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Cytotoxic Activity and Phase G_2-M Cell Cycle Inhibition Active Fraction of *Mimusops elengi* Stem bark on T47D Cells

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Abstract: Some research suggest *Mimusops elengi* stem bark has anticancer activity, but there is no data showing anticancer activity of this plant against breast cancer. Fractionation is one method that allows the extraction of soluble compounds based on the polarity, so it may be a reference to determine the fraction that have anticancer activity, especially breast cancer. Breast cancer cells used in this study is T47D cells. T47D cells are easy to handle and still sensitive on anticancer agents and is suitable for preliminary testing of breast anticancer. Fractions obtained from the fractionation of the *Mimusops elengi* stem bark ethanolic extract resulting fractions *n*-hexane, ethyl acetate fraction and aqueous fraction (residu). To calculate the IC_{50} values of each fraction obtained by the cytotoxic test on T47D cells using MTT [3 - (4,5 - dimetiltiazol - 2 -il) -2,5 difeniltetrazolium bromide] method. The most active fractions then proceed to test the mechanism of the cell cycle inhibition with flowcytometry method. Phytochemical screening showed steroids/triterpenoids in *n*-hexane fraction; flavonoids, tannins, glycosides in ethyl acetate fraction; flavonoid, saponins, and glycosides in aqueous fraction. Cytotoxic assay results IC_{50} values of *n*- hexane, ethyl acetate, and aqueous fraction to T47D cells respectively 5796.218 µg/ml; 398.236 µg/ml, and 145.068 µg/ml, and the aqueous fractionis the most active fraction. Aqueous fraction inhibits the cell cycle at the G2-M phase.
Keywords : fractions , T47D , cytotoxic , MTT , flowcytometry.

Introduction

Breast cancer is a type of cancer that often affects women. Breast cancer cases in the United States recorded nearly 200.000 women diagnosed each year and there are more than 40.000 died because this disease[1.] Recent data from the American Cancer Society has calculated that in 2013, there were 64.640 cases of breast cancer[2]. Approximately 39.620 women die each year because this disease. Pathology Based Cancer Registry data working with the Indonesian Cancer Foundation, show breast cancer in Indonesia was on second ranked of all types of cancer[3].

Cancer treatment can be done through surgery, radiation or chemotherapy. The ideal anticancer drugs that have selective toxicity, meaning can eliminate the cancer cells without damaging normal cells. Interest to use

traditional medicine for cancer, especially these days tended to increase. This is caused fears of side effects by conventional drugs and other reason, traditional medicine easily available and cheap[4].

Mimusops elengi is a kind of originated tree from India, Sri Lanka and Burma. It including in the family Sapotaceae [5], has been traditionally used by people in several countries of Asia and Indonesia as antibacterial drugs, antiviral, analgesic, antihiperlipidemia, and anticancer[6].

Based of explanation, the researcher is interested in performing fractionation of the *Mimusops elengi* stem bark ethanolic extract aimed at separating active compounds are efficacious as anticancer properties based on polarity so that the data obtained fractions that have the most active activity against T47D breast cancer cells by cytotoxic testing and inhibition of cell cycle flowcytometry.

One type of breast cancer cell line is T47D cells. These cells are continuous cell line isolated from the tumor tissue of breast ductal 54-year-old woman. These cells are used in cancer research in vitro because easy to handling, has unlimited replication capability, high homogeneity and easily to replaced with frozen stock in the event of contamination[7].

Materials and Methods

Materials

Mimusops elengi stem bark obtained from Johor region, Medan, North Sumatra, Indonesia. T47D breast cancer cells that is a collection of the Laboratory of Parasitology of the Faculty of Medicine Gadjah Mada University, Yogyakarta, Indonesia. Fetal Bovine Serum (FBS) 10% (v/v) (FBS qualified, Gibco, Invitrogen™ USA) and penisillin-streptomisin 1% (v/v) (Gibco, Invitrogen Corporation, Grand Island, NY, 14072, USA). MTT [3-(4,5-dimetiltiazol-2-yl)-2,5 difenil tetrazolium bromide] obtained from (Sigma Aldrich), and propidium iodide (PI) (Sigma Aldrich Chemie GmBH, Steinheim, Germany. All other ingredients, reagents and solvents were of analytical grade.

Methods

Preparation the ethanolic extract

Preparation the extracts was done by percolation using 96% ethanol. 400 g Powdered botanicals are put into the percolator, add 96% ethanol until botanicals are submerged. Percolator covered with aluminum foil and left for 24 hours. Then opened and allowed to drip liquid extract at 20 drops per minute and 96% ethanol added repeatedly to taste and set the hatching fluid velocity equal to the speed drops the percolat, so that there is always a layer of liquid on top penyari botanicals.

Percolation is stopped when percolat the last exit evaporated, no leaving residue. Percolat then distilled and evaporated at reduced pressure at a temperature of not more than 50°C using a rotary evaporator to obtain a viscous extract.

Preperation of fractions

A total of 20 g of ethanolic extract was dissolved in 96% ethanol until dissolved then add 40 ml of distilled water, put into a separating funnel, then added 100 ml of *n*-hexane and allowed to stand until there are two separate layer. *N*-hexane layer (upper layer) was taken with a streamed manner, and fractionation performed until a layer of *n*-hexane gave negative results with Lieberman-Baurchard reagent. Then add 100 ml of ethyl acetate, allowed to stand until there are two separate layers, ethylacetate layer (upper layer) was taken by way of streamed, and fractionation performed until a layer of ethyl acetate gave negative results with $FeCl_3$ reagent, and aqueous fraction (residu) was taken and all fraction obtained as evaporated with a vacum rotary evaporator to obtain a viscous fluid.

Phytochemical screening

Phytochemical screening fraction of *n*-hexane, ethyl acetate, and aqueous is done to obtain information class of secondary metabolites were dissolved in the respective fractions of different polarity. class of secondary

metabolites tested are alcaloids, flavonoids, saponins, glycosides, tannins, anthraquinone glycosides, and steroids / triterpenoids.

Preparation of stock solutions of test material

The test material is weighed 5 mg, followed by retrieval of DMSO to 5 ml (stock solution concentration of 1 mg/ml) and stored as stock solutions for subsequent use in research. Cytotoxic concentration of extract to a test carried out by using the dilution medium.

Cytotoxic assay

T47D cells were cultured in RPMI medium (Roswell Park Memorial Institute) containing Fetal Bovine Serum 10% (v/v) and penisillin-streptomisin 1% (v/v). Cells (1×10^4 cells/well) were transferred to 96-well plate (Iwaki, Japan) and incubated for 24 h (70–80% confluent). Cells were treated by test material with concentration 250; 125; 62.5; 31.25; and 15.625 µg/ml, and incubated for 24 h. At the end of the incubation, 5 mg/ml solution of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] were added to each wells and the cells were incubated for 4 h in 37°C. Viable cells reacts with MTT to form purple formazan crystal. After 4 h, stopper sodium dodesil sulphate 10% in 0,1N sulphuric acid solution were added to dissolve formazan crystal. Cells were incubated over night and protected from light. Cells were shaken for 10 min before read by ELISA reader at λ 595 nm.

Absorbance of each well converted to percentage of viable cells:

$$\% \text{ viable cells} = \frac{\text{treated cells abs} - \text{medium control abs}}{\text{Celsl control abs} - \text{medium control abs}}$$

Flowcytometry assay

Cells (1×10^6 cells/well) were transferred into 6-well plate (Iwaki, Japan) and incubated until the cells return to normal condition. Cells were treated by sample test (Active fraction) and incubated for 24 h. At the end of the incubation, the media containing free cells suspension were taken and transferred into 1.5 ml eppendorfs. The eppendorfs were centrifugated (2.000 rpm, 3 min) and the supernatant were removed. The cells in 6-well plate were added by phospate buffered saline (PBS), and the PBS were transferred into previous eppendorfs. The eppendorfs were centrifugated and the supernatant wer removed again. This steps were repeated before the cells harvested by trypsin-EDTA 0.25%. Harvested cells were transferred into the other eppendorfs and centrifugated (2.000 rpm, 30 s). The remaining cells in the 6-well plate were rinsed with PBS and transferred into the eppendorfs. The eppendorfs were centrifugated and the supernatant were removed. Pellet cells in eppendorfs washed by cold PBS and added by propidium iodide (PI). The eppendorfs were wrapped in aluminum foil and incubated in 37°C for10 min. After 10 min, cell suspension were homogenated and transferred into the flowcyto-tube to be analyzed by flowcytometer FACS using ModFit LT 3.0 program.

Results and Discussion

Phytochemical screening results

The results of the screening are then used as a reference for identifying which class of compounds that have the most active activity against T47D breast cancer cells. Results of phytochemical screening fraction of *n*-hexane, ethyl acetate, and aqueous (Table 1).

Cytotoxicity of fractions

Cytotoxic test is a preliminary parameter to determine the potential toxicity of a test substance is mainly expressed by cancer cells IC_{50} parameters. Based on Table 2, the cytotoxic activity on T47D cells showed the IC_{50} values of *n*-hexane fraction 5796.218 µg/ml, ethyl acetate fraction 398.236 µg/ml, and aqueous fractions 145.068 µg/ml. treatment with test samples of aqueous fraction showed the smallest IC_{50} values, while the *n*-hexane fraction showed the largest IC_{50} values. Therefore, the aqueous fraction can be said to have the most

active anticancer activity with IC_{50} values of the smallest 50 % have been able to inhibit the growth of T47D cells. Based on the value of the aqueous fractionsaid to be active as anticancer as an extract is considered active if $IC_{50} > 500$ µg/ml[8]. Therefore the aqueous fractionused as a test sample for testing advanced anticancer.

Ability aqueous fraction inhibits growth of cancer cells is suspected due to the content of chemical compounds in them. If referring to the phytochemical screening showed that aqueous fraction containing saponins and flavonoid. Flavonoid compounds inhibit cell proliferation in various human cancer cells through the inhibition of oxidative processes that can lead to cancer initiation. This mechanism is mediated decrease xanthin oxidase enzyme, Cyclooxygenase (COX) and Lipooxygenase (LOX) required in the process prooxidation thereby delaying cell cycle. Flavonoids also inhibit the expression of topoisomerase I and II enzymes that play a role in catalyzing DNA screening. Topoisomerase enzyme inhibitor complex will stabilize DNA topoisomerase and cause cuts and damage [9].

Saponins can recognize cancer cells, because cancer cells have cell membranes and structures are different from normal cells. Cancer cell membranes contain more compounds such as cholesterol. Saponins can bind cholesterol contained in the membrane of cancer cells, thereby disrupting membrane permeability[10]. Saponins also reduce the occurrence of reactive oxygen species such as H_2O_2 and inhibit signaling pathways phosphatidyl-inositol-3 kinase which may be the reason for the prevention of damage chromosome[11].

Table 1. **Phytochemical screening results of *Mimusops elengi* stem bark**

Secondary metabolites	***n*- hexane**	**ethyl asetate**	**aqueous**
Alkaloid	-	-	-
Flavonoid	-	+	+
Glikosida	-	+	+
Saponin	-	-	+
Antrakuinon glikosida	-	-	-
Tanin	-	+	-
Triterpenoid/ Steroid	+	-	-

Table 2 IC_{50} values from each fraction of *Mimusops elengi* stem bark

Fractions	**IC_{50} (µg/ml)**
n- hexane	5796.218
ethyl asetate	398.236
aqueous	145.068

Induction of T47D cell cycle arrest by active fraction of *Mimusops elengi* stem bark

Inhibition of the cell cycle is one of the main targets of anticancer agents. In this observational study was conducted using cell cycle flowcytometry. T47D cell cycle profile and distribution of T47D cells after treatment is shown in Figure 2 and 3, the control group showed accumulation of T47D cells in G_0-G_1, S and G_2-M phase respectively 47.49%, 27.78%, and 20.72% (Table 3). After giving the aqueous fraction with IC_{50} concentrations increased accumulation of cells in G_2-M phase becomes 58.91%, while in the G_0-G_1 phase, and S decrease 18.98% and 17.59% (Table 3). The same is also indicated in the treatment of the aqueous fraction ½ IC_{50} concentration, where the largest cell accumulation also occurs in G_2-M phase is equal to 41.80%. So, it can be said that the mechanism of inhibition of the cell cycle is the aqueous fractionin the G_2-M phase. Checkpoint at G_2-M phase is a potential target of cancer therapy. When DNA is damaged the cell enters mitosis. Checkpoint on the G_2 will control the entry of cells from G_2 to M phase Complex binding between Cdk1 (cdc2) (Cyclin-dependent kinase) with Cyclin B1 is required for the development of G_2 to M phase B1-cdc2 complex binding involving the inhibition of phosphate on a pair of amino acids activated by Wee1. Dephosphorylation by cdc25C enzyme (cell division cycle 25 homolog C) will increase the phosphatase activity of cdc2-B1 binding. When DNA damage, Chk1 (checkpoint kinase-1) inactivates cdc25C lead cdc2-B1 binding becomes inactive so that the cells fail to enter into the phase of mitosis that undergo apoptosis [12]. Based on observations of the aqueous

fraction mechanisms are thought to inhibit Cyclin B1, cdc2, and cdc25C protein that is important in relation to the G_2-M phase. This is likely mediated by the flavonoid content of chemical compounds in the aqueous fractions. In studies looking at the expression of proteins that regulate the G_2-M phase transition by Western blot method, flavonoids showed decreased levels of the protein Cyclin B1, cdc2, and cdc25c dose dependent[13].

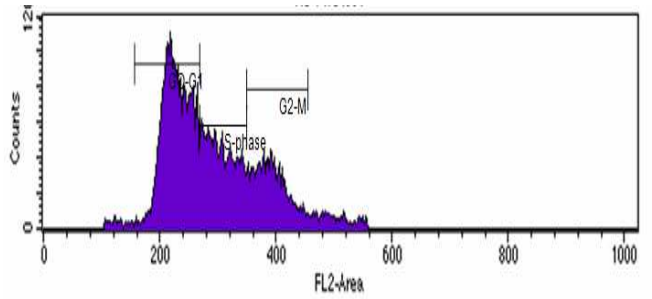

Figure 1. cell cycle profile on T47D cells control

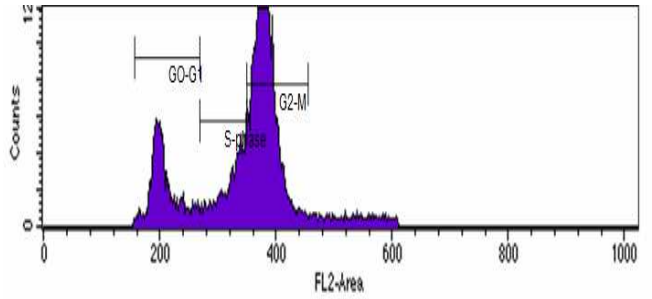

Figure 2. cell cycle profile on T47D after treatment with concentration IC_{50} (145 µg/ml) of aqueous fraction

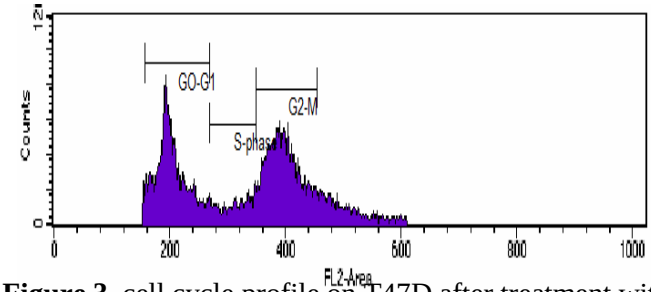

Figure 3. cell cycle profile on T47D after treatment with concentration ½ IC_{50} (72,5 µg/ml) of aqueous fraction

Table 3 Cell accumulate on each phase

Treatment	**concentration**	**Cell cycle phase (%)**		
		G_0-G_1	**S**	**G_2-M**
Control	-	47,49	27,78	20,72
Aqueous fraction	1 IC_{50}	18,98	17,59	58,91
Aqueous fraction	½ IC_{50}	36,68	10,10	41,80

Conclusion

Aqueous fraction of *Mimusops elengi* stem bark showed the most active cytotoxic activity fraction than *n*-hexane and ethyl acetate. Aqueous fraction also could inhibit T47D cell cycle at G2-M phase.

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