Cytotoxic Activity and Antiproliferation of Soursop Seed Protein *(Annona muricata)* Against 4T1 Cells

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Abstract. Current cancer treatments still have a lot of negative impacts, so it is necessary to find alternative treatments that come from nature or plants that have anti-cancer activity. Plants that have the potential to be anti-cancer agents are plants that contain *Ribosome-Inactivating Proteins* (RIPs). Soursop plants are candidates for this experiment because the same genus as soursop, sugar apple (Annona squamosa L.), has shown RIPs activity. This study aimed to determine the antiproliferative activity of soursop seed protein *(Annona muricata)* against 4T1 cells. Protein isolation used the ion-exchange chromatography method. Cytotoxicity and antiproliferation tests used the MTT assay method, after obtaining the IC_{50} value, an antiproliferative test was conducted with concentrations below IC_{50} with incubation times of 24, 48, and 72 h. The cytotoxicity test results of the soursop protein fraction were 73.96 ug/mL ± 25.10, and doxorubicin had an IC_{50} value of 5.067 ug/mL. The results of the control antiproliferative test were 100.33 h, while the test group was 77.44 h. The results of the test indicate that the soursop seed fraction was not able to inhibit the proliferation of 4T1 cells.

Keywords: Annona muricata · MTT assay · 4T1 cells · antiproliferative assay · cytotoxic assay

1 Introduction

Breast cancer is a malignancy in breast tissue originating from the ductus epithelium or its lobules. Based on *Globocan,* in 2020, the number of people with cancer in Indonesia is 396,914, and breast cancer itself ranks first by 65,858 sufferers or by a percentage of 16.6% [1]. Current cancer therapy can be radiation, chemotherapy, and surgery [2]. Chemotherapy actively fights cell division and multiplication, but chemotherapy may affect normal cells. Thus, it can affect a person's quality of life [3]. Chemotherapy treatment can cause toxic effects in hematological, gastrointestinal, skin, and hair follicle toxicity, nervous system toxicity, local toxicity, metabolic disorders, liver toxicity, urinary tract toxicity, cardiac toxicity, pulmonary toxicity, and gonad toxicity [2]. These side effects encourage researchers to find alternative treatments derived from plants or

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natural ingredients as anti-cancer therapy. Breast cancer cells 4T1 are breast cancer cells derived from mammary gland tissue from the BALB/c strain of mice. Metastasis in 4T1 cells occurs via the hematogenous route to the liver, lungs, bone, and brain, similar to metastatic breast cancer in humans [4]. The 4T1 cells have several characteristics that make them a suitable experimental animal choice for breast cancer in humans. Specifically, the first tumor cells are easily transplanted into the mammary gland so that the primary tumor grows in the correct anatomical site. The second advantage is that, as in human breast cancer, 4T1 metastatic disease develops spontaneously from the primary tumor. The third advantage is that the progressive spread of 4T1 metastases to draining lymph nodes and other organs is similar to that of human breast cancer. Another advantage of 4T1 cells is their resistance to 6-thioguanine. In addition, 4T1 cells are easy to manipulate in vitro and in vivo [5].

Soursop plants are known as soursop (English), *graviola* (Portuguese), *guana´bana* (Latin America), while in Indonesia, it is known as *Sirsak*, *Nangka Belanda*, and *Nangka sabrang*. Soursop *(Annona muricata)* is a plant belonging to the genus *Annona*. The soursop plant contains alkaloids, megastigmanes, flavonol triglycerides, phenolics, cyclopeptides, and essential oils. The Indonesian people have long known soursop plants as herbal plants to treat arthritis, high blood pressure, snake bites, diarrhea, headaches, and malaria. In addition, it is referred to as an antibacterial drug, an anti-diabetic drug, anti-inflammatory, protozoan, antioxidant, insecticidal, and larval drug**.** Plants belonging to the same genus as soursop, namely sugar apple (*Annona squamosa* L.), have shown RIPs activity. The RIPs in sugar apple are ARIPs *(Annona squamosa Ribosome inactivating proteins)* with band sizes of 21 kDa and 28 kDa [6]. ARIPs (*Annona squamosa Ribosome inactivating proteins*) have been studied to show antimicrobial, antimutagenic, and cytotoxic activities [6].

RIPs are widely distributed in higher plants, and some have been found in some fungi and bacteria. *Ribosome-inactivating proteins* (RIPs) are enzymes that inhibit eukaryotic ribosomes through the activity of N-glycosidase. They cleave the specific adenine residues of 28S RNA in the 60S ribosomal subunit, thereby inhibiting synthesis [7]. Plants with RIPs are classified into three main categories based on their physical properties. Type I RIPs are single-chain proteins of about 30 kDa with N-glycosidase activity [8]**.** Type II RIPs consist of two distinct domains: a 30-kDa enzymatic A chain (similar to type I RIPs) linked to a slightly larger B chain with lectin properties and specificity for sugars having a galactose-like structure**.** So far, type III RIPs, also considered atypical type I RIPs, have only been described in maize and barley, and the function of their additional domain remains unknown [9].

The cytotoxic test is an in vitro test that uses cell culture to determine whether a compound has antineoplastic activity. Cell culture is one way of determining in vitro to obtain cytotoxic drugs. This system is a quantitative test by determining cell death [10]. A cytotoxic test was accomplished to obtain the value of *Inhibition Concentration* 50 (IC_{50}) using the MTT assay method. The IC_{50} value is used to determine the potential toxicity of a compound to test [11]. There are several categories in the grouping of IC_{50} values for anticancer activity, namely a compound can be classified as very active (value 20 μg/mL), moderately active (value 21–200 μg/mL), weak (value 201–500 μg/mL), and > 501 g/mL inactive [12]. The antiproliferative test is used to see the ability of

a sample to maintain living cancer cells (cell viability) to survive or not to proliferate (grow to multiply) on a fixed % viability scale. Antiproliferative activity is expressed in % the viability of living cells [13]. Antiproliferation is the percentage (%) of the inhibitory activity of the number of cells growing to less than 50% [14]. The antiproliferative test uses the MTT method. The MTT method is based on the tetrazolium salt (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide), which is cleaved by the succinate tetrazolium reductase system through the mitochondrial respiratory pathway of living cells, resulting in a purple formazan crystal. In addition, it is insoluble in water. This formazan crystal is directly proportional to the number of living cells; the higher the intensity of the purple color in the formazan crystal, the more the number of living cells [15].

2 Method

2.1 Tools

The equipment used in this study were Buchner funnel, analytical balance (Ohaus), oven (BINDER), micropipette (Socorex) 20, 200 and 1000 L, (Olympus, type CKX41), incubator (Binder, type COincubator$_2$), nanodrop, autoclave, ELISA reader (Elx800 Bio-Tech), haemocytometer (Assistant), Cytotoxic Safety Cabinet (ESCO, cytoculture type), hand tally counter (Kenko, model HT-302), 100 ml and 250 ml reagent bottles, falcon conical 15 ml tube, oven (Memmert), *96-well plate*, petri dish, 50 ml measuring cup, mortar and stamper, eppendorf, 15 ml conical tube, pH meter, dropper, test tube, knife, measuring flask and camera for documentation.

2.2 Material

The materials used in this study were soursop seeds obtained from Pasar Gedhe, Surakarta, sodium phosphate, phosphoric acid, 0.14 M NaCl, 2 M NaOH, 2M HCl, 70% alcohol, sterile distilled water, the column with DEAE matrix, *Phosphate Buffer Saline* (PBS), trypsin-EDTA, doxorubicin, 4T1 cell cultures obtained from the stock of the UMS Pharmacy Laboratory of mammalian cell culture, RPMI 1640 media: RPMI 1640 (Sigma), DMEM media, sodium bicarbonate, and hepes; Cell culture media: RPMI 1640 media, Fetal bovine serum (Gibco) 10% v/v, penicillin-streptomycin (Gibco 1% v/v and fungison (Gibco) 0.5% (v/v); DMSO; MTT (Sigma)), stopper reagent: sodium dodecyl sulfate 10% v/v in 0.01 N HCl.

2.3 Material Preparation, Protein Extraction, and Isolation

The plant part used in this study was soursop seeds *(Annona muricata).* Soursop seeds were washed, weighed 50 g, then cut into small pieces. Next, it was mashed using a mortar and stamper. Following that, the extraction was achieved with 0.14 M sodium chloride at 4 °C in 5 mM sodium phosphate buffer pH 7.2 as much as 50 ml. Subsequently, the extract was squeezed using a screen printing filter measuring small liquid obtained in a cold centrifuge at 14,000 rpm for two minutes. The supernatant obtained is a protein extract later stored at 4 °C [16].

The first step to complete protein isolation was to prepare sterile aqua dest for stroking DEAE matrix up to five times the volume or about 50 mL. After that, it was continued to use 10 mL of 5 mM sodium phosphate buffer pH 7.2. Then, it was inserted up to 10 mL of the supernatant sample into the column. The eluent was prepared in the form of a NaCl solution whose molar concentration was increased from 0.2 M; 0.4 M; 0.6 M; 0.8 M; 1.0 M. It was added up to 10 mL of each NaCl solution of low molarity into the column, and the eluent was collected in a labeled test tube. The result of each reservoir was a protein fraction, which was then measured for protein content. The fractions were selected from the protein fraction that had the highest absorbance value. Furthermore, the fraction with the highest absorbance value was stored at 4 °C or directly used as a sample in the DNA cutting process and cytotoxicity testing [17]. After obtaining the protein fraction, the total protein content was measured by taking 2 μL, and later its absorption was measured with nano drops at a wavelength of 260/280 nm using a 5 mM sodium phosphate buffer pH 6.5 [17]. Measurement of protein content was conducted at a wavelength of 260/280 nm because at a wavelength of 280 nm is the maximum absorption region of the protein and at a wavelength of 260 nm is the maximum absorption of nucleic acids [18].

2.4 Cytotoxic Test

The cells were taken from the CO_2 incubator, and then observed using a microscope; when the cell is in the condition of 80% confluent, the cells are ready to be harvested. The growth medium was removed using a sterile micropipette or pasteur pipette. Cells that had been successfully separated from the growth medium were washed with 5 ml of PBS. Afterward, trypsin-EDTA (trypsin 0.25%) was added evenly on all cell surfaces as much as 450 μL, then incubated in an incubator for five minutes at 37°C. The addition of trypsin-EDTA is helpful for releasing cells from the matrix. The ± 5 ml media serves were added to inactivate trypsin. Cell resuspension was accomplished using a pipette until the cells were released one by one or did not clump together. It was confirmed by observing under a microscope that there were no clustered cells. The cells that had been successfully separated one by one were transferred into a sterile conical tube [19].

To calculate the percentage of cells, 10 μL of the harvested cells were taken and pipetted into a hemocytometer under a microscope with a counter. Calculation of cells using the formula:

The number of cells counted:

$$\frac{\sum \text{room cell A} + \sum \text{room cell B} + \sum \text{room cell C} + \sum \text{room cell D}}{4} \times 10^4$$

The volume of harvested cells taken:

$$\frac{\text{The total number of cells required}}{\text{Count cell count}}$$

The number of cells needed for the test:
10^4 cells x 100 wells = 10^6 cells.
Total suspension volume:
100 wells x 100 μL = 10,000 μL [20].

The first step of sample preparation, namely the soursop seed fraction, was diluted using 0.2 M NaCl to obtain a concentration of ± 50 μg/mL. The second step was after the concentration of ± 50 μg/mL was received, a series of concentrations of the soursop seed fraction was made by taking 1 ml of the soursop seed fraction with a concentration of ± 50 μg/mL and placing it in mini tube and marking it as mini tube 1. The third step was to take a 500 μL Soursop seed fraction with a 50 μg/mL concentration added to culture media up to 1 mL and placed in mini tube and marked as mini tube 2. The same steps in the third step were performed until five mini tube with different concentrations were obtained.

In the preparation of doxorubicin, five different concentrations were made using graded dilution. The first step was to take a stock solution of doxorubicin with a concentration of 2 mg/mL as much as 25 μg and add up to 1 mL of culture media to obtain a doxorubicin concentration of 50 μg/mL and marked it as eppendorf 1. The second step was to take 500 μg of doxorubicin solution from mini tube 1, and culture medium was added to 1 mL to obtain doxorubicin levels of 25 μg/mL and marked as mini tube 2. The same thing was repeatedly done until five mini tube were obtained.

The 4T1 cells were distributed into 96 well plates (Nunc) with a total of 10,000 cells per well, which were then incubated together with the test sample (soursop seed protein fraction) with five concentration series (64.4; 32.2; 16.1; 8.05).; and 4.025 μg/mL) for 24 h in a CO_2 incubator (Heraceus). Cell control or negative control (media + 4T1 cells), media control (medium only), doxorubicin control (media + doxorubicin), control of the test compound (culture medium + cells + test sample), and positive control (media + 4T1 cells + doxorubicin). At the end of the incubation process, 100 μL MTT (Sigma) was added in RPMI medium (Gibco) for 4T1 cells into each well. Next, the plate was incubated for four hours at 37 °C to form formazan crystals (see under an inverted microscope (Zeiss)). Living cells would react with MTT to produce a purple color. After four hours, the MTT reaction was stopped by adding 10% SDS stopper reagent, 100 μL, into each well, then incubated overnight at room temperature and covered with aluminum foil. Absorption was read with an ELISA reader (Bio-Rad) at a wavelength of 550 nm [21].

2.5 Antiproliferation Test

Observation of the inhibition of proliferation by the active protein fraction on cells was completed by the MTT method. The sample concentration was below the IC50 value, which was 65.60; 32.8; 16.4; 8.2; and 4.1 μg/mL. Cell viability observations were carried out at 24, 48, and 72 h [22]. Absorbance results obtained using Elisa were used to obtain cell viability values. The doubling time value was obtained from the graph between the incubation time, and vs. the log of the number of cells that would obtain a linear regression equation $Y = Bx + A$, then the Y value was the log value two times the number of initial live cells, and the X value was the doubling time value [23].

3 Result and Discussion

Soursop *(Annona muricata)* seed extraction was *conducted* at cold temperatures to ensure that the protein contained in it does not experience denaturation due to temperature. A

Table 1. The IC_{50} value of soursop seed fraction and doxorubicin

Sample	Concentra-tion (μg/mL)	% of Living Cells Average	IC_{50} (μg/mL)
Soursop	64.40	48.02	73.96
	32.20	73.33	
	16.10	88.25	
	8.05	99.77	
	4.025	113.12	
Doxoru- bicin	50	9.21	5.07
	25	26.22	
	12,50	44.23	
	6,25	45.72	
	3,125	53.82	

cytotoxic test was completed to obtain the value of Inhibition Concentration 50 (IC_{50}) using the MTT assay method. The IC_{50} value is used to determine the potential toxicity of a compound to test [11]. The potential for toxicity of the test compound is more significant if the IC_{50} value is getting smaller [24]. The MTT method has the advantage that the MTT test is fast, sensitive, accurate, and many samples can be tested [25]. The MTT method is based on the tetrazolium salt (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide), which is cleaved by the succinate tetrazolium reductase system through the mitochondrial respiratory pathway of living cells, resulting in a purple formazan crystal and insoluble in water. This formazan crystal is directly proportional to the number of living cells; the higher the intensity of the purple color in the formazan crystal, the more the number of living cells [15]. Cytotoxicity assay results from the soursop seed protein fractions are shown in Table 1..

There are several categories in the grouping of IC_{50} values for anti-cancer activity, namely a compound, which can be classified as very active (IC_{50} value 20 μg/mL), moderately active (IC_{50} value 21–200 μg/mL), weak (IC_{50} value 201 -500 μg/mL), and inactive > 501 μg/mL [12]. Based on the IC_{50} classification, the cytotoxicity test results using an ELISA reader with a wavelength of 550 nm in this study were moderately active against 4T1 cells with an IC_{50} value of 73.96 ± 25.10 μg/mL, and IC_{50} values in the positive control using doxorubicin were very active. Meanwhile, the results of the soursop seed fraction test used cells Vero and Hela were non-toxic (Sari and Indrayudha, 2021 unpublished data), and in the test using MCF-7 cells, they were quite active with an IC_{50} value of 429.90 ± 9.77 μg/mL (Rahma and Indrayudha, 2021 unpublished data). A summary of the IC_{50} values in this study can be seen in (Table 1.).

The antiproliferative test used the MTT assay method, and the value would be obtained by doubling time. Doubling time is the time the cells take to develop into a double in number [26] The formula doubling time is: $Doubling\ time = \frac{Y-A}{B}$ x 100%. The concentration of the test solution used is below the IC_{50} value, which is 65.60; 32.8;

Table 2. Doubling time of soursop seeds

Sample	Concentration (μg/mL)	Y	Regression Linear	Slope	Intercept	Doubling Time
Control Cell	-	4.301029996	$y = 0.0046x + 3.8395$	0.0046	3.8395	100.33
Soursop	3.75	4.301029996	$y = -1.9762x + 157.34$	−1.9762	157.34	77.44

Description Y = log (2x the initial number of living cells); A = Intercept; B = Slope.

16.4; 8.2; and 4.1 μg/mL obtained from the cytotoxicity test so that the growth and morphology of cells can be observed so that not many cells die due to the toxic effects of the test compound [27] and the concentration of doxorubicin is 50; 25; 12.5; 6.25; 3.125 μg/mL. Table 2. shows the doubling time calculation. The results of the control antiproliferative test were 100.33 h, while the test group was 77.44 h. The doubling time value of control cells is more significant than the doubling time of the soursop seed sample, which means that the soursop seed sample does not have antiproliferative activity against 4T1 cells.

4 Conclusion

Based on the research recently conducted, the soursop seed fraction has the potential to 4T1 cancer cells seen from the IC_{50} value, but further research is needed on this topic, considering the poor antiproliferative results.

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