Cytoxicity of Melinjo (*Gnetum Gnemon* L.) Seed Protein on MCF-7 and Vero Cells and Its Antiproliferation Activity

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Abstract. Melinjo seeds are one of the plants that have the potential to be developed as anticancer drugs. This study aimed to determine the cytotoxic activity of melinjo seed protein against MCF-7 and vero cells, and to determine the antiproliferative activity of melinjo seed protein against MCF-7 cells. The method used for the isolation of melinjo seed protein was SDS-PAGE. Cytotoxic and antiproliferative activities were carried out using the MTT assay method and the absorbance was read on an ELISA reader. The results showed that melinjo seed protein had cytotoxic activity against MCF-7 cells with IC_{50} of 125.89 μg/mL. The IC_{50} results of melinjo seed protein against vero were 3.37 μg/mL. Melinjo seed protein was not selective against vero cells where the SI values were 0.03. In the antiproliferative activity test of melinjo seed protein, the doubling time at levels of 1.875 μg/mL was 86.55 h, while the control cell doubling time was 112.32 h. These results indicated that melinjo seed protein was not able to inhibit the proliferation of MCF-7 cells.

Keywords: Melinjo · MCF-7 · Vero · Cytotoxic · Antiproliferative · Selectivity

1 Introduction

Cancer is a non-communicable disease characterized by abnormal and uncontrolled cell growth that can damage the surrounding tissue, besides that cancer cells can spread to places far from their original cells (metastasize). Malignant cancer cells can grow from any cell in the human body [2].

Cancer is a disease that causes the highest death in Indonesia with 204.059 deaths. Of the many types of cancer, the most common cancer found in Indonesia is breast cancer with the number of cases around 16.6% [23]. Of the many cases, to reduce the death rate from cancer, one alternative that can be done is to develop a new drug as an anticancer.

One of the plants in Indonesia that has the potential to be developed as an anticancer drug is melinjo. Melinjo seeds contain stilbenoids that have the potential as anticancer, namely transresveratrol and gnetin C which can induce apoptosis of cancer cells [17]. Medicinal plants containing Ribosome Inactivating Proteins (RIPs) are more toxic to

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A. Sri Wahyuni et al. (Eds.): ICB-Pharma 2022, AHCPS 3, pp. 339–348, 2023.
https://doi.org/10.2991/978-94-6463-050-3_29

cancer cells than normal cells, so they have the potential to be developed as anticancer drugs [14]. Cell proliferation test is a cell division cycle, in which the parent DNA divides into two daughter cells under normal conditions [12]. This study tested the active protein isolated from melinjo seeds using the DEAE matrix for its ability to inhibit the proliferation of MCF-7 cells attack the tyrosine kinase receptor specifically so that there is no stimulation of cell proliferation [6].

New anticancer agents can be developed by utilizing RIPs (Ribosome Inactivating Proteins) in a plant. RIPs are toxic proteins that are widely distributed in higher plants and some are found in fungi and bacteria [7]. The presence of RIPs in a plant can be identified through their enzymatic activity in cutting double-stranded supercoiled DNA into nick circular DNA or linear DNA in vitro [8]. The potent cytotoxic activity of RIPs can be used as candidates for the development of immunotoxins for cancer therapy [9].

2 Method

The materials used for extraction, fractionation and isolation were melinjo seeds obtained from Tlobong Delanggu Klaten, Sodium phosphate buffer, ammonium sulfate buffer, NaCl solution, DEAE matrix. The materials used for the cytotoxic test were MCF-7 cell cultures and vero cells obtained from the Mammal Cell Culture Laboratory of the Pharmaceutical Biology Division of UMS; Roswell Park Memorial Institute (RPMI), Dulbecco's Modified Eagle Medium (DMEM), Phosphate Buferred Saline (PBS), Sodium Dodecyl Sulfate (SDS) 10% in 0.01 N HCL (Stopper solution), MTT solution (3-(4.5-Dimethylthiazol-2-yl)-2.5 diphenyl-tetrazolium bromide), Doxorubicin, Fetal Bovine Serum (FBS), trypsin-EDTA.

The tools used in this study were Laboratory glassware, CO_2 incubator (binder), 96 well plate (Nunc), cytotoxic safety cabinet (ESCO), analytical balance (ohaus), centrifugator (PLC series), ELISA reader (Epoch biotek), nanodrop (biodrop), inverted microscope and camera (opti lab), pH meter (Lutron pH-206), hemocytometer.

2.1 Melinjo Seed Protein Extraction

Melinjo seeds were washed, then weighed as much as 50 g and mashed. After that, it was extracted with 0,14 M sodium chloride at 4 °C in 50 mL of 5 mM sodium phosphate buffer pH 7.2. Furthermore, the extract was squeezed, the liquid obtained was centrifuged cold at a speed of 7,000 rpm for 5 min. Then the results of the centrifuge were stored at 4 °C [15].

2.2 Melinjo Seed Protein Isolation with DEAE Matrix

The DEAE matrix was eluted with 50 mL sterile distilled water. Then continued to be eluted with 5 mM sodium phosphate buffer pH 7,2 as much as 10 mL. The sample is inserted into the column as much as 10 mL. Prepared eluent in the form of NaCl solution with increasing molarity of 0.2 M and 0.4 M. Added NaCl solution into the column as much as 10 mL starting from the low molarity. The resulting eluent is accommodated in a test tube, then the protein content is measured [4].

2.3 Protein Level Measurement

Two μL of isolated protein fraction was taken with a DEAE column, then the absorption was measured with nanodrops at a wavelength of 260/280 nm using a 5 mM sodium phosphate buffer pH 6.5 blank [19]. The melinjo seed fraction was found to be 5286.8 μg/mL and then diluted to 47.4 μg/mL. The results of the dilution levels were used for samples in the cytotoxic test treatment.

2.4 Analysis of Melinjo Seed Protein Profile with SDS-PAGE

According to Coligan et,al [8] the Laemmli method used the Laemmli buffer system. The concentration of polyacrylamide gel used was 12%. Prepared 2 glass plates for molding polyacrylamide gel. Transfer the separating gel solution that has been made, then put into the mold, then add distilled water into the mold so that the surface of the gel is flat. The gel that has dried, discarded the distilled water and the remaining water in the mold. Put the stacking gel solution into the mold, put a comb on the surface of the gel and let it sit until the gel hardens. Then the gel mold is transferred to the electrophoresis device. The melinjo seed fraction was put into Eppendorf and NaCl buffer was added. Then heated at 100 °C for 5 min. Then, 10 μL of the mixture of melinjo and buffer fractions was put into the well that had been imprinted and then electrophoresed at a voltage of 200 V for 55 min to reach the end of the gel. After electrophoresis, it was visualized with a staining solution containing coomassie brilliant blue overnight and moved with a shaker. Then the gel was washed with a destaining solution for 15 min, and was washed twice.

2.5 Harvest and Cell Counting

Cell cultures that were 80% confluent were harvested. The media in the culture flask was discarded, and the cells were washed using 5 mL of PBS, and added 450 μL of trypsin-EDTA 0.25%, then incubated for 5 min. After incubation, 5 mL of MK was added and resuspended. 5 mL of the cell suspension was transferred into the conical tube, then 10 μL was taken to be counted in a hemocytometer.

$$\text{Count of cells count} : \frac{\sum \text{cell A} + \text{cell B} + \sum \text{cell C} + + \sum \text{cell D}}{4} \times 10^4$$

$$\text{Transferred cell volume} : \frac{\textit{Total number of cells required}}{\textit{count cell count}}$$

Number of cells needed for the test = 10^4 cells × 100 wells = 10^6.
Total volume of cell suspension = 100 wells × 100 μL = 10.000 μL [6].

2.6 Cytotoxicity Test Against MCF-7 Cells and Vero Cells

Cytotoxicity test using MTT assay. MCF-7 cells were distributed into each of 96 different well plates (Nunc) with the number of 10,000 cells per well and incubated with the melinjo seed fraction with 5 concentration series for 24 h. After incubation, 100 μL

of MTT in DMEM medium for MCF-7 cells was added to the wells. Then, the plate was incubated again for 4 h at 37 °C to form formazan crystals (see under an inverted microscope). After 4 h, 100 μL of 10% SDS stopper reagent was added to each well. Then it was incubated overnight at room temperature and covered with aluminum foil. The absorption was read by ELISA reader at $\lambda = 595$ nm [13]. Do the same way for vero cells at $\lambda = 550$ nm.

2.7 Proliferation Observation of MCF-7 Cells

Antiproliferation observations by the protein fraction of melinjo seeds on MCF-7 cells were carried out using the MTT assay method. The concentration of the sample used is below the IC_{50} value, namely ½, ¼, 1/5 and 1/8 of the IC_{50} for MCF-7 cells. Then observed at 0, 24, 48 and 72 h [6]. Then calculated the inhibition of proliferation with the formula:

$$\text{Doubling time} : \frac{Y - A}{B}$$

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

2.8 Selectivity Index

To determine the selectivity value of melinjo seed isolate, samples were tested for their cytotoxic effect on vero cells. Based on the results of the MTT test on the Vero cell, the selectivity index is determined by the formula:

$$\text{SI} : \frac{IC50\ vero\ cells}{IC50\ cancer\ cells}$$

The extract is said to be less selective if the SI value is < 3 and selective if the SI value is ≥ 3 [22].

3 Discussion

3.1 SDS-PAGE Analysis

In this study, the protein analysis of melinjo seeds was carried out using the SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) method which aims to determine the presence or absence of protein contained in melinjo seeds. The principle of SDS PAGE analysis is an analysis that involves the initial denaturation of component proteins with anionic detergents that bind to proteins, giving the proteins a negative charge proportional to the molecular mass of the protein [21]. Molecules that have a smaller mass will move faster on the gel, while molecules that have a larger mass will move slowly, resulting in a band adjacent to the well on the gel [1].

The results of the SDS-PAGE analysis of the melinjo seed fraction showed the presence of protein bands in the melinjo seeds with different thicknesses (Fig. 1). The thickness and thinness of the protein bands seen on the polyacrylamide gel is an illustration of the amount of protein contained in the profile of a protein. Based on the picture, the thickest band is at melinjo DEAE 50 μg with a molecular weight of 25 kD.

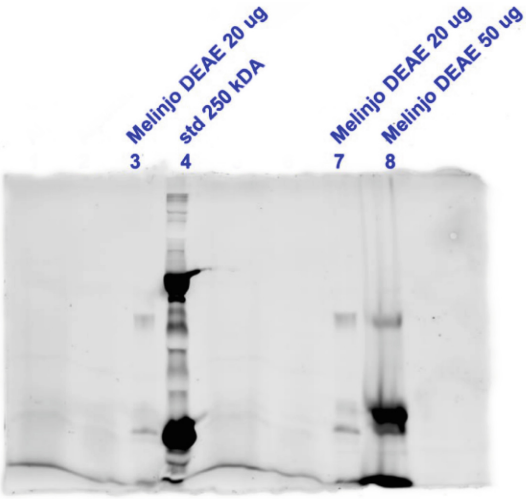

Fig. 1. SDS-PAGE results of melinjo seed protein

Table 1. Melinjo seed protein content reading data

Sample	A_{260}/A_{280}	Concentration (μg/mL)	IC_{50} (μg/mL)
Melinjo seed fraction 1*	1.000	47.40	125.89
Melinjo seed fraction 2**	0.750	78.40	3.37

**concentration used for cytotoxic assay on MCF-7 cells

3.2 Protein Isolation

In this study, the isolation technique used was ion exchange chromatography. The ion-exchange chromatography technique relies on the charge of the molecule in the mobile phase and the charge of the bound group on the stationary phase. The functional groups on the surface of the stationary phase will experience neutralization due to the opposite ion charge in the mobile phase [16]. The mobile phase used in this study was NaCl, and the stationary phase used was the DEAE matrix.

Based on Table 1 the levels of the melinjo seed fraction were 47.40 μg/mL for MCF-7 cells and 78.40 μg/mL for vero cells, with values A260/A280 for MCF-7 and vero cells respectively 1.000 and 0,750. The purity value is of good quality if it has an A_{260}/A_{280} ratio of 1.7–2.0 [20]. Measurement of protein content was carried out at λ = 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 [19]. Based on these results, the value of A_{260}/A_{280} for both cells did not have a good quality purity value. This is probably because the melinjo seeds used do not come from the same tree.

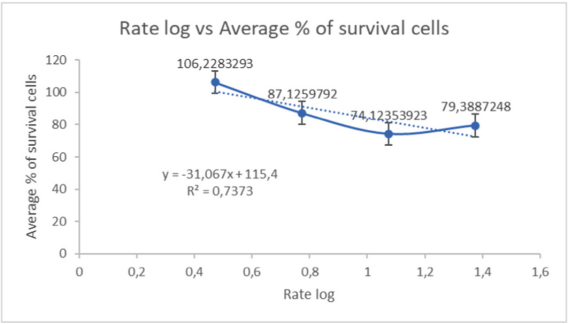

Fig. 2. Correlation curve of log concentration vs average % of survival cells melinjo seed fraction on MCF-7 cells

Table 2. Melinjo seed protein cytotoxic test data isolated using DEAE −650 M (NaCl 0.2 M) against MCF-7 cells at 595 nm

Sample	n*	Rate (μg/mL)	Average % of survival cells	IC_{50} ((μg/mL)
Melinjo seed fraction	2	23.7	79.389	125.89 ± 23.10
		11.85	74.124	
		5.925	87.126	
		2,9625	106.228	
Doxorubicin	2	25	38.038	12.88 ± 12.33
		12.5	56.980	
		6.25	61.314	
		3.125	58.328	

*n = number of replication (IC_{50} data is only 1 data used because the other data are far apart)

3.3 Cytotoxicity Test of Melinjo Seed Protein Against MCF-7 Cells

In this study, a cytotoxicity test was conducted to determine the effect of melinjo seed protein toxicity on MCF-7 cancer cells. Cytotoxicity test is a test that can be used to determine the ability of an extract or fraction to give a toxic effect to a cell at a certain concentration. And to determine the level of the test sample that can inhibit cell growth up to 50% (IC_{50}). The cytotoxicity method commonly used is the MTT method (3-(4.5-dimethylthiazole-2-yl)-2.5-diphenyl tetrazolium bromide) [18]. The principle of the test is the reduction of the yellow MTT tetrazolium salt by the reductase enzyme, tetrazolium succinate which enters the respiratory chain in the mitochondria of living cells and forms purple formazan crystals and is insoluble in water [11]. In this study, doxorubicin was used as a positive control (Fig. 2).

The results of the cytotoxic test of melinjo seeds against MCF-7 showed IC_{50} values of 125.89 μg/mL. Meanwhile, the IC50 values for the positive control were 12.88 μg/mL (Table 2). According to the American National Cancer Institute (NCI), the criteria for the toxicity of a compound to cancer cells are IC_{50} 20 g/ml = very active, IC_{50} 21–200 g/ml

Table 3. Selectivity Index

Sample	IC_{50} (μg/mL)		Selectivity Index
	MCF-7 cells	Cell Vero	
Melinjo seed fraction	125.89 ± 23.10	3.37 ± 2.59	0.03

Table 4. Melinjo seed protein antiproliferation test data isolated using DEAE -650 M (NaCl 0.2 M) against MCF-7 cells at 595 nm

Sample	Concentration (μg/mL)	Number of Living Cells				Similarity between incubation time vs log number of live cells	Dou-bling Time (Hours)
		0 hour	24 hour	48 hour	72 hour		
Cell Control	-	10000	0.770	0.861	0.978	Y = 0.0045x + 3.7956	112.32
B5	1.875	10000	114.65	100.33	134.04	Y = 0.0059x + 3.7904	86.55

= moderately active, IC_{50} 201–500 g/ml = weak. Based on the results of the study, it showed that the melinjo seed group was in the moderately category, and the positive control, namely doxorubicin, was in the very toxic category.

3.4 Selectivity Test of Melinjo Seed Protein Against Vero Cells

Melinjo seed cytotoxic test was carried out on Vero cells to see the selectivity index. Vero cells are cultured cells obtained from mammals, these cells are derived from the kidneys of African green monkeys [3]. The results of the cytotoxic test of melinjo seeds against Vero cells showed IC50 values of 3.37 μg/mL. If a fraction can only inactivated cancer cells and not cause to normal cells, the fraction is said to be selective [5]. To find out a selective or non-selective fraction in inactivated cancer cells, it can be known by using the selectivity index (SI). The sample is said to be selective if the SI value ≥ 3, and not selective if the SI value < 3 [22].

The SI value of oriented melinjo seed protein was 0.03 (Table 3). According to Demirgan *et,al* [9], the higher the SI value, the more selective a compound in cancer cells and the smaller the effect on normal cells. From these results, it can be concluded that the protein isolate of melinjo seeds is not selective against normal cells, which means that the melinjo seed fraction can inactivated cancer cells and normal cells. In this research, only 1 experiment was carried out due to time constraints and limited materials in the laboratory.

3.5 Antiproliferation Test of Melinjo Seed Protein Against MCF-7 Cells

In the observation test for inhibition of MCF-7 cell proliferation, a concentration below the IC_{50} value was used, which was 30 μg/mL. The antiproliferative test was only carried out once due to time constraints and limited materials in the laboratory. In this research,

the results of the melinjo seed fraction with levels of 1.875 μg/mL obtained doubling time values of 86, 55 h. While the value of doubling time in the control cell is 112.32 h (Table 4). A fraction has antiproliferative activity, if the doubling time value of the fraction is longer than the negative control (control cell) [10]. From the results of the study, it can be concluded that the melinjo seed fraction did not have MCF-7 cell growth inhibition activity, this was shown in the results of the sample doubling time value which was smaller than the control cell doubling time value.

4 Conclusion

From the results of this study, it can be concluded that the melinjo seed fraction has a cytotoxic effect on MCF-7 cells with IC_{50} values of 125.89 μg/mL. The selectivity index value of the melinjo seed fraction on cancer cells compared to Vero cells showed a result of < 3, meaning that the melinjo seed fraction was not selective for normal cells. The melinjo seed fraction could not inhibit the proliferation of MCF-7 cells.

Acknowledgments. Thank you to the Universitas Muhammadiyah Surakarta for supporting this research through the PUPS grant.

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