



The cytotoxic activity of *Annona muricata* Linn Leaves Ethanolic Extract (AMLEE) on T47D breast cancer cell line

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Abstract: Breast cancer is the most common cancer in women throughout the world, with new cases and deaths which continue to increase. Soursop leaves (*Annona muricata* L) have been used extensively in traditional medicine, including cancer. Acetogenin, alkaloids, and phenols contained in soursop leaves are known to have anti-cancer effects. Among them, acetogenin has the most dominant role and reported to have a cytotoxic effect on various cancer cell lines. This study aims to determine the cytotoxic activity of soursop leaf ethanol extract on T47D breast cancer cell line. Measurement of cytotoxic activity was carried out by the MTT method, and the viability percentage of T47D cells was calculated based on the absorbance values in the treatment, cell control, and media control groups of each replicate. The correlation between extract concentration and viability percentage of the T47D cell line was outlined in the regression equation to obtain the IC₅₀ value. IC₅₀ values of 109.91 ± 3.04 with R values 0.975 and R² 0.9508 obtained. R values close to 1 indicated a strong correlation between extract concentration and the percentage of living T47D cells. Meanwhile, the amount of R² suggested that the level of AMLEE had a 95.08% influence on the rate of cell viability, and the other 4.92% influenced by factors other than the AMLEE dose. These results indicated that the ethanol extract of soursop leaves has a cytotoxic effect and has the potential to inhibit T47D cell proliferation in vitro.

Keyword: *Annona muricata*; cytotoxic activity; T47D

INTRODUCTION

Cancer remains a global health problem. In 2015, 8.8 million deaths due to cancer reported worldwide. In Indonesia, the estimated prevalence of the disease in 2013 was 1.4 ‰ or approximately equal to 347,792 people (Pusat Data dan Informasi, 2015).

Breast cancer is the most common cancer in women throughout the world. Nearly 1.38 million cases of breast cancer newly diagnosed in 2008, of which 60% of deaths occurred in low-income countries (Shah et al., 2014). Data from GLOBOCAN/IARC (International Agency for Research on Cancer) in 2012 showed that breast cancer ranked first in Indonesia, with an estimated incidence of 40 per 100,000 women (Pusat Data dan Informasi, 2015). To date, various cancer therapies, including therapies from natural ingredients, are currently being developed to reduce mortality rates and increase the average life expectancy of cancer patients. One of the natural ingredients that have been used extensively in the traditional medicine of cancer is soursop (*Annona muricata* L).

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Soursop is a species of the Annonaceae family. This plant is commonly found in Indonesia because it is suitable to grow in the tropics. Various parts of the soursop plant can be used for the treatment of multiple diseases such as malaria, hypertension, diabetes, diarrhea, respiratory and gastrointestinal disorders, as an antispasmodic, sedative, anti-inflammatory, anti-oxidant, and the treatment of cancer (Moghadamtousi et al., 2015; Nik Mat Daud et al., 2016; Pieme et al., 2014). Besides, the leaves of the soursop plant are widely used by society for the treatment of cancer. Various compounds have been isolated from *A. muricata* leaves, including acetogenin, alkaloids, phenolics, tannins, terpenoids, glycosaponins, and coumarin. Among them, several compounds that are known to have anti-cancer effects are acetogenin, alkaloids, and phenols. However, acetogenin has the most dominant impact as an anti-cancer. Qualitative analysis of phytochemical screening of *A. muricata* leaf extract indicates that *A. muricata* leaf is a highly nutritious material and a potential source of phytomedicine (Gajalakshmi et al., 2012; Gavamukulya et al., 2014; Kim et al., 2016; Wahab et al., 2018).

Research on the cytotoxic activity of *A. muricata* in several types of breast cancer cell lines with the MTT method shows different results. A study by Arifianti et al., which performed MTT assay on T47D cells with a 24-hour incubation period, showed IC_{50} values of 20.36 ± 1.58 . But in this study, the soursop plant that was processed into extracts was the seeds. Investigations have been carried out on the leaves of *A. muricata* as the leaves are the most utilized parts used for a wide array of ethnomedicinal uses. Besides that, among the components of *A. muricata*, acetogenin is found in high amounts in the leaves (Wahab et al., 2018). Contrastingly, in another study which used soursop leaf samples obtained from 19 different locations, MTT assay was carried out with 72-hour incubation time. The results showed that crude extract of soursop leaves from sample B1 had the highest cytotoxic activity against MCF7, MDA-MB-231, and 4T1 cells with IC_{50} values of 221.67 ± 1.67 ; 350 ± 5.77 and 251.67 ± 6.01 , respectively. This suggested that soursop leaves obtained from different locations have various cytotoxic effects on cancer cells. The existing differences possibly influenced this in the level of secondary metabolites of each plant obtained from different locations despite the same species (Syed Najmuddin et al., 2016). Therefore, this study aims to determine the cytotoxic activity of *Annona muricata* Linn Leaves Ethanol Extract (AMLEE) on T47D cells.

MATERIALS AND METHOD

This study used a true experimental design with a post-test only control group design using samples divided into five groups with different concentrations on T47D cells. Each level of the sample, including a group of cell control and media control, tested with three replications, where 1×10^4 cells were used for each replication. A sample used in this study was ethanol extract of soursop leaves, which was tested for cytotoxic activity to T47D cell lines.

Tools and materials needed in this study were extraction tools, T47D cell cultures, and MTT assay kit. Soursop leaves were obtained from Materia Medica Batu, Technical Service Unit/Unit Pelayanan Teknis (UPT) of the East Java Provincial Health Office, with soursop determination letter number 074 / 123A / 102.7 / 2019. Extracts were made by the maceration method using 96% ethanol. T47D cell line was obtained from the parasitology laboratory of the Faculty of Medicine, Gadjah Mada University, Yogyakarta. Cells were incubated in a 5% CO₂ incubator. Other materials used were Rosewell Park Memorial Institute (RPMI 1640) (Gibco), Fetal Bovine Serum

(FBS) (Sigma), penicillin-streptomycin (Sigma), fungizone, Dimethyl sulfoxide (DMSO), and trypsin-EDTA (Sigma). Materials used in cytotoxic tests were 3-(4,5-dimethylthiazol-2-yl) diphenyltetrazolium bromide (MTT) (Sigma), Sodium Dodecyl Sulfate (SDS) as a stopper, and an Elisa reader to analyze MTT results.

Preparation and phytochemical screening of ethanol extract of soursop leaves, soursop leaves were cleaned and dried using an oven at 40°C and ground to form a powder. Furthermore, the dust was soaked with 96% ethanol and allowed to stand for 24 hours in a tightly closed condition. The precipitate was filtered to form a liquid extract, then evaporated three times over a water bath until a concentrated extract formed. Phytochemical screening carried out to determine the compounds contained in the extract. Detection carried out on syntheses of alkaloids, flavonoids, tannins, steroids, and terpenoids.

The cytotoxic activity of soursop leaf extract was measured by the MTT method to determine the percentage of living cancer cells after being treated and the IC₅₀ value. Previously, T47D cells were cultured on RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.5% fungizone. Cells harvested by separating the cells attached to the petri dish using trypsin-EDTA (0.25% trypsin) and subsequent counting in the counting chamber. Next, 100 µl of cell solution was put into a 96-well plate and incubated in a 5% CO₂ incubator at 37°C for 24 hours. Cell condition after incubation firstly observed using an inverted microscope (Olympus CKX41). If the cell condition had recovered and there was no contamination, cell media discarded, and extracts that had dissolved using DMSO at concentrations of 200, 100, 50, 25, and 12.5 µg / ml added. Each level, together with cell control and media control, was prepared as three replications (triple), and was re-incubated for 24 hours. The next day, the solution in the plate removed and 100 µl of MTT solution (5 mg/ml) was added and incubated for 4 hours until purple formazan crystals formed. The MTT reaction then stopped by adding 100 µl of 10% SDS to 0.1 N HCl, and 96 well-plate was wrapped in paper or aluminum foil and left overnight. MTT results read with an Elisa reader (Bio-Rad, Benchmark) at a wavelength of 595 nm.

Alkaloids, flavonoids, tannins, steroids, and terpenoids identified in phytochemical screening, positive results were assessed based on changes in color and sediment formed after extracts reacted with specific reagents.

The percentage of T47D cells viability was calculated based on absorbance values in the treatment, cell control, and media control groups, using the following formula:

$$\% \text{ viability} = \frac{(\text{absorbance of treatment} - \text{absorbance of media})}{(\text{absorbance of cells} - \text{absorbance of media})} \times 100 \%$$

The correlation between AMLEE concentrations and the percentage of living T47D cells determined through a linear regression equation, based on the regression equation obtained, the IC₅₀ value of each replication was determined; thus, the average IC₅₀ and standard deviation (SD) obtained.

RESULTS AND DISCUSSION

A total of 1 kg of dried soursop leaves used for making extracts with the maceration method using 96% ethanol solvent, resulting in the final result of 100 grams thick extract. Furthermore, phytochemical screening performed to determine the compound content in the extract, as shown in Table 1.

Table 1. Phytochemical Screening of *A. muricata* Leaves Ethanolic Extract

Compound name	Result	Note
Alkaloid	positive	Formation of precipitates
Flavonoid	positive	Orange colour
Tanin	positive	Dark green colour
Steroid	Positive	Bluish green colour
Terpenoid	negative	No reddish purple colour

Annona muricata contains various active compounds. As of February 2017, 212 bioactive compounds and multiple minerals have identified in *A. muricata* (Gavamukulya et al., 2017). Among all bioactive compounds, acetogenin (found in high amounts in leaves) followed by alkaloids and phenols were the three main compounds (Gavamukulya et al., 2017; Wahab et al., 2018; Yajid et al., 2018). Tannins, steroids, and terpenoids also found in soursop leaves; however, this study showed negative results of terpenoids and positive effects of alkaloids and flavonoids as the main compounds in *A. muricata*. The results of this study were not too different from the other research by Setyorini et al. phytochemical screening results in the study showed that *A. muricata* leaves ethanolic extract contained alkaloid, flavonoid, tannin, and steroid (Setyorini et al., 2016).

Bioactive compounds isolated from the leaves of *A. muricata* with anti-cancer activity were acetogenin, alkaloids, and phenols (flavonoids including phenol groups). In addition to their anti-cancer activity, flavonoids also exhibit anti-oxidant, anti-inflammatory, and immunomodulatory activities (Kim et al., 2016; Mohamad Rosdi et al., 2015; Wahab et al., 2018).

Cytotoxic activity of AMLEE tested against T47D breast cancer cell line (Figure 1). Before cytotoxic testing performed, cells must have reached 70 -80% confluent to make sure that they were ready for harvest.

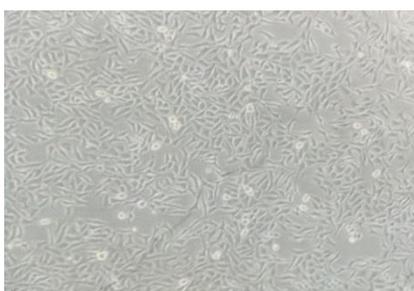


Figure 1. T47D Cell

The MTT method used to assess cell proliferation or viability, while the cytotoxic potential of a sample was determined based on IC_{50} values. Determination of IC_{50} value of each replication based on a regression equation between the AMLEE dose (x-axis) and the percentage of living T47D cells (y-axis) in the group. The IC_{50} value was the value of x if y was equal to 50. Furthermore, the average IC_{50} and standard deviation were calculated (Table 2). Regression equation on the graph, R

and R square (R^2) value obtained using the regression analysis in 2013 excel program.

Table 2. AMLEE Cytotoxic Test Result on T47D Cells

AMLEE Dose ($\mu\text{g/ml}$)	T47D Viability (%)			Mean	SD
	1	2	3		
200	9,43	8,65	9,04	9,04	0,39
100	38,25	43,18	47,47	42,97	4,61
50	90,31	89,53	91,99	90,61	1,26
25	93,55	92,64	91,61	92,60	0,98
12,5	89,66	95,24	98,36	94,42	4,41
Regression Equation	$y=-0,482x + 101,6$	$y=-0,494x + 104,13$	$y=-0,497x + 106,21$	$y=-0,491x + 103,98$	
IC_{50}	107,05	109,58	113,10	109,91	3,04

From this study, IC_{50} values of 109.91 ± 3.04 with R values 0.975 and R^2 0.9508 obtained (Table 3). This IC_{50} value indicated that AMLEE had cytotoxic activity. It was because IC_{50} value less than $1000 \mu\text{g} / \text{ml}$ after 24 hours of contact with cancer cells is an indicator of the cytotoxic activity of a specific extract (Arifianti et al., 2014). However, the IC_{50} value of this sample belongs to the medium category based on the cytotoxic level in the study. Cytotoxic effect is said to be very strong if it has an IC_{50} value $< 10 \mu\text{g/ml}$, cytotoxic is active if IC_{50} between $10 - 100 \mu\text{g/ml}$, moderate cytotoxic if IC_{50} between $\mu\text{g/ml}$, and low cytotoxic if IC_{50} between $500 - 1000 \mu\text{g/ml}$ (Diba et al., 2019).

IC_{50} value from this sample was relatively higher compared to the study by Pieme et al. (2014), which performed cytotoxicity tests of *A. muricata* leaves extract with an incubation period of 48 hours in HL-60 cell line. The study conducted by Abdullah et al. showed a significant result of the cytotoxic value of *A. muricata* leaf extract based on incubation time, which can inhibit cell proliferation up to 45% in 48 hours after the treatment given. Henceforth, the doubling time method can performed to assess the effect of *A. muricata* leaves extract on cell proliferation at 0, 24, 48, and 72 hours

Table 3. Regression Statistic of AMLEE Cytotoxicity on T47D Cells

AMLEE Dose ($\mu\text{g/ml}$)	Mean of T47D Viability (%)	Regression Statistics	
		Multiple R	R Square
200	9,04		
100	42,97		
50	90,61	0,975	0,9508
25	92,60		
12,5	94,42		

R values close to 1 indicated a strong correlation between extract concentration and cell viability. This can see in this study that the use of a higher

level of the extract led to a smaller proportion of living cancer cells (Figure 2). This was consistent with the survey conducted by Syed Najmuddin et al., where an increase in the dose of *A. muricata* leaves crude extract caused a decrease in MCF7, MDA-MB-231, and 4T1 cell viability. However, the increased percentage of viability of T47D cells was quite high at concentrations of 200, 100, and 50 $\mu\text{g} / \text{ml}$. It recommended that further studies perform cytotoxicity tests with a smaller concentration range of 50 -200 $\mu\text{g} / \text{ml}$. Meanwhile, the value of R^2 suggested that the concentration of AMLEE had a 95.08% influence on the percentage of cell viability, and the other 4.92% influenced by factors other than the AMLEE dose.

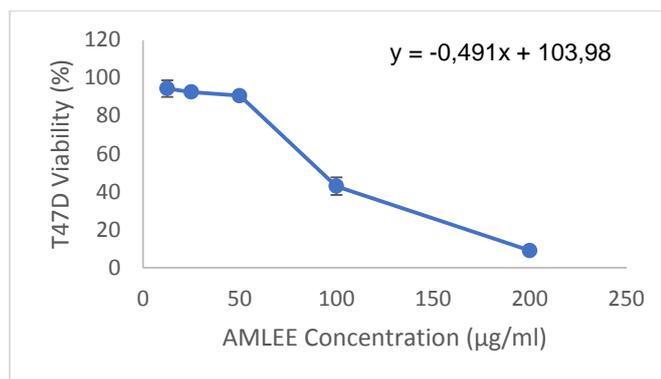


Figure 2. Linearity Graph of AMLEE Concentration to Viability Percentage of T47D Cells

Cytotoxic activity of soursop leaves caused by the content of bioactive compounds that have anti-cancer effects. Among the bioactive compounds isolated from *A. muricata* leaves which has the most dominant anti-cancer activity, is acetogenin, and it has also reported having anti-proliferative effects on various cancer cell lines (Moghadamtousi et al., 2015; Mohamad Rosdi et al., 2015; Wahab et al., 2018). Besides, various studies assessing the anti-cancer effects of this plant showed that cytotoxic effects of *A. muricata* on cancer cells were by decreasing Bcl2 protein regulation, increasing Bax, inhibition of mitochondrial complex I, and inhibition of ubiquinone-oxidase NADH in the cancer cell plasma membrane resulting in apoptosis (Gavamukulya et al., 2017). Interestingly, the active cytotoxicity of *A. muricata* leaves on cancer cells did not affect healthy cells. This evidenced in the cytotoxic test of ethanol extract of *A. muricata* leaves on normal splenocytes, which showed very high selectivity or no cytotoxic effect on normal splenocytes at all extract concentrations tested. The high selectivity of this extract against cancer cells is a crucial aspect for its use in therapy because normal cells not targeted (Gavamukulya et al., 2014; Wahab et al., 2018; Yang et al., 2015).

The results of this study showed that AMLEE had cytotoxic activity against T47D cells. However, the extract still contains various types of bioactive compounds so that it was not possible to know the direct cytotoxicity of acetogenin, which has the most dominant anti-cancer effect and found in high amounts in the leaves. Besides, AMLEE cytotoxic tests on normal cells not carried out in this study.

CONCLUSION

Annona muricata leaves ethanolic extract to have the potential to inhibit T47D cell proliferation in vitro with an IC_{50} value of $109,91 \pm 3,04$.

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CONFLICT OF INTEREST

There were no conflicts of interest with related parties in this study.

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