

GEOGRAPHICAL EFFECT ON THE CYTOTOXIC ACTIVITY OF *Annona muricata* L. LEAVES ETHANOL EXTRACT AGAINST MCF-7 CANCER CELL

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ABSTRACT

Many studies have shown the anti-cancer activities of the chemical compounds extracted from the leaves of *Annona muricata* or soursop plant. Cianjur and Sukabumi are quite large soursop producing area in Indonesia. This study was carried out to determine the difference of cytotoxic activity of soursop leaves ethanolic extract which were harvested from three different areas of Cianjur (I, II, III) and Sukabumi (I, II, III). The Soursop leaves were macerated with 70% ethanol using microwave assisted extraction (MAE) method. The extract was tested *in vitro* on breast cancer cell line MCF7 and its constituent was identified using GC-MS apparatus. The results showed that the highest cytotoxic activity with IC_{50} values of $9.12 \mu\text{g ml}^{-1}$ was determined on the extract of soursop leaves harvested from Cianjur III area. Qualitative identification of chemical constituent shows that the soursop leaves contain alkaloid, flavonoid, triterpenoid, tannin and saponin compounds. No steroid compound was detected in the extract. It can be concluded that the geographical regions affected the biochemical properties of soursop leaves.

Keyword : Anticancer, breast cancer, cytotoxic, MCF-7, soursop leaf extract

AKTIVITAS SITOTOKSIK EKSTRAK ETANOL DAUN SIRSAK (*Annona muricata* L.) BERDASARKAN EFEK GEOGRAFIS TERHADAP SEL KANKER MCF-7

ABSTRAK

Berbagai penelitian menunjukkan bahwa ekstrak daun sirsak (*Annona muricata*) mengandung senyawa-senyawa yang memiliki aktifitas anti kanker. Sukabumi dan Cianjur merupakan daerah-daerah penghasil sirsak yang cukup besar di Indonesia. Penelitian ini bertujuan untuk melihat perbedaan aktifitas sitotoksik ekstrak daun sirsak yang diperoleh dari tiga daerah berbeda di Cianjur (I, II, III) and Sukabumi (I, II, III). Daun sirsak dimaserasi menggunakan metode *microwave assisted extraction* (MAE) dengan pelarut alcohol 70%. Senyawa-senyawa yang terkandung dalam ekstrak diidentifikasi menggunakan alat GC-MS. Ekstrak selanjutnya diujikan aktifitas sitotoksiknya terhadap sel kanker galur MCF-7. Hasil penelitian menunjukkan aktifitas sitotoksik terlihat pada sel kanker dengan perlakuan ekstrak daun sirsak dari daerah Cianjur III dengan nilai IC_{50} $9,12 \mu\text{g ml}^{-1}$. Identifikasi senyawa kimia secara kualitatif menunjukkan bahwa ekstrak daun sirsak mengandung senyawa-senyawa alkaloid, flavonoid, triterpenoid, tannin dan saponin, dan tidak mendeteksi adanya senyawa golongan steroid. Dari penelitian ini dapat disimpulkan bahwa letak geografis tempat tanam mempengaruhi kandungan senyawa kimia pada tanaman.

Kata Kunci : Antikanker, kanker payudara, sitotoksik, MCF-7, ekstrak daun sirsak

INTRODUCTION

Breast cancer is the second leading cause of cancer death in women. The chance that a woman will die from breast cancer is about 1 in 38 (about 2.6%). The breast cancer is more common found in developed countries and is more than 100 times more common in women than in men. Based on data from the World Health Organization in 2012 patients with cancer in Southeast Asia as much as 1.171 million people died and 1.728 million new findings of cancer (IARC 2012). Breast cancer is a cancer that occurs in the mammary gland, duct glands, and other breast supporting tissues (Purwatiningsih *et al.*, 2008; Raymond 2007). Worldwide, breast cancer is the leading type of cancer in women, accounting for 25% of all cases. In 2012 it resulted in 1.68 million new cases and 522,000 deaths (Shah *et al.*, 2013). Breast cancer is treated in several ways including surgery, chemotherapy, hormonal therapy and radiation therapy depend on the kind of breast cancer and how far it has spread.

Chemotherapy treatment of breast cancer can cause both temporary side effects that stop soon after treatment finishes and longer-term side effects. On going side effects, such as hot flushes fatigue and extreme tiredness (Bruton *et al.*, 2005; Wamidh 2011). Doxorubicin is a chemotherapy drug used to treat many different types of cancer. It slows or stops the growth of cancer cells by blocking an enzyme called topoisomerase 2. Cancer cells need this enzyme to divide and grow. However, this anticancer drug also affects other organs like brain, kidney and liver (Bruton *et al.*, 2005). Considering the negative effect of chemotherapy, the discovery of new anticancer agents with fewer side effects still need to be pursued particularly with the use of natural products as a platform for drug development.

Soursop (*Annona muricata* L.) which

belongs to *Annonaceae* family is a plant that grows in tropical areas. The chemical compounds extracted from the leaves of this plant exhibit pharmacological properties such as anti-cancer, anti-inflammatory, anti-diabetic and free radical scavenging activity (Moghadamtousi *et al.*, 2015). Many studies show that soursop leaves has a range biological activities such as wound healing, antimicrobial, induces apoptosis, antioxidant and cytotoxic activity on cancer cell without affecting a normal cells (Solomon-Wisdom *et al.*, 2014; Artini *et al.*, 2012; Moghadamtousi *et al.*, 2014; Paarakh *et al.*, 2009). Soursop has also been reported to have significant anti-cancer effects in a number of cancer cell lines both *in vitro* and *in vivo* including anti-proliferative effects of HL-60 cells and MCF-7 tumor growth (Dai *et al.*, 2011). Other study of Rodriguez *et al.* (2010) also stated that the soursop leaves can be used in the treatment of cancer case and prevent cancer growth.

In this study, the leaves of soursop fruit cultivated in Cianjur dan Sukabumi regions was prepared, extracted and furthermore tested on the breast cancer cell line MCF-7.

MATERIAL AND METHOD

Material

Fresh soursop leaves collected from six different areas namely Sukasirna (Cianjur I), Sukasarana (Cianjur II), Ciherang (Cianjur III), Coral Pakpak (Sukabumi I), Coral Pakpak (Sukabumi II), and Sukasari (Sukabumi III). The leaves then were cleaned under running tap water in the oven at 50 °C. The MCF7 cancer cells was obtained from the Laboratory of Microbiology and Immunology, Wildlife and Primate Study Center, Indonesia.

Extraction of Sample

Each dried leaves of samples were extracted with 70% v/v ethanol using microwave assisted extraction method (MAE). The MAE method has a number of advantages such as shorter extraction time, less solvent, higher extraction rate and lower cost. The extracts obtained were then filtered through a Whatman No. 1 filter paper. The collected filtrates were evaporated to dryness under vacuum at 40°C using a rotary evaporator. The samples were prepared according to the method of AOAC (1984), Hasan (2013) and Herman *et al.* (2013) with a minor modification. All chemical used were of analytical grade.

Qualitative analysis of chemical components of the extract

The qualitative analysis of chemical constituents was carried out to detect the presence of alkaloids, flavonoids, steroids, triterpenoids, tannins and saponins in the ethanol extract of soursop leaves. The qualitative analysis was performed according to Harbone (1987), Gajalakshmi *et al.* (2012) and Hasan (2013) methods. A total 100 mg of dry extract were dissolved in 1 mL of 99% ethanol. The 1 mL solution then injected into capillary column HP-5 (Agilent 19091J-433: 0:25 mm × 30 mm × 0:25 µm containing 5% diphenyl 95% dimethylpolysiloxane) of GC-MS. The flow rate used was 1.0 mL / min, the injection temperature was 300 °C, the pressure was 10:47. The GC-MS was running using split mode with the He (Helium) was used as a mobile phase. MS parameter was used to detect compounds with masses 50-800. MS quad was set at temperature of 150-200 °C and MS source at 250-300 °C. The chromatogram resulted was analyzed by comparing it to the available database.

Determination of Total Phenolic Compound

The 0.5 ml of 10% extract solution was added with 5 ml of Folin-Ciocalteu reagent and 4 ml of 10% Na₂CO₃ 1 M. The mixture solution was shaken using vortex then incubated for 15 min at room temperature. Phenolic compounds were measured at a wavelength of 765 nm. The standard curve was made using a series of gallic acid solution 0, 50, 100, 150, 200, 250 and 300 mg l⁻¹ in methanol:water (1:1 v/v). The amount of total phenolic compounds for each extract were calculated using linear regression equation obtained from the absorbance values of each extract plotted on equation of gallic acid standard curve.

$$y = a + bx \quad (1)$$

Description:

y : the dependent variable

x : the independent variable

a : intercept

b : regression coefficient/slope

MTT test against cancer cells MCF-7

The MTT test is a colorimetric assay for assessing cell metabolic activity. The NAD(P)H-dependent cellular oxidoreductase enzymes under defined conditions could reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT use 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. MTT test on MCF-7 cancer cells was performed based on Lin *et al.*, (1991) and Hasan *et al.*, (2013) method. Soursop leaf extract was dissolved in DMSO (dimethyl sulfoxide) to prepare a 10% solution stock solution. The stock solution then diluted with RPMI-1640 medium (Roswell Park Memorial Institute) to obtain 1% solution. A series of solution in different concentration ranging from 250, 100, 50, 10, and 1 mg ml⁻¹ were then prepare from the 1% stock solution. The

20 mL of solution was taken from each concentration then added to the microplate containing 100 mL sustainable cell cancer (7.5×10^4 cells ml^{-1}) and incubated for 24 hours at 37°C in an incubator with 5% CO_2 . DMSO (0.05%) was used as a negative control and doxorubicin (0.5 mg ml^{-1}) as positive control. The number of survived cells were measured using ELISA reader at a wavelength of 570 nm. Data obtained were converted into percentage (%) of living cell using equation below:

$$\begin{aligned} \text{The percentage of Living Cells (\%)} &= \\ &= \frac{(\text{Treatment absorbance} - \text{media control absorbance})}{(\text{control cell absorbance} - \text{media control absorbance})} \times 100 \end{aligned} \quad (2)$$

RESULTS AND DISCUSSION

Extracts

The extraction of soursop dry leaves using MAE methods show that the highest yield of extract was obtained from the leaves collected from Cianjur III area and lowest yield was obtained from the soursop leaves collected from Sukabumi III area with total yield 13.48% and 8.68% respectively as shown in Table 1. The differences of extract yield indicated the differences in content and variety of

chemical compounds contained in each sample. The data confirmed that the geographic area influences the chemical characteristics of plant compounds.

Table 1. The Yield of Soursop Leaves Extracted With 70% Ethanol Using MAE Method

Location	Yield (% w/w)		
	Site I	Site II	Site III
Cianjur	9.32	10.00	13.48
Sukabumi	10.92	11.40	8.68

Qualitative Test of Chemical Compounds In Soursop Extract

The results of qualitative test detected the presence of alkaloids, flavonoids, triterpenoids, saponins and tannins compounds in soursop ethanol extracts (Table 2). No trace of steroid compounds found in the extract. The absence of steroid compounds in line with the previous research by Artini (2012) which showed similar results but different from the results of Solomon *et al.* study (1999) that found the steroid compounds in the extract of soursop leaves. This difference is probably related to differences in the method and solvent used in the study.

Table 2. The Compounds Detected In Soursop Leaves Ethanol Extract

Location	Type test							
	Alkaloids			Triterpenoid	Steroid	Flavonoid	Tanin	Saponin
	Dragendorff	Meyer	Wegner					
Cianjur I	+++	++	++	+++	-	+	++	++
Cianjur II	+++	++	+++	+++	-	+++	+	++
Cianjur III	+++	++	+++	++	-	+++	+++	++
Sukabumi I	+++	++	+++	+	-	+	+++	+++
Sukabumi II	+++	+++	+++	+++	-	++	+	++
Sukabumi III	+++	+++	+++	+++	-	+++	++	+++

note : - : not detected , + : less detected, ++ : well detected, +++ : very well detected

Identification of Chemical Compounds In Soursop Extract

The identification of chemical compounds in soursop extract was performed using GC-MS apparatus. The chemical compounds found in the extracts was determined by comparing the similarity of pattern resulted from GC-MS reading with the pattern of available database. From the GC-MS reading, it was identified that ethanol extract of soursop leaves contains many identified compounds at different concentration as shown in Tabel 3. The compounds contained in soursop extract commonly belong to alkaloids, flavonoids, and phenols group. According to many literatures, these groups of compound have an activity as antidiabets, antioxidant and anticancer.

The active compounds expected to inhibit cancer cell proliferation are piperine, piperidine, stigamsterol, and cysterol. Piperine and piperidine are alkaloid amide compounds which working by activating the apoptosis of cancer thus cell division is controlled (Bezerra *et al.*, 2006; Abraham *et al.*, 2013).

Table 3. Chemical Compounds of Soursop Leaf Extract

Location	Compounds
Cianjur I	Hydrofucosterol, Ergosterol, Palmitic acid
Cianjur II	Methyl lenoleic, Tetromethyl hexadecenol, cytosterol
Cianjur III	Piperine, Tertomethyl hexadecenol, Palmitic acid
Sukabumi I	Piperine, Tocopherol, Cholesterol
Sukabumi II	Piperidine, Benzemida, Piperine
Sukabumi III	Gamma cytosterol, Piperin, Tetromethyl hexadecenol

Determination of Total Phenolic Compounds

The total phenolic compounds was calculated base on equations derived from a standard curve prepared from the standard

solution. The equation obtained was $y = 0.0069x + 0.0242$ and the regression value was 0.999. From the calculation, it is found that the highest phenolic compound occur in extract of soursop leaves collected from Sukabumi I (4.94%) and lowest phenolic compound occur in extract of soursop leaves collected from Cianjur II regions (1.90%) as shown in Table 4. The difference of total phenol content in soursop leave extracts was due to many environmental factors as altitude, temperature, soil nutrients, harvesting time and plant maintenance methods (Mendez 2001).

Table 4. Total Phenolic Compound of Soursop Leave Extracts

Location	Total phenol	Percentage	
Cianjur	I	32.14 ± 0.807	2.26%
	II	18.96 ± 0.753	1.90%
	III	29.83 ± 1.150	2.98%
Sukabumi	I	49.40 ± 1.045	4.94%
	II	21.66 ± 0.549	2.10%
	III	22.04 ± 0.886	2.63%

Cytotoxicity of Soursop Extract On Breast Cancer Cell Line MCF-7 Based On MTT Test

The MTT test was performed to determine the cytotoxicity activity of soursop leaf extract on MCF7 cancer cell line *in vitro*. The parameter measured in the MTT test is value of (IC_{50}). The IC_{50} or half maximal inhibitory concentration is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function, also defined as the concentration or **amount** of drug needed to kill 50% the cell population.

Each extract of soursop leaves have shown inhibitory activity on the proliferation of MCF-7 cancer cell. The IC_{50} value of the soursop leaves extract collected from Cianjur III occur at concentration of $9.12 \mu\text{gml}^{-1}$ MCF-7. The

cancer cells were severely damage when the concentration increase to $250 \mu\text{g ml}^{-1}$. As shown in Figure 1, compared to the soursop leaves extracts collected from other regions, the soursop leaves extract from Cianjur III region have the highest cytotoxic activity to inhibit the growth of MCF-7 cancer cells. The difference of IC_{50} values are related to variation of biochemical content found in the extracts of soursop leaves, but do not show any correlation with the total phenol content of the extract. Overall, the soursop leaves extracts exhibit the cytotoxic properties to inhibit the proliferation of MCF-7 cancer cells *in vitro* at concentration higher than the doxorubicin positive control ($0.5 \mu\text{g ml}^{-1}$). The morphological appearance of MCF7 cancer cells when treated with soursop leaves extract at concentration of $250 \mu\text{g ml}^{-1}$ can be seen in Figure 2. The ability of soursop extracts to diminish the rate of cancer cells proliferation also have been confirmed by many inhibitory capability inhibition. Inhibition of cancer cells from soursop leaves extract previous studies from Moghadamtousi *et al.* 2014; Moghadamtousi *et al.* 2015. The results suggest that the soursop leaves extract is a potential candidate to be further developed for cancer treatment especially the breast cancer and deserves further research as an alternative to conventional drugs while also stressed out the selection of soursop sample which plays a significant role in determining its potential therapeutic effect on cancer.

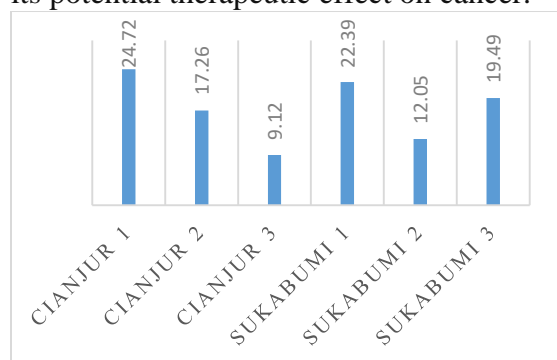


Figure 1. The IC_{50} Value of Soursop Leaves Extracts Collected From Six Different Regions



Figure 2. The morphological appearance of MCF7 cancer cells when treated with soursop leaves extract: 1. DMSO 2. doxorubicin $0,5 \mu\text{g ml}^{-1}$ 3. $1 \mu\text{g ml}^{-1}$ extract 4. $100 \mu\text{g ml}^{-1}$ extract 5. $250 \mu\text{g ml}^{-1}$ extract

CONCLUSION

The soursop leaves extracts collected from different regions in west java, Indonesia contain alkaloids, flavonoids, tannins, triterpenoids, and saponins. The MTT test have confirmed that all extracts exhibit the cytotoxic activity against MCF7 cancer cells. The highest activity was shown by is the extract of soursop leaves from collected from Cianjur III with IC_{50} values of $9.12 \mu\text{g ml}^{-1}$. The Cianjur III soursop leaves extract also contain highest piperine compound with levels reach 8.73%. Finally, it can be concluded that the geographical region affected the biochemical properties of soursop leaves.

CONFLICT OF INTERESTS

The authors declare no conflict of interest regarding the publication of this paper.

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