Cytotoxic Activity and Antiproliferation of Soursop Seed (*Annona muricata* L.) Protein on MCF-7 Cells

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Abstract. One of the new anticancer treatments that can be developed is a protein similar to RIPs. RIP is an enzyme found in various parts of plants that is toxic and can inhibit protein synthesis. Soursop seeds (*Annona muricata* L.) may be developed into anticancer. The plant of the same genus, *Annona Squamosa* L., has been proven to have a protein content similar to that of RIPs because it can supercoil DNA into circular nicks at low levels. Another study reported that soursop seed protein had moderate cytotoxic activity against 4T1 cells but did not show any antiproliferative activity. This study was conducted to determine the cytotoxic and antiproliferative activity of soursop seed (*Annona muricata* L.) protein against MCF-7 cells. Cytotoxic and antiproliferative activity tests were carried out using the MTT method on the protein fraction of soursop seeds. Cytotoxicity test was performed within 24 h. The antiproliferative test was carried out for 24, 48, and 72 h with the test parameter, doubling time. The results of the cytotoxicity test obtained that the protein fraction had weak cytotoxic activity with an IC_{50} value of 429,90 μg /mL. The antiproliferative results implied that there was no growth inhibition as indicated by the concentration of the protein fractions of 7,5 μg/mL and 3,75 μg/mL, which were unable to prolong the time twice compared to control cells. The results of this study showed that the protein fractions of soursop seeds did not have antiproliferative activity against MCF-7 cells.

Keywords: Soursop Seed · RIPs · Cytotoxic · Antiproliferation

1 Introduction

Cancer is one of the leading causes of death worldwide. One of the most common prevalence in Indonesia is breast cancer. Based on data from The Global Cancer Observation in 2020, breast cancer ranked as the second leading cause of cancer death in Indonesia with total of 22,430 cases [1]. It is expected to increase in the future due to changes in lifestyle, diet, stress, and environmental changes. Cancer treatments that are currently developing such as surgery, chemotherapy, and radiation entail disadvantages, such as high cost, adverse drug reactions, and significant side effects. Currently, various anticancer treatments using natural ingredients have been advanced. One available instance

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as an anticancer is a protein similar to RIPs (Ribosome Inactivating Proteins) from a plant. RIPs (Ribosome Inactivating Proteins) is an enzyme identified in plants, fungi, algae, and bacteria, containing toxic due to the activity of N-glycosylase which can inhibit protein synthesis [2].

Several RIPs (Ribosome Inactivating Proteins) exhibit activity against cervical cancer, choriocarcinoma, leukaemia/lymphoma, stomach cancer, and breast cancer [3]. The RIPs isolated from *Annona Squamosa* L. were reported to be able to kill tumor cells stronger than normal cells [4]. Global extract from *Annona Squamosa* L. was reported to incorporate protein RIPs as indicated by the presence of supercoiled DNA cutting activity into circular nicks at low levels, namely pUC19 DNA cutting activity during the incubation with sugar apple global extract at 25 °C supercoiled DNA could separate at the total protein concentration 25 μg/mL [5].

The cytotoxic activity of ARIPs (*Annona squamosa* L. Ribosome Inactivating Proteins) against cancer cells in the stomach showed an IC_{50} value of 43,02 μg/ml, which was investigated as an anticancer potential [6]. [4] reported that the purification of sugar apple seed protein (*Annona Squamosa* L.) at a concentration of 0,1 μg/mL had a cytotoxic effect of 90% on EVB/NPS B-LCl cancer cells. From the data above, a potential method for further development is soursop seeds because the sugar apple plant (*Annona Squamosa* L.) is similar genus to the soursop plant (*Annona muricata* L.), therefore it is possible to possess corresponding properties within, one of which is a protein resembling RIPs.

Meanwhile, in the relevant study of soursop seed protein, the results of Indrayudha and Dewi (2021, data unreleased) found moderate cytotoxic activity in the soursop seed protein fraction against 4T1 cells with an IC_{50} value of 73,960 μg/mL $\pm$ 25,11, however it failed to inhibit 4T1 cell proliferation as shown by the inability of soursop seed protein fraction to prolong the doubling time value of control cells. The results of another study by Indrayudha and Sari (2021, data unreleased) did not confirm cytotoxic activity against HeLa cells or Vero cells.

Based on the data aforementioned, *Annona muricata* L. is promising to be developed into an anticancer treatment. This study aimed to examine the cytotoxic and antiproliferative activities of soursop seed protein *(Annona muricata* L.) isolated using DEAE matrix against MCF-7 cells. In this study, protein fraction was isolated from *Annona Muricata* L. seeds and tested for its cytotoxic activity against MCF-7 cells, and its activity in inhibiting the proliferation of the MCF-7 cell culture was observed.

2 Method

2.1 Tools and Materials

The tools used consist of glasses, stands and clamps, small test tubes, test tube racks, conical tubes, autoclaves, high speed cooled centrifuges, micropipettes (20, 200, 1000 L), analytical balances (Libra-Shimadzu EB-330), pH meter (Electrofac merrohm), UV spectrophotometer (Backman) (NanoDrop), yellow tip, blue tip, white tip, Ependorf tube (Biorad), Incubator thermostat, Ultra Violet lamp (Biorrad), refrigerator, magnetic stirrer, CO2 incubator (Heraceus), 96 well plates (Nunc), inverted microscope (Zeiss),

ELISA reader (Bio-Rad), Cyto Culture (ESCO) and a disposal site for used media, and PBS.

The materials required are soursop seeds procured from Pasar Gede Surakarta; sodium chloride in 5 mM phosphate buffer pH 7,2; sterile distilled water; sodium phosphate buffer 5 mM pH 7.2; NaCl 0.2 and 0.4; ammonium sulphate and column with DEAE matrix; MCF-7 cell cultures were obtained from the stock of the UMS Pharmaceutical Mammal Cell Culture Laboratory; phosphate buffered saline 1×; trypsin-EDTA 0.25%; culture media (RPMI/DMEM); DMSO; MTT 5 mg/mL PBS (50 mg MTT and 10 mL PBS); SDS 10% in 0.01 N HCl; tissues; aluminum foil; doxorubicin (positive control).

2.2 Research Procedure

2.2.1 Soursop Seed Protein Extract

Soursop seeds were cleansed, weighed of 50 g, cut into small pieces, crushed, and then extracted with sodium chloride 0,14 M at 4 °C in 25 mL sodium phosphate buffer 5 mM pH 7,2. The extract was extracted using a small size filter, and the liquid was centrifuged at 14.000 rpm for 2 min. The supernatant obtained was sapwood extract and then stored at 4 °C [7].

2.2.2 Soursop Seed Protein Isolation with DEAE Matrix

Prepared DEAE matrix was eluted with 50 mL of sterile distilled water. It was followed by elution with 5 mM sodium phosphate buffer pH 7, 2 amounting 10 mL. Following that, the sample in the form of the supernatant was incorporated as much as 10 mL in the column. Eluent was prepared in form of NaCl solution with a molarity of 0, 2 M and 0,4 M. Then, it was placed in a column as much as 10 mL of NaCl solution, starting from the molarity of 0,2 M and 0,4 M. Next, the eluent was accommodated in a marked test tube. The results of the accommodated eluent are protein fractions, then the protein content was measured. The active fraction was selected from the protein fraction with the highest absorbance, which was then stored at 4 °C or could be used as a sample in DNA cleavage and cytotoxic assays [8].

2.2.3 Protein Level Measurement

Total protein content was measured by drawing 2 μL of sapwood extract, isolated protein fraction using a DEAE column, and measured the absorption with NanoDrops at a wavelength of 260/280 nm using a 5 mM sodium phosphate buffer blank at pH 6, 5 [8]. Measurement of protein content was performed at a wavelength of 260/280 nm because the wavelength of 280 nm is the maximum absorption region of protein and at a wavelength of 260 nm is the maximum absorption of nucleic acids [9].

2.2.4 Cell Harvest and Cell Counting

Cell cultures with 80% confluent were harvested. The media was discarded in the culture flask and the cells were cleansed using 5 mL of PBS. Then, the cells were poured together

with 450 μL of trypsin-EDTA 0,25% and incubated in a CO_2 incubator for 5 min. The incubated cells were added with 5 mL of MK and resuspended to remove the cells from the culture flask. A total of 5 mL of the obtained cell suspension was transferred to a conical tube and 10 μL was taken to be counted in a hemocytometer consisting of 4 counting chambers.

$$\text{Number of calculated cells} = \frac{\sum \text{sel A} + \sum \text{sel B} + \sum \text{sel C} + \sum \text{sel D}}{4} \times 100$$

$$\text{Transferred cell volume} = \frac{\text{total number of cell require}}{\text{total cell count}}$$

Number of cells required for the test = 10^4 cells × 100 wells = 10^6 cells. Total volume of cell suspension = 100 wells × 100 μL = 10,000 μL [10].

2.2.5 Cytotoxic Test

Cytotoxic test applied the MTT assay. The MCF-7 cells were distributed into 96 well plates (Nunc) with a total of 10.000 cells per well and incubated with the test sample (soursop seed protein isolate) with 5 concentration series (64,40; 32,20; 16,1; 8,05; 4,025 μg/mL) for 24 h in a CO_2 incubator (Heraceus). Cell control (media + MCF-7), media control (media only), solvent control (media + buffer), and positive control (media + MCF-7 cells + doxorubicin) were used. At the end of incubation process, 100 μL MTT (Sigma) was included in RPMI medium (Gibco) in each well. Then, the plate was incubated for 4 h at 37 °C to form formazan crystals [see under an inverted microscope (Zeiss)]. Live cells will react with MTT to form a purple color; the more purple the color, the more cells are alive [11]. After 4 h, The MTT reaction was stopped by appending 10% SDS stopper reagent, 100 μL in each well, then incubated overnight at room temperature covered by aluminum foil. The absorption was interpreted using an ELISA reader (Bio-Rad) at a wavelength of 595 nm [12].

2.2.6 Proliferation Inhibition Observation

Observation of proliferation inhibition by active protein isolate on cells was conducted by means of the MTT method. The concentration of the sample used is below the IC_{50} value (60 μg/mL). Observations were carried out at 0, 24, 48, and 72 h [13].

2.3 Data Analysis

2.3.1 Calculating IC_{50} Value

The IC_{50} value determination based on the results of the MTT test was conducted using the following formula:

If the absorbance of the control solvent is similar to the control cell, then:

$$\text{Percentage of cell viability} = \frac{\text{Absorption of treatment} - \text{Absorption of media control}}{\text{Absorption of cell control} - \text{Absorption of media control}} \times 100\%$$

If the absorbance of the control solvent is lower than the absorbance of the control cell then:

$$\text{Percentage of cell viability} = \frac{\text{Absorption of treatment} - \text{Absorption of media control}}{\text{Absorption of solvent control} - \text{Absorption of media control}} \times 100\%$$

Subsequently, a graph of the concentration vs percentage of live cells was created. Hence, it obtained a linear regression equation from the graph. Based on the value of r in the linear regression equation, if r is greater than r table, the linear regression equation fulfils the standard for calculating the IC_{50} value. Then, enter y = 50% in the linear regression equation and the x value was acquired. After that, the antilog of the concentration was calculated so the IC_{50} value was obtained [14].

2.3.2 Antiproliferation Test

To assess the results of the anti-proliferative test, it was enumerated by the following formula:

$$\text{Doubling time} = \frac{Y - A}{B} \times 100\%$$

Description: Y = log (2× the initial number of live cells); A = Intercept; B = Slope [15]. The doubling time value can be obtained from the linear regression equation (Y = Bx + A) between incubation time vs log number of live cells by entering the log value 2 times the initial cell as the y value so that the doubling time value is obtained as the x value [16].

3 Results and Discussion

3.1 Soursop Seed Protein Extract

Purification of protein in a solution can be done by ion-exchange column chromatography method. Prior to protein purification, the protein contained in *Annona Muricata* L. seeds was separated by extraction using 0, 14 M NaCl in 5mM phosphate buffer pH 7, 2. This was intended to increase protein solubility so it could be separated during filtration [17]. Soursop seed extraction with 5 mM sodium phosphate pH 7, 2 containing 0,14 M sodium chloride at 4 ºC yielded approximately 36 mL of sapwood extract. Centrifugation was carried out to separate the precipitated protein. Separated proteins from the extracted global extract were split using a DEAE column, which is an anion exchange chromatography that has a positive charge on the surface used to bind negatively charged proteins [18]. The protein fraction was obtained by integrating 5 mM phosphate buffer pH 7, 2 to break proteins that were unbound to the stationary phase followed by elution with 0,2 M and 0,4 M NaCl solutions (Fig. 1). The protein fraction separated by the DEAE column was measured to find its protein content at a wavelength of 260/280, which is the maximum absorption region of protein and nucleic acids [9]. The results of the measurement of protein fraction level of soursop seeds are shown in Fig. 1:

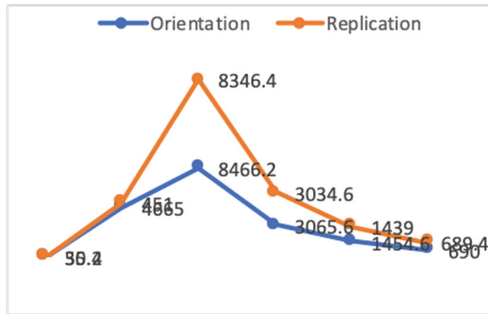

Fig. 1. Soursop seed protein fraction profile separated by DEAE650M column at a wavelength of 260/280

Based on Fig. 1, the results of the measurement of protein fraction levels at a wavelength of 260/280 obtained 6 peaks, with the highest peak at the beginning of elution with 0, 2 M NaCl solution. In protein purification, there were 2 forms of active proteins, which are those eluted without a sodium chloride gradient and eluted using 0, 2 M and 0,4 M sodium chloride [19]. The highest peak concentration at the beginning of elution with 0, 2 M NaCl contained protein bound to the stationary phase or the active protein fraction. A similar pattern was also reported in the purification of the protein fraction from *Mirabilis japala* L. leaves with the CM Sepharose CL-6B column which discovered the highest cytotoxic activity in the protein fraction bound to the stationary phase with the percentage of HeLa cell death of 29,23%, as a result of the active protein fraction [8].

Shen [20] reported the pattern of mechanisms in anion exchange chromatography containing a positively charged resin which will attract negatively charged solutes. In low salt solutions, a protein with a negative surface charge will be strongly bound to a positively charged anion exchange column, then buffers of higher salt concentrations will elute negatively charged proteins bound in the stationary phase. This condition is referred to as active protein fraction.

Based on the graph shown in (Fig. 1), the active protein fraction was carried out for cytotoxicity experiments on MCF-7 cells which are representative of breast cancer cells in order to determine that the active protein fraction bound to stationary phase has cytotoxic activity indicated by IC_{50} value, a parameter of toxicity to cancer cells [21].

3.2 Cytotoxic Test

In this study, a cytotoxicity test was conducted to measure the potential toxicity of the soursop seed protein fraction to MCF-7 breast cancer cells, which is a common measurement implemented in in vitro biological studies. Cytotoxic test on MCF-7 cells was administered to ascertain the potential toxicity of the soursop seed protein fraction which was used to determine the dose in the doubling time test [22]. The parameter in the cytotoxic test is the IC_{50} value. The IC_{50} value is a concentration that may cause the death of 50% of the cell population, therefore the level of toxicity can be anticipated [23]. The method in the cytotoxicity test is the MTT method.

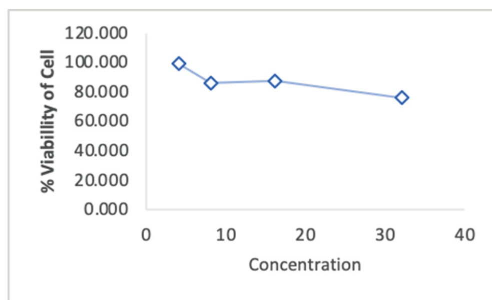

Fig. 2. Cytotoxic effect profile of *Annona Muricata* L. protein fraction on MCF-7 cells.

The principle of the test is to decide cell viability through the determination of mitochondrial function of cells which is done by measuring the activity of mitochondrial enzymes, such as succinate dehydrogenase, then MTT will be reduced to purple formazan using NADH [24]. The purple formazan crystals were not soluble in water, thus a stopper reagent was added to dissolve the crystals and then measured for absorbance with an ELISA reader at a wavelength of 550 nm ($\lambda = 550$–600 nm) [14]. The absorbance value obtained was calculated by finding the percentage of viable cells and the IC_{50} value. Soursop seed protein fraction treatment with a series content of 32,20; 16,10; 8,05; 4,025 μg/mL against MCF-7 cells was depicted in the curve of the relationship between the concentration vs the percentage of cell viability (Fig. 2). It can be understood that the cell growth at a concentration of 32,20 μg/mL decreased % of the number of cell viability, or in general the higher the concentration of protein fraction, the lower percentage of cell viability will be. It can be implied that the protein fraction had cytotoxic activity against MCF-7 cells. The results of the treatment of soursop seed protein fraction on MCF-7 cells were indicated by the IC_{50} value. The IC_{50} value indicated the potential toxicity of the test material, the smaller the IC_{50} value, the higher the potential of the test material as a cytotoxic agent. The IC_{50} value of soursop seed protein in cancer cells is shown in Table 1:

The results of the cytotoxic test denoted that the *Annona Muricata* L. protein fraction had weak cytotoxic activity in inhibiting the growth of MCF-7 cells, as shown by an IC_{50} value of 429,90 $\pm$ 9,77 μg/mL with an IC_{50} value of doxorubicin as a positive control 36,17 $\pm$ 26,08 μg/mL (Table 1). Classification of cytotoxic activity based on [25] is categorized as follows: very active cytotoxic activity $IC_{50} = 20$ μg/mL, moderately active = IC_{50} 21 μg/mL – 200 μg/mL, weak = IC_{50} 201 μg/mL – 500 μg/mL, and inactive = $IC_{50} > 501$ μg/mL. Based on these categories, the protein fraction of soursop seeds in this study contained weak cytotoxic activity against MCF-7 cells. Meanwhile, the results of Indrayudha and Dewi (2021, unreleased data) found potent cytotoxic activity in the protein fraction of soursop seeds against 4T1 cells with an IC_{50} value of 73,960 μg/mL $\pm$ 25,11. Thus, the protein fraction of soursop seeds are possibly developed into anticancer. It is necessary to conduct an antiproliferative test to determine the mechanism of inhibition of the growth of MCF-7 cells.

Table 1. Cytotoxic test of soursop seed protein isolated from DEAE matrix against MCF-7 cells at λ 550 nm

Sample	Concentration (µg/mL)	%Average viability Cells	IC_{50} (µg/mL)
NaCl fraction 0,2 M	32,2	75,393	429,90 ± 9,77
	16,1	87,309	
	8,05	86,418	
	4,025	99,296	
Doxorubicin (Positive Control)	50	33,392	36,17 ± 26,08
	25	57,741	
	12,5	67,875	
	6,25	20,901	
	3,125	19,564	

3.3 Antiproliferation Test

Antiproliferative activity was carried out to measure the ability of soursop seed protein to inhibit the proliferation of MCF-7 breast cancer cells. Antiproliferation is the percentage of inhibiting activity in the number of cells growing to less than 50% [26]. The antiproliferative test applied the MTT method to obtain the doubling time value, which is the time cancer cells require to multiply by 2 times [16]. The IC_{50} value obtained from the cytotoxic test was used as a parameter in the antiproliferative test. The concentration of the protein fraction used was below the IC_{50} value, 7, 5 µg/mL and 3, 75 µg/mL. Both concentrations were less than the IC_{50} value, which was 429, 90 µg/mL. In the antiproliferative test, 2 (two) concentrations below the IC_{50} value were implemented so less cells died during observation for 72 h due to the cytotoxic effect of these compounds [27]. In the graph of the relationship between incubation time and the number of cell viability, it indicated that the antiproliferative effect, namely in the treatment of protein fraction (Fig. 3), increased in the number of viable cells at 24 h, which were deemed undergone an adaptation phase (lag time) [28]. At 48 h, an increase in the number of cell viability and a decrease at 72 h were noticed. It was suspected that there was inhibition of cell proliferation, or the longer the incubation time for the protein fraction, the less the number of cell viability will be. The inhibitory effect of cell growth was also proportional to the concentration that the greater the concentration of the protein fraction tested, the more significant the inhibitory effect is. It was indicated by the declining number of cell viability. Meanwhile, in (Fig. 3) the results display that the greater the concentration, the higher the number of viable cells are, and it will be above the number of cells in the control cell. The number of cells in the protein fraction treatment was higher than the control cell. This implies that the protein fraction was unable to inhibit cell proliferation (Fig. 3). Furthermore, a linear regression equation was created between the log of the number of cell viability and the incubation time to obtain the doubling time value, which is the test parameter in the antiproliferation test [16]. From the value of the doubling time (Table 2), it obtained the speed of response inhibition of the protein fraction against

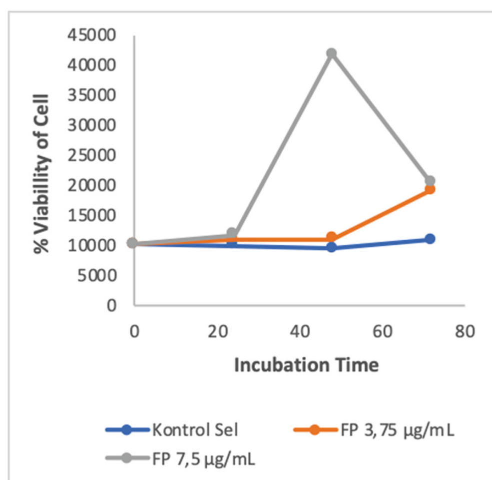

Fig. 3. MCF-7 cell growth profile at 7, 5 g/mL protein fraction concentrations and 3,75 g/mL protein fraction concentrations with incubation times of 24, 48, and 72 h

the speed of control cells to proliferate by comparing the doubling time of the control cells vs protein fraction.

The doubling time value of the protein fraction concentration of 3,75 µg/mL was 84, 37 h, meaning at 84, 37 h the cells will divide into 2 and in control cells will divide into 2 at 319, 21 h. A comparable situation happened in the protein fraction concentration of 7, 5 µg/mL.

Statistically, the treatment of protein fraction did not show a significant difference. Based on [22] antiproliferative properties, it implies that the doubling time of cells in the treatment of protein fraction is greater than the control indicating a direct inhibition. Given this statement, it can be considered that the rate of cell antiproliferation cannot be inhibited by administering protein fraction concentrations of 3,75 g/mL and 7,5 g/mL, as indicated when the doubling time of control cells was longer than the administration of protein fractions. From the linear regression equation, it is known that the inhibitory effect of the protein fraction on the proliferation of MCF-7 cells from the slope of the linear regression equation at the concentration of the protein fraction tested was smaller than the slope in the control cells [22]. The slope value of the cell control obtained was 0,0011 and the protein fraction concentration of 7,5 µg/mL and 3,75 µg/mL was 0,005 while the results obtained the slope value of the protein fraction was greater than that of control cells (Table 2).

Meanwhile, based on the results of research by Indrayudha and Dewi (2021, unreleased data) reported that the protein fraction of soursop seeds did not have antiproliferative activity against 4T1 cells as indicated by the doubling time value of 77, 44 h, which was no longer than control cells of 100,33 h. Based on this study, it showed that the protein fraction of soursop seeds with concentrations of 7, 5 and 3,75 µg/mL did not have antiproliferative activity against MCF-7 cells. So it is important to conduct further research on optimizing protein isolation techniques, therefore the active protein fraction

Table 2. Based on a linear regression equation, below are the results of the doubling time of MCF-7 cells of the effect of soursop seed protein fraction

Treatment	Equation of line	Slope	Linearity	Double Time (hours)
Cell Control	y = 0,0011x + 3,9499	0,0011	0,7203	319,21
FP 7,5 μg/mL	y = 0,005x + 4.0897	0,005	0,1882	42,27
FP 3,75 μg/mL	y = 0,005x + 3.8792	0,005	0,7446	84,37

FP: Protein Fraction

contains high purity, evaluation of the cytotoxic and antiproliferative test methods, and characterization of active protein from soursop seeds.

4 Conclusion

Based on the results of this study, it concludes that: Soursop seed protein fraction (*Annona muricata* L.) contained weak cytotoxic activity against MCF-7 cells with an IC50 value of 429.90 g/mL and did not show antiproliferative activity against MCF-7 cells as indicated by the inability of soursop seed protein fraction (*Annona muricata* L.) to prolong the doubling time of control cells. For further research, it is encouraged to optimize protein isolation techniques in order to contain high purity, evaluate protein storage to preserve protein, cytotoxic and used antiproliferative tests.

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Authors' Contributions. The following individuals contributed to the preparation of this manuscript: Itsna Sofia Rahma prepared the data collection. Analysis, and interpretation of the results of the paper, which was examined and approved by Peni Indrayudha, Maryati, Azis Saifudin, and Cita Hanif Muflihah.

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