THE BREAST OF ANTICANCER FROM LEAF EXTRACT OF ANNONA MURICATA AGAINTS CELL LINE IN T47D

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Abstract

The breast cancer is the second leading cause of death in women after cervical cancer. The soursop (Annona muricata) is a traditional medicinal plant which is empirically by the people of Indonesia are used for anti-inflammatory and anti-tumor. This study aims to determine the cytotoxic effects from extracts of leaves of soursop and fraction results in cancer cells T47D. The research was carried out by extraction using ethanol and fractionation by column chromatography method that used various solvents were n-hexane, chloroform, ethyl acetate and methanol. Cytotoxic test performed by the method of MTT assay and apoptosis tests performed by the method of Double Stainning. The parameters obtained from the cytotoxic test was IC_{50} values, ie values that produce inhibitory concentrations of cancer cells by 50%. Apoptosis assay results are analyzed in a qualitative description. The results showed that the ethanol extracts of leaves of the soursop has a cytotoxic activity with IC_{50} values of 17.149 µg / mL. The results of the four fractions obtained by fractionation and the fraction F3 were the fraction that has the best cytotoxic activity with IC_{50} values of 30.112 µg / mL. Apoptosis assay results showed that the fraction F3 were able to induce apoptosis of cells.

Key words: breast cancer, T47D cell line, soursop (*Annona muricata*), cytotoxic, apoptosis.

INTRODUCTION

The breast cancer is the cancer with the highest incidence in Indonesia in the year of 2005, that is amounting to 39.23% of all cancer patients (MOH, 2007). The number of patients and the number of deaths were caused by cancer that is continued to increase, must be accompanied by curative efforts. Cancer treatment is medically still caused by problems because of its side effects are great.

The plant that is empirically trusted by societies to have anticancer properties are the leaves of the soursop (*Annona muricata Linn.*). Based on chemotaxonomy approach, some plant family Annonaceae that have been studied have anticancer activity. The results of the plant family Annonaceae have been carried out. *A. Montana* contains monotetrahydrofuranic acetogenins which have toxicity to liver cancer in Hep G2 cells (Liaw, et.al, 2005). The seeds of *A. crassiflora* have high antioxidant activity (Roesler, 2007). *A. squamosa* containing ribosome-inactivating protein (RIP), an immunotoxin for the treatment of cancer (Sismindari 1998). Based on studies chemotaxonomy plants that have close kinship likely contain similar compounds (Princess, 2008) so that data from previous studies had showed that the plant *Annona muricata* (soursop) is a potent anticancer Annonaceae family.

MATERIALS AND METHODS

a. Collection of plant material

The plant was collected from the side of Purwokerto, Indonesia. The collection was made in July. The plant was identified in the Laboratorium of Taxonomy, Department of Biology, Jenderal Soedirman University, Indonesia.

b. Preparation of simplicia

The material taken is soursop leaf. The material is washed with running water, then dried with dioven at 60 ± 1ºC. Simplicia soursop leaf then powdered using by grinder.

c. Extraction and fractionation of soursop leaf

The powder was extracted using maceration with ethanol for 3 x 24 hours. Once filtered, the filtrate was evaporated to obtain ethanol extract of leaves of soursop. Ethanol extract and then fractionated by column chromatography, respectively, using solvent were n-hexane, chloroform, ethyl acetate and methanol. The results obtained by fractionation of fractions F1, F2, F3, and F4. Each extract tested cytotoxicity against T47D cells.

d. Preparation of stock solutions of test material

The soursop leaf extract 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. Tamoxifen concentrations obtained by dilution with medium. As a control solvent, used 2% DMSO (v/v), ie the highest concentration of DMSO in the test compound.

e. Cytotoxic test with soursop leaf extract on T47D breast cancer cells by MTT assay

i. Preparation of RPMI 1640 medium.
ii. Activation, culturing, and harvesting cells T47D.
iii. Preparation of soursop leaf extract and tamoxifen.
iv. Cytotoxic activity assay using 96 wells culture medium.

Cytotoxic test performed using 96 wells microcultures. The number of wells is divided into 8 lines (A, B, C, D, E, F, G, H). Each row contained 12 wells (no. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12). Microcultures charging scheme can be seen in Figure 1.

Microcultures is filled with the following steps:

Each of the columns mikroplate pitting of the 1-3, 9-11, AE line, column and row G 1-7 filled T47D cell suspension of 1.5 x 104 cells dissolved in culture medium RPMI 1640. Cells is then incubated for 24 hours in 5% CO_2 incubator at 37°C. After incubation, wells with columns 1-3, rows AE and medium, with a concentration of 100; 50; 25; 12.5; 6.25 ug / ml. Columns 9-11 AE line tamoxifen plus 100 mL, with a concentration of 300; 150; 75; 37.5; 18.75 ug / ml. Line G plus 100 mL RPMI medium without treatment (control). H lines 1-7 column filled with 100 mL RPMI (control medium). Microcultures were then stayed on incubation for 24 hours in 5% CO_2 incubator at 37°C. After that, the media removed, any sinks coupled with 100 mL of new media and 10 mL MTT reagent (10µl/100µl per wells), then incubated for 4-6 h in 5% CO_2 incubator at 37 ° C. After that, add 100µl of sodium dodecyl sulfate (SDS) 10% in HCl 0.01%. Then mikroplate rocking at room temperature for 5 minutes. After that, wrap with aluminum foil microcultures, incubation at room temperature overnight. Mikroplate is then read using an ELISA at a wavelength of 595 nm (Lippman, 2004).

f. Apoptosis using the method of double stainning ethidium bromide-acridine orange

Apoptosis tested in ethanol extract. Apoptosis test performed using microcultures 24 wells. The number of wells is divided into four lines (A, B, C, D). Each row contained six wells (no. 1, 2, 3, 4, 5, 6). Microcultures charging scheme can be seen in Figure 2. The Test carried out by preparing mikroplate with 24 wells and cover slip, slip cover inserted into the wells using tweezers. 1000 mL cell suspension pipetted on to the cover slip that had been inserted into the wells in row B No. 2, 3 and 4, then the cells were observed under a microscope to see the cell distribution. Tues incubated in an incubator overnight, taken mikroplate with 24 wells which already contain cells, culture media and then discarded. Cells in the wells were washed with PBS each 500 mL, and then removed from the wells with PBS Pasteur pipette and then gently inserted soursop leaf ethanol extract with IC50 concentrations of ½ as much as 1000 mL in column B2. In the same way, with concentrations of tamoxifen IC_{50}, a half as much as 1000 mL in column B3 and control cells (without treatment) in column B4 then on incubation for 10 hours. Media from wells removed by pasteur pipette mikroplate slowly at the wall. Cells in wells were washed with 500 mL PBS. PBS from the wells is slowly removed. Furthermore, the cover slip placed on the object glass, then dropped into 10 mL reagent mixture of ethidium bromide-acridine orange on the cover slip and then observed under a microscope flouresen.

Cells that showed green berfluoresens living cells, and cells that berfuoresens red indicates dead cells. Red berfluoresens whole cells showed cell necrosis and cells that showed fragmented cells undergoing apoptosis.

RESULTS AND DISCUSSION

A. Determination of Plant

The determination of the soursop leaf plants performed at the Laboratory of Applied Biology Faculty of PKA used a reference book Flora of Java, Volume I (Bakhuizen van De Backer and Brink, 1963). The determination made to avoid mistakes of the plants to be used in the study. The determination results was explained that plants used in the study are *Annona muricata* Linn.

B. Preparation Simplicia

The soursop leaves obtained from Purwokerto Indonesia collected in June 2011. The leaves are washed with running water to remove dirt or dust attached to the leaf. Leaves a clean cut into small pieces to speed up the drying process. Drying process using the oven with a temperature of 60ºC, to ensure that no damage occurs an unstable compound by heating (Gunawan and Mulyani, 2004). Simplicia was dried and then carried out pollination to increase the surface area of particles, so that the extraction process can be more effective and easier solvent in attracting compounds contained in the cell.

C. Preparation of Extracts

Soursop leaf powder of 250 g performed maceration using ethanol solvent during 3x24 hours. The selection method is chosen in addition to being easy, simple and expected to reduce the risk of damage to the content of the compounds so it is a suitable method used in the study. The extract obtained was 37.16 g which is mean the rendemen about 14,86%.

D. Cytotoxic Test Ethanol Extract

Cytotoxicity test is a qualitative and quantitative tests to determine how cell death. The method used to see cytotoxic effects of ethanol extract of leaves of the soursop on T47D breast cancer cells is the MTT assay. The principle of the MTT assay is a spectroscopic method is by determining the absorbance value of formazan. MTT will be absorbed into the cell and entered into the system of cell respiration in mitochondria. The action of the enzyme active mitochondria in cells was metabolize tetrazolium salts, resulting in termination of tetrazolium ring by dehydrogenase enzymes which lead to tetrazolium formazan transformed into water-insoluble but soluble in SDS 10% and the purple coloured. Formazan formed is colored purple will be proportionate to the number of living cells (Pebriana et al., 2008). Cells that die dissolved in water and remain yellow because the mitochondria of cells that die are not respiration tetrazolium ring is disconnected so it can not reduce MTT reagent to formazan and the color is still yellow.

The observations made by microscopic showed that the number of formazan formed in control wells with media more than the formazan formed in the wells treated test compound. This suggests that the treatment of ethanol extract of leaves of the soursop on T47D breast cancer cells can lead to death. Cells that are dead will not be affected by the MTT reagent. Characteristic morphology of living cells is round with a protected cell wall that shines and stuck to the bottom plate, while the dark-colored cells that die and are not attached to the base plate. After addition of MTT and incubated for 4 hours of diving, added SDS in 10% HCl. The reason the use of SDS 10% as it can dissolve the formazan crystals and the results of MTT reaction did not cause precipitation. After settling for a night, then used an ELISA reader to determine absorbance values. 595nm wavelength is used because it is the maximum wavelength in order to obtain a sensitive and specific measurements. Absorbance value of each test compound can be seen in Table 1.

The results of Table 1 showed that the higher of the concentration of test compound, is the lower absorbance values. This may imply that the test compound has a potency in inhibiting or killing the T47D cells. The stronger intensity of the color purple is obtained the greater the absorbance. The graph can be seen in Figure 3. From the graph above showed that the percentage inhibition of T47D cell growth is increased with increasing concentrations of test compound. Absorbance data obtained, is used to calculate IC_{50} values. IC_{50} value indicates the value of concentration that can inhibit proliferation of T47D cancer cells by 50%. IC_{50} value of ethanol extract is 17.149 µg/mL which is indicates that the concentration 17.149 µg/mL, ethanol extract inhibit proliferation of T47D cancer cells by 50%.

Figure 4 shows the IC_{50} value of each test compound, shows that the ethanol extract and tamoxifen has cytotoxic effects due to both the test compound IC_{50} value was 17.149 µg/mL and 13.38 µg/mL. An extract is said to have cytotoxic activity if the IC_{50} value of less than 1000 µg/mL after 24 hours contact time (Meiyanto et al., 2008). The smaller the IC_{50} value of a test compound the more toxic compound.

E. Apoptosis Test

Apoptosis is programmed cell death mechanism that is important in multicellular organisms to maintain equilibrium. Tests performed to determine the mechanism of apoptotic cell death is through the mechanism of apoptosis. In T47D cells that were given the test compound indicates that berflouresens orange cells, whereas cells that are not given the test compound indicates green berfloures cells as shown in figure 5. The apoptosis assay results by the method of double stainning above show that cancer cells are treated T47D ethanol extract of leaves of soursop (A) and tamoxifen (B) some berflouresens orange. This indicates that the test compound can induce apoptosis. In control cells berflouresens still look bright green oval which means the cells do not undergo apoptosis because the cells live only absorb acridine orange.

F. Separation of Ethanol Extracts of Active Fraction by Column Chromatography

Fractionation of the compounds contained in the ethanol extract was conducted by column chromatography. In this method previously conducted to determine the orientation of the stationary phase to be used. After doing orientation, the best of the stationary phase is obtained by silica gel GF 254 0.2-0.5 mm. The mobile phase used in the coloum chromatography ethanol extract was successively hexane, chloroform, ethyl acetate and methanol, each of which produces fractions of hexane, chloroform, ethyl acetate and methanol fractions. Each fraction was then performed tests such as the cytotoxic ethanol extract.

G. Soursop Leaf Fraction Cytotoxic Test

Performed the same test method to test cytotoxic cytotoxic ethanol extract of leaves on soursop. Absorbance value of each fraction can be seen in Table 3. The results in Table 3 showed that the higher the concentration of test compound have the lower absorbance values. The four factions have differences IC_{50} value. The percentage inhibition of T47D cell growth increased with increasing concentrations of test compound as shown in the graph 6. Absorbance data obtained, is used to calculate IC_{50} values. The smaller value of IC_{50} have the greater potential cytotoxic against to T47D cell lines. The fractions that have a better cytotoxic potency, respectively consecutive are hexane fraction, chloroform, methanol and etyl acetate fraction with IC_{50} are 143.077; 120.718; 44.987 and 31.268 µg/mL. Etyl acetate fraction has the best potency of cytotoxic among other fractions against to T47D cell lines. Figure 7 shows the IC_{50} values of each fraction. Ethyl acetate fraction has the smallest IC_{50} value, which means having the greatest cytotoxic effect than the three other fractions.

CONCLUSIONS

Ethanol extract of leaves of soursop (Annona muricata) has a cytotoxic activity in T47D breast cancer cell lines with IC_{50} of 17.149 µg/mL and can induce apoptosis. Etyl acetate fraction has the best potency of cytotoxic among other fractions against to T47D breast cancer cell lines with value of IC50 was 31.268 µg/mL.

Acknowledgements

The authors acknowledge the financial support provided by University of Jenderal Soedirman, Indonesia for Institutional Research (No. 1947/H.23.9/PN/2011).

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	1	2	3	4	5	6	7	8	9	10	11	12
A	**EK**	**EK**	**EK**						**T**	**T**	**T**	
B	**EK**	**EK**	**EK**						**T**	**T**	**T**	
C	**EK**	**EK**	**EK**						**T**	**T**	**T**	
D	**EK**	**EK**	**EK**						**T**	**T**	**T**	
E	**EK**	**EK**	**EK**						**T**	**T**	**T**	
F												
G	**K**	**K**	**K**	**K**	**K**	**K**	**K**					
H	**M**	**M**	**M**	**M**	**M**	**M**	**M**					

Figure 1. Microcultures charging scheme for the cytotoxic test

Descriptions:

EK = ethanol extract, T = Tamoxifen, K = control cells (cancer cells and medium),

M = medium

	1	2	3	4	5	6
A						
B		S	T	K		
C						
D						

Figure 2. Charging scheme to test apoptosis microcultures
Descriptions:
S: Ethanol extract of leaves of soursop (*Annona muricata*)
Q: Tamoxifen
C: Control cells (T47D cells and media)

Table 1. The mean absorbance, percentage inhibition of T47D cells and IC_{50} values from ethanol extract of leaves of *A.muricata*

Test Materials	Concentration (µg/mL)	Mean absorbance	Living Cells (%)	Retardation (%)	IC_{50} (µg/mL)
Ethanol extract *A.muricata*	500 250 125 62.5 31.25	0.161 0.129 0.141 0.138 0.457	7.71 2.34 4.36 3.80 57.28	92.29 97.66 95.64 96.20 42.72	17.149
Tamoxifen	50 25 12.5 6.25 3.125	0.253 0.262 0.889 1.070 0.167	0 0.19 71.62 92.29 91.95	100 99.81 28.38 7.71 8.05	13.38

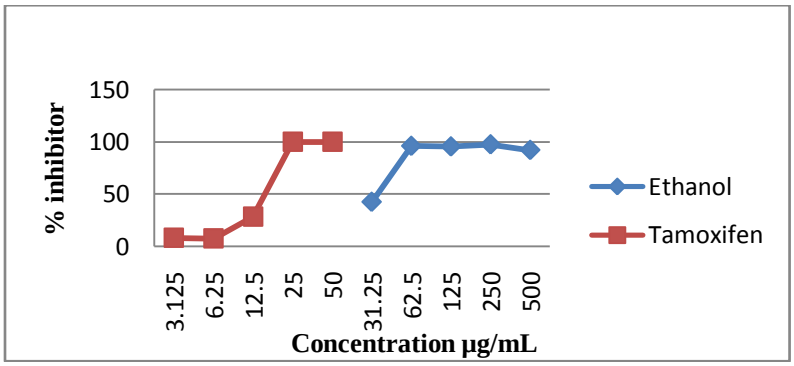

Figure 3. Graph showing the relationship between the percentage inhibition of T47D cells with various concentrations of test compound

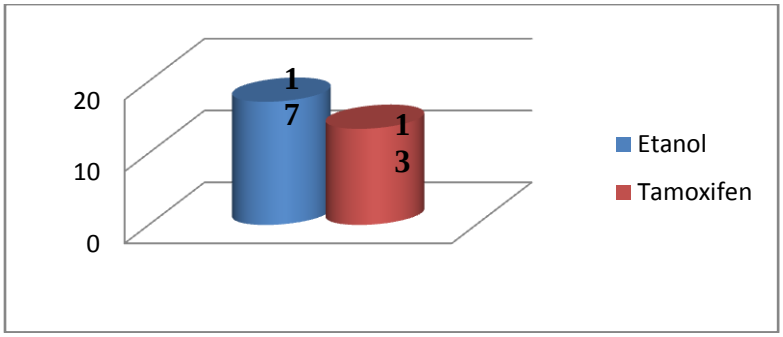

Figure 4. IC50 values of each test compound

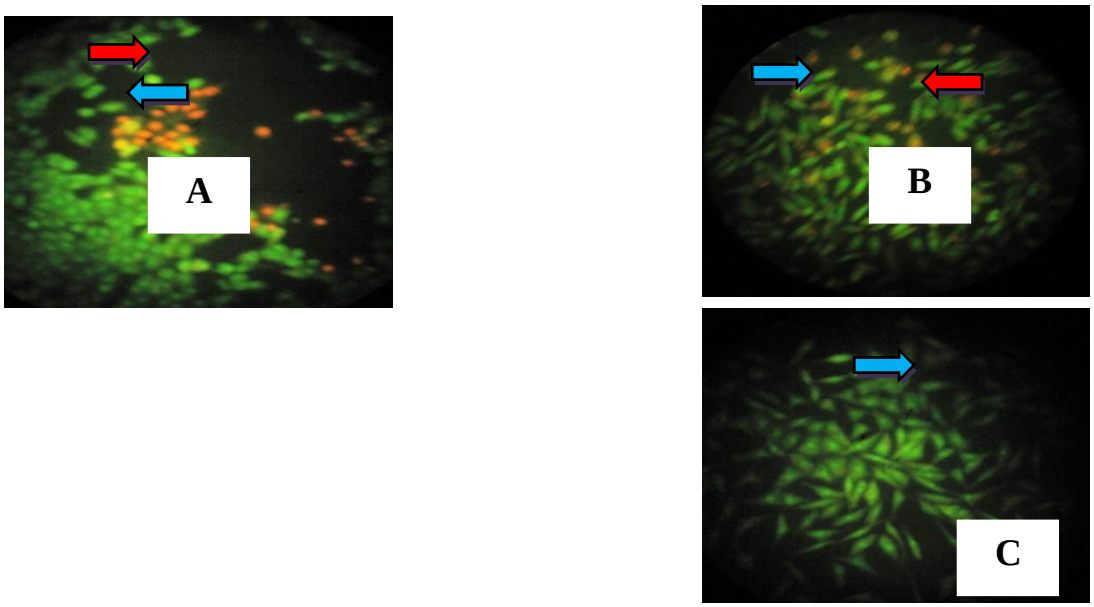

Figure 5. Apoptosis assay results soursop leaf ethanol extract of T47D cancer cells. Ethanol extract of leaves of soursop (A), tamoxifen (B), control cells (C). Red arrows indicate cells undergoing apoptosis and blue arrows indicate the cells not undergoing apoptosis.

Table 3. The mean absorbance, percentage inhibition of T47D cells and IC50 values after administration of test compound

Fraction	Consentration (µg/mL)	Mean Absorbances	Living Cells (%)	Retardation (%)	IC_{50} (µg/mL)
Hexan Fraction	500	0.263	0.23	99.77	143.077
	250	0.266	0.65	99.35	
	125	0.987	82.75	17.25	
	62.5	1.098	95.48	4.52	
	31.25	1.107	96.47	3.53	
Chloroform Fraction	500	0.264	0.42	99.58	120.718
	250	0.296	4.03	95.97	
	125	0.838	65.81	34.19	
	62.5	0.992	83.40	16.60	
	31.25	1.098	95.44	4.56	
Etyl Acetat Fraction	500	0.292	3.53	96.47	31.268
	250	0.269	0.99	99.01	
	125	0.261	0	100	
	62.5	0.283	2.55	97.45	
	31.25	0.856	67.90	32.10	
Methanol Fraction	500	0.279	2.15	97.95	44.987
	250	0.245	0	100	
	125	0.264	0.38	99.62	
	62.5	0.579	32.25	63.75	
	31.25	0.886	71.32	28.68	
Tamoxifen	50	0.253	0	100	13.38
	25	0.262	0.19	99.81	
	12.5	0.889	71.62	28.38	
	6.25	1.070	92.29	7.71	
	3.125	0.167	91.95	8.05	

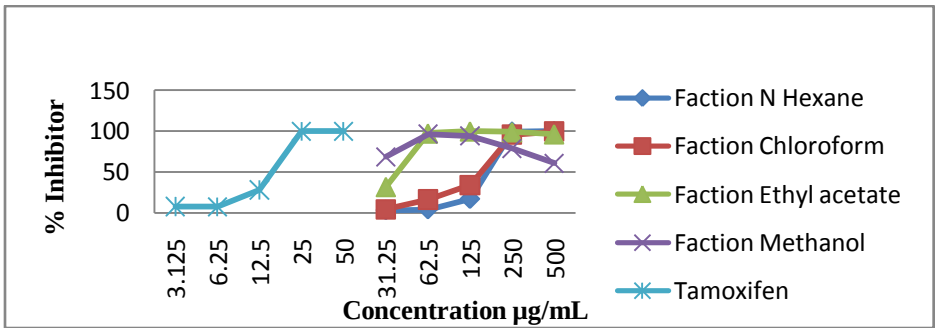

Figure 6. Graph showing the relationship between the percentage inhibition of T47D cells with various concentrations of test compound

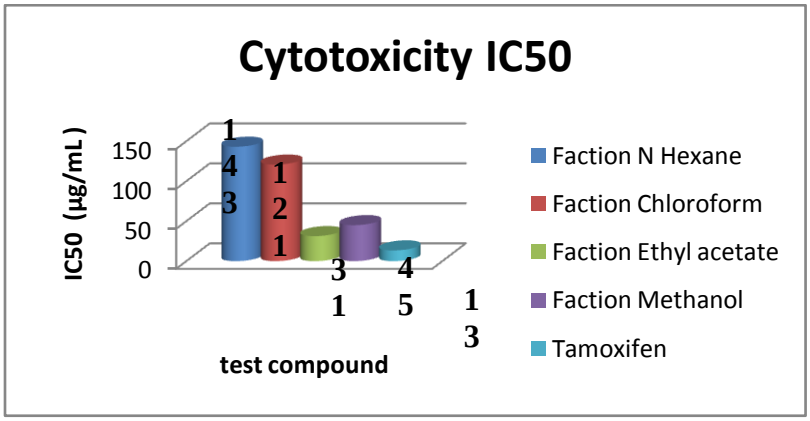

Figure 7. IC_{50} values of each test compound