Cytotoxic Activity of Melinjo Seed Protein (*Gnetum Gnemon* L.) Against 4T1 Cells and Hela Cells, and Antiproliferation Test on 4T1 Cells

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Abstract. Melinjo (*Gnetum gnemon* L.) is an easily discovered plant in Indonesia. The previous researchers documented that the Melinjo seed has anticancer activity. This plant contains a high concentration of Ribosome Inactive Proteins (RIPs), which could inhibit protein synthesis. The research aims to determine the cytotoxic and antiproliferative activity of Melinjo seed extract against cervical cancer (HeLa) and breast cancer (4T1) cell lines. The extract was collected using the ion exchange DEAE matrix isolation method. The bioactivity test was done by MTT assay. The cytotoxic activity was determined by the IC_{50} value of 361,1 μg/mL and 939,723 μg/mL against 4T1 and HeLa cells, respectively. The antiproliferative activity was determined by doubling the time value on the concentration of 15 μg/mL and 7,5 μg/mL at 24, 48, and 72 h of the test. The result showed no inhibition activity of the extract because the doubling time of the control cells group was higher than the tested group. It can be concluded that the protein fraction of Melinjo seed does not have the cytotoxic and antiproliferative activities against HeLa and 4T1 cell lines.

Keywords: Antiproliferation · Melinjo seed protein (Gnetum gnemon L.) · cytotoxic · 4T1 cells · HeLa cells

1 Introduction

Cancer is an abnormal growth of cells in the body that can cause organ damage. Cancer is the third leading cause of death in Indonesia following heart disease and stroke. The high prevalence of cancers in women is breast and cervical cancer. Based on data from the International Agency for Research on Cancer (IARC), it is identified that in 2020 there will be 2.261.419 new cases of breast cancer and 604,127 new cases of cervical cancer in the world. Cancer prevalence continues to grow and is predicted to increase along with the deteriorating pattern of human life [2]. The occurrence of metastasis and disease recurrence are the leading causes of casualty in cancer patients [3].

The 4T1 cancer cells are a type of murine breast cancer with triple-negative immunological characters with strong metastatic abilities. This type of cancer covers 12–17% of

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the total breast cancer patients in the world. Triple-Negative Breast Cancer (TNBC) has a higher rate of aggressiveness and recurrence compared to other breast cancer subtypes [4]. HeLa cells are cervical cancer cells due to infection with Human Papillomavirus (HPV 18). They have different characteristics from normal cervical cells. Cervical cancer cells infected with HPV are known to express two oncogenes, namely E6 and E7 [5].

Cancer treatment options include chemotherapy, immunotherapy, radiotherapy, and surgery [6]. Chemotherapy is one option that is frequently used in cancer management; however, it has severe side effects. Therefore, efforts are needed to minimize these side effects and increase their anticancer activity. The screening and development of anticancer agents from natural ingredients need to be continuously researched to be a safer, more effective, and selective cancer treatment option. Anticancer compounds from plants can be obtained from plant extracts or single bioactive compounds obtained from plant isolation [7]. One of the bioactive compounds from plants that can indicate anticancer activity is Ribosome Inactivating Proteins (RIPs) [8]. RIPs are proteins with the ability to depurination rRNA from mammalian, eukaryotic, and bacterial ribosomes by RNA N-glycosidase activity. RIPs from several plants are documented to have tumor cell killing activity [9]. Interest in RIPs is increasing because of their potential to become an immunotoxin to be developed as an anticancer [10].

Melinjo (*Gnetum gnemon* L.) as one of the plant commodities with high availability in Indonesia contains a protein concentration of 9–11% and various other active compounds that are useful for human health [11]. Melinjo has antibacterial, antioxidant activity, inhibits angiogenesis, inhibits premature aging, and stimulates the immune system [12]. Melinjo has been internationally widespread and has begun to be developed as a health support product. Unfortunately, this potential is actually being explored more in other countries than in Indonesia itself. Melinjo seed extract (*Gnetum gnemon* L.) has been shown to have pharmacological activity in inhibiting the proliferation of cancer cells without affecting normal cells. It was indicated by its cytotoxic activity on PANC-I, PC-3, MCF-7, and HT-29 cells with IC_{50} values sequentially 61.27; 38.26; 37.3 and 39.33 μg/mL [12]. Previous studies have reported that Melinjo seed protein extract has supercoiled DNA cleaving activity (pBSKS) and has potential as an anticancer agent (unpublished data).

Thus, the potency of Melinjo seed protein as an anticancer need to be investigated further to ensure its activity in inhibiting cancer cells by isolating protein using DEAE ion exchange matrix to obtain the active protein fraction. The cytotoxicity of the protein fraction of Melinjo seeds was tested using an MTT assay. The results of this study are expected to provide scientific data related to the potential of Melinjo seed protein as an anticancer agent, which is safer, more effective, and selective.

2 Method

2.1 Tool

The tools were glassware, autoclave, high-speed refrigerated centrifuge, electrophoresis (Mupid J), micropipette (Gilson), analytical balance (Libra-Shimadzu EB-330), pH

meter (Electrofac merrohm), water bath, vortex machine (Genei 2), UV spectrophotometer (Backman), cuvette, yellow tip, blue tip, ependorf tube (Biorad), Bunsen burner, thermostat incubator, Ultra Violet lamp (Biorrad), ose, refrigerator, magneict strirer, orbital sheker incubator, CO_2 incubator (Heraceus), 96 well plates (Nunc), inverted microscope (Zeiss), and ELISA reader (Bio-Rad).

2.2 Ingredient

2.2.1 Extraction, Fractionation, and Isolation of Melinjo Seeds

Melinjo seeds (*Gnetum gnemon* L.) were obtained from Tlobong Village, Delanggu, Klaten. Other ingredients included sodium phosphate, NaCl, ammonium sulfate, and column with DEAE matrix.

2.2.2 Cell Cytotoxic Test Material 4T1

The 4T1 and HeLa cell cultures were obtained from the stock of BPTO Laboratory, Karanganyar; RPMI 1640 media: RPMI 1640 (Sigma), sodium bicarbonate and hepes; Cell culture media: RPMI 1640 media, penicillin-streptomycin (Gibco 1% v/v and fungison (Gibco) 0.5% (v/v), Fetal bovine serum (Gibco) 10% v/v; MTT (Sigma); stopper reagent: sodium dodecyl sulfate 10% v/v in 0.01 N HCl.

2.3 Research design

2.3.1 Plant Determination

Determination was carried out to ensure plant identity and avoid errors in plant collection, which was completed by matching the state of plant morphology based on the determination key using the literature.

2.3.2 Melinjo Seed Protein Extraction

Fifty grams of crushed Melinjo seeds were 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 using a small screen-printing filter, the liquid obtained was centrifuged cold at 14,000 rpm for two minutes. The supernatant obtained was stored at 4 °C [13].

2.3.3 Melinjo Seed Protein Isolation with DEAE Matrix

Prepare the DEAE matrix by eluting it first using five times the volume of the sterile distilled water or about 50 mL. Afterward, it was continued by using 5mM sodium phosphate buffer pH 7.2 as much as 10 mL. The 10 mL sample in the supernatant form was then added to the column. Prepared eluent in the form of NaCl solution with increasing molarity, starting from 0.2 M; 0.4 M; 0.6 M; 0.8 M; 1.0 M. Each NaCl solution starting from the lowest molarity was put into a column of 10 mL, and the eluent was accommodated in a marked test tube. The results from each reservoir are protein fractions that are then measured for protein content, carried out by taking 2 μL of extract, isolated

protein fraction with DEAE column. Then, the absorption was measured by nanodrop at a wavelength of 260/280 nm using a five mM sodium phosphate buffer pH 6.5 blank. The active fraction was selected from the protein fraction with the highest absorbance and stored at 4 °C or directly used as a sample in the cytotoxic test treatment [14].

2.3.4 Cell Harvest and Cell Counting

Cell cultures that were 80% confluent were harvested. The media in the culture flask was removed, and the cells were washed using 5 mL of PBS. Then, the cells were poured with 450 μL of trypsin-EDTA 0.25% and incubated in a CO_2 incubator for five minutes. The incubated cells were added 5 mL of media culture and resuspended to remove the cells from the culture flask. A total of 5 mL of the obtained cell suspension was transferred into a conical tube, and 10 μL was taken to be counted in a hemocytometer consisting of four counting chambers. Number of calculated cells $= \frac{\sum \mathbf{selA} + \sum \mathbf{selB} + \sum \mathbf{selC} + \sum \mathbf{selD4}}{4} \times 10^4$.

Transferred cell volume $= \frac{\text{Totalnumberofcellsrequired}}{\text{Totalcountcells}}$

Number of cells needed for the test $= 10^4$ cells x 100 wells $= 10^6$ cells.

Total volume of cell suspension = 100 wells x 100 μL=10.000 μL [15]

2.3.5 Cytotoxic Test on 4T1 Cells and HeLa Cells

Cytotoxicity test was performed using MTT assay. The 4T1 cells and HeLa cells were each distributed into 96 well plates (Nunc) with a total of 10,000 cells per well and incubated with the test sample (Melinjo seed protein isolate) with five concentration series (0.0625; 0.125; 0.25; 0,5, and 1 μg/mL) for 24 h in a CO_2 incubator (Heraceus). Cell control (media + cancer cells), media control (media only), solvent control (media + buffer), and positive control (media + cancer cells + doxorubicin) were used. At the end of incubation, 100 μL MTT (Sigma) in RPMI medium (Gibco) was added to each well. Then, the plate was incubated again for four hours at 37 °C to form formazan crystals (see under an inverted microscope (Zeiss). After four hours, the MTT reaction was stopped and 10% SDS stopper reagent added, 100 μL to each well, then incubated overnight at room temperature covered with aluminum foil. Next, the absorption was read with an ELISA reader (Bio-Rad) at a wavelength of 595 nm [16].

2.3.6 Proliferation Inhibition Observation

Observation of proliferation inhibition by active protein isolate on cells was conducted using the MTT method. The sample concentration was below the IC_{50} value, namely ½, ¼, dan 1/8 from the IC_{50} for both cells. Observations were made at 0, 24, 48, and 72 h (CCRC, 2009).

2.4 Data analysis

2.4.1 Calculating IC_{50}. Value

Determining the IC_{50} value based on the results of MTT test was completed by calculating using the following formula:

If the absorbance of the solvent control is the same as the control cell, then the percentage of live cells is.

$$= \frac{\text{Treatmentabsorbance} - \text{Controlmediaabsorbance}}{\text{Contolcellabsorbance} - \text{Controlmediaabsorbance}} \text{ x } 100\%$$

If the absorbance of the control solvent is lower than the absorbance of the control cells, the percentage of live cells is

$$= \frac{\text{Treatmentabsorbance} - \text{Controlmediaabsorbance}}{\text{Solventcontrolabsorbance} - \text{Controlmediaabsorbance}} \text{ x}100\%$$

Next, a graph of the concentration vs. percentage of live cells was made. Then, it obtained a linear regression equation from the graph. Based on the value of r in the linear regression equation, if r is more significant than r table, the linear regression equation meets the standard for calculating the IC_{50} value. Next, enter y = 50% in the linear regression equation, and the x value will be obtained; then, the antilog of the concentration is calculated so that the IC_{50} value is obtained [17].

2.4.2 Antiproliferative Test

It is calculated by the following formula to assess the results of the antiproliferative test:

$$= \frac{Y - A}{R} \times 100\%$$

Where Y = log (2x the initial number of living cells); A = Intercept; B = Slope [18]. The difference in the value of doubling time between the control and treatment groups was statistically analyzed using the paired T-Test method, using the SPSS application.

3 Results and Discussion

3.1 Protein Isolation

The supernatant extract of Melinjo seed was isolated by DEAE matrix ion-exchange chromatography method to obtain the protein fraction. This method is based on the electrostatic attraction of the charge on the surface of the stationary phase with the sample. The use of ion-exchange chromatography began with elution using a predetermined pH buffer. The treatment is done expecting that the protein can be firmly bound to the column and other compounds can be eluted first. Column chromatography can release protein bonds by changing the pH of the buffer or the ionic strength of the solvent. DEAE matrix is a cation matrix that binds a functional group with a positive charge covalently [19].

The highest protein concentration was obtained in the mobile phase of 0.2 M NaCl, which was 5286.8 μg/mL for further use in cytotoxic and antiproliferative tests. The high concentration of 0.2 M NaCl mobile phase indicates that the protein fraction tends to be positively charged (cationic) because the protein eluted at the initial addition of the mobile phase with the lowest concentration.

3.2 Cytotoxic Test

Cytotoxic test with MTT assay was used to observe the cytotoxic activity of the protein fraction of Melinjo seeds as seen from the inhibition of cancer cell viability. Cell viability was described by the formation of a purplish-blue formazan product due to the reduction of the tetrazolium compound (MTT), which was originally yellow. The tetrazolium ring is cut by the activity of mitochondrial enzymes in the living cells; therefore, the formation of the formazan indicates the presence of living cells in the wells (cell viability). IC_{50} was measured, IC_{50} value defined the sample's ability to cause cell death to 50% of the cancer cell population. The smaller the IC_{50} value, the higher the potential of the test compound as an anticancer agent [20].

Doxorubicin is an anticancer agent that is commonly used in the treatment of cancer. However, the use of doxorubicin has been limited due to side effects such as hepatotoxicity, cardiotoxicity, hematologic toxicity, [21] and the risk of resistance. Based on Table 1, doxorubicin has an active cytotoxic ability with $IC_{50} < 10$ µg/Ml,

The cytotoxic activity of the extract against cancer cells is categorized as very active if the IC_{50} value is < 10 µg/mL, the active category if the IC_{50} value is 10–100 µg/mL, and the category is quite active if the IC_{50} value is 100–500 µg/mL. [22]. Results showed that the protein extract of Melinjo seeds had no activity to inhibit HeLa cells with IC_{50} values of 939.72 µg/Ml. Meanwhile, it was classified as quite active in the cytotoxic test on 4T1 cells with an IC_{50} value of 361.1 µg/mL Table 2.

Table 1. The data of the cytotoxic test results from the isolation of Melinjo seed protein and Doxorubicin against HeLa cells and 4T1 cells.

Sample	IC_{50} (µg/mL)	
	HeLa cells	4T1 cells
Melinjo seed protein fraction	939.72	361.10
Doxorubicin	2.36	5.45

* data obtained with three replicates for each concentration.

Table 2. The 4T1 cell doubling time result data

Sample	Concentration (µg/mL)	Live Cell Count Log				Rregression linear	Doubling Time
		0 hour	24 h	48 h	72 h		
Control cell	-	4.30103	3.88052	4.1559	4.20601	$y = 0.0068x + 3.7553$	80.25
B2	15	4.30103	3.8364	4.0806	4.4603	$y = 0.013x + 3.5018$	61.48
B3	7.5	4.30103	3.9042	4.103	4.4939	$y = 0.0123x + 3.5772$	58.85

* data obtained with three replications for each concentration

3.3 Antiproliferation Test

The main parameter in measuring the proliferative nature of a cancer cell is Cell cycle progression; inhibition of the cell cycle progression is completed by doubling time test using MTT method. [23]. The concentration of the test solution used refers to the previous cytotoxic test, which is below the IC_{50} value. If levels above IC_{50} are used, it is possible that cell death will occur before 72 h of incubation and the growth profile is not visible, so cell proliferation kinetics cannot be observed [20]. The absorbance results of live cells in each treatment sample concentration were 15 and 7.5 μg/mL.

No antiproliferative test was conducted on HeLa cells because, in the previous cytotoxic test, the IC_{50} value was too high. The ability of a compound to inhibit the proliferation of cancer cells is indicated by the more extended the doubling time compared to control cells [24]. The protein extract of Melinjo seed could not suppress the growth of 4T1 breast cancer cells, seen from the doubling time value of the control cells, which was more significant than after being given the test compound.

4 Conclusion

It was concluded that the protein extract of Melinjo seeds could not inhibit the growth of HeLa cervical cancer cells and has cytotoxic activity against 4T1 breast cancer. Cells with an IC_{50} value of 361.1 μg/mL. However, the protein extract of Melinjo seeds could not inhibit the proliferation of 4T1 cancer cells.

Based on these results, further research is needed to ensure the anticancer activity of the active protein fraction of Melinjo seeds.

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