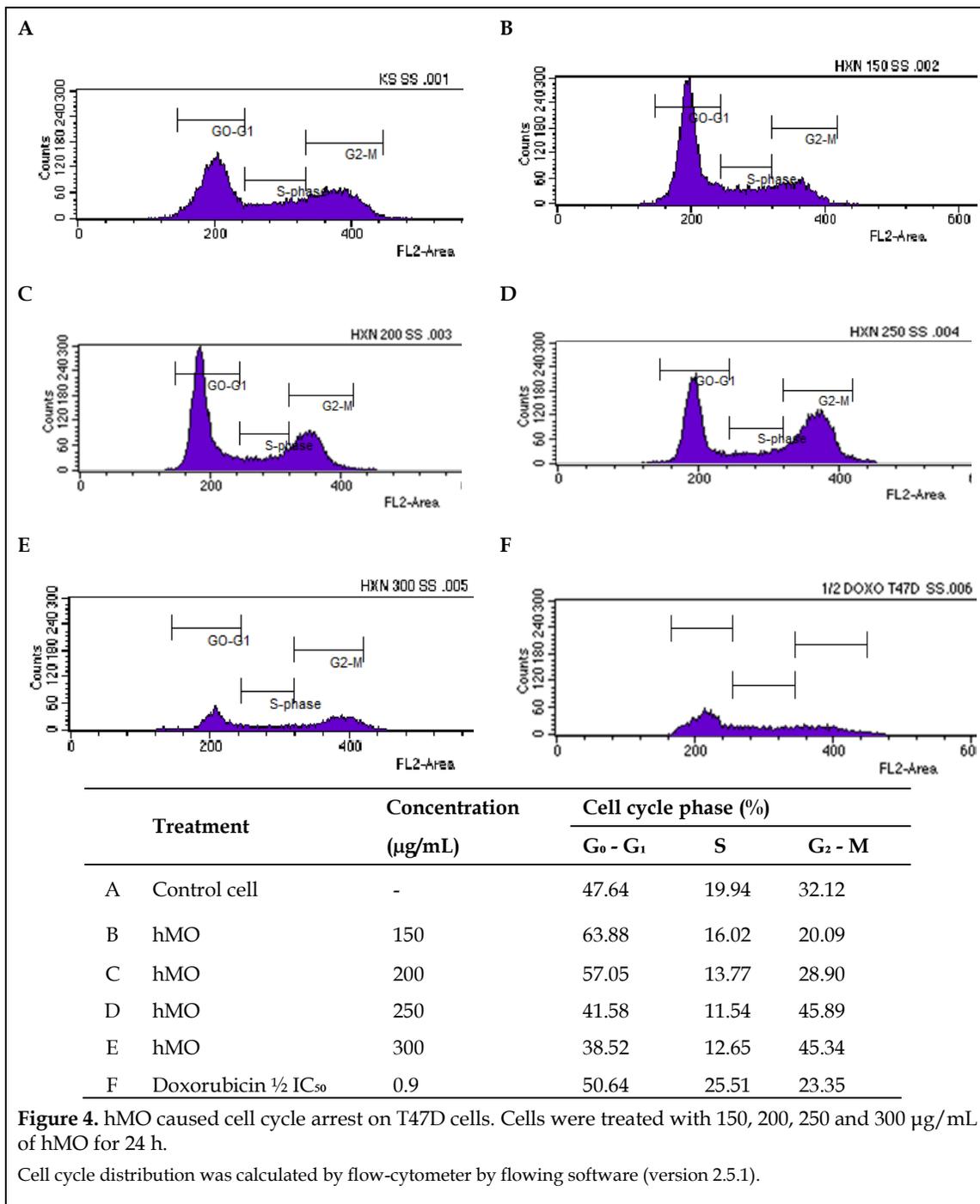

Figure 3. Determination of Bcl-2 expression on hMO treated T47D cells using immunocytochemistry method.
(A) control cells; **(B)** hMO 150 µg/mL; **(C)** hMO 200 µg/mL; **(D)** hMO 250 µg/mL.

Figure 4. hMO caused cell cycle arrest on T47D cells. Cells were treated with 150, 200, 250 and 300 μg/mL of hMO for 24 h.

Cell cycle distribution was calculated by flow-cytometer by flowing software (version 2.5.1).

	Treatment	Concentration (μg/mL)	Cell cycle phase (%)		
			$G_0 - G_1$	S	$G_2 - M$
A	Control cell	-	47.64	19.94	32.12
B	hMO	150	63.88	16.02	20.09
C	hMO	200	57.05	13.77	28.90
D	hMO	250	41.58	11.54	45.89
E	hMO	300	38.52	12.65	45.34
F	Doxorubicin ½ IC$_{50}$	0.9	50.64	25.51	23.35

To confirm the action of hMO in cell cycle arrest, the expression of cyclin D1 was also investigated using an immunocytochemistry method. As shown in Fig. 5, immunocytochemistry evaluation indicated that cyclin D1 level decreased significantly on T47D cells treated with 150 and 200 ug/mL hMO. While on T47D cells treated with 250 the level of cyclin D1 was increase.

Cyclin D1 is synthesized in a response to growth factor stimulation through Ras/Raf/ERK signaling pathway. Cyclin D1 will associated with

CDK 4 and CDK 6 that will be able to enter the restriction point on the cell cycle. If there is no growth factor stimulation, Cyclin D1 level will decrease and could not associated with CDK 4 and CDK 6. On the T47D cells treated with 150 and 200 μg/mL hMO, the cyclin D level was decreased, cells cannot pass the restriction point and will accumulate in the G0 phase. Whereas, treated the T47D cells with 250 and 300 μg/mL hMO induced cell cycle arrest on G2/M phase, however there was no decrease in cyclin D1 expression. Our result suggests that hMO has apoptotic effect to T47D cells. Even though the necrosis effect of hMO to T47D cells need to be explore. The mechanism of action of cytotoxic drug does not solely include features.

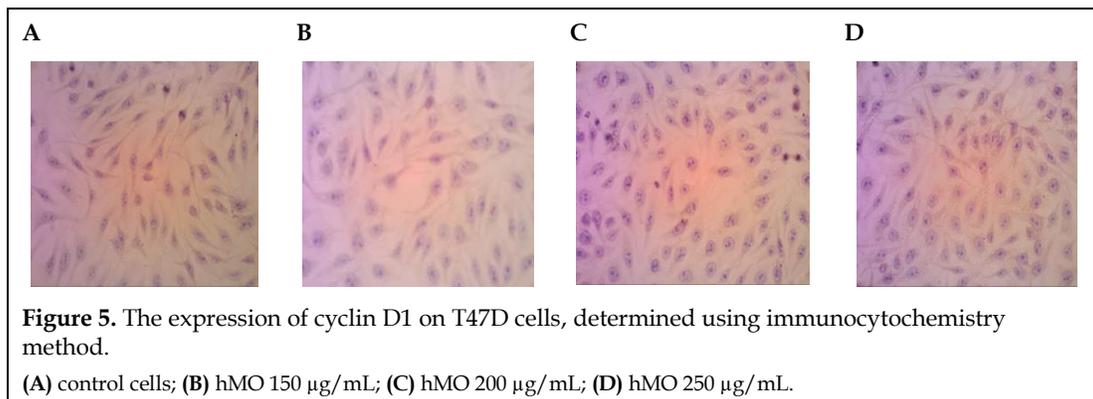However, this research didn't analyze the effect of hMO to normal cell as a control, there are so many research show that *M. oleifera* extract showed greater cytotoxicity for tumor cells than for normal cells strongly suggesting that it could potentially be an ideal anticancer therapeutic candidate specific to cancer cells (Jung et al., 2014). Study of toxicology effect of *M. oleifera* also indicated that the LD_{50} was estimated to be 1585 mg/kg therefore leaf extract of *M. oleifera* is relatively safe (Awodele et al., 2012).

CONCLUSIONS

hMO has properties to induce apoptosis and cell cycle arrest on T47D cells. Treatment of T47D cells with hMO, decrease expression of Bcl-2 and cyclin D. hMO has a potential compound to be explored and developed as a chemo preventive agent for breast cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Figure 5. The expression of cyclin D1 on T47D cells, determined using immunocytochemistry method.
(A) control cells; **(B)** hMO 150 μg/mL; **(C)** hMO 200 μg/mL; **(D)** hMO 250 μg/mL.

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Contribution	Gaffar S	Apriani R	Herlina T
Concepts or ideas	x		x
Design	x		x
Definition of intellectual content	x	x	x
Literature search	x	x	x
Experimental studies	x	x	
Data acquisition	x	x	x
Data analysis	x	x	
Statistical analysis	x		
Manuscript preparation	x	x	
Manuscript editing	x		x
Manuscript review	x	x	x