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Melinjo (Gnetum Gnemon) Seed Protein Activity Against pBSKS DNA Cleavage and Its Cytotoxicity in T47D and 4T1 Cells

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Abstract.
The plant protein of melinjo (*Gnetum gnemon*) in seeds is known to have antioxidant properties. Apart from being an antioxidant, melinjo seed protein also has the potential to be developed as an anticancer compound. One of the potential anticancer compounds has been screened based on supercoiled DNA cleavage activity (pBSKS). The purpose of this study was to determine the activity of the protein fraction isolated with DEAE and BUTYL matrices against supercoiled DNA cleavage of pBSKS. Supercoiled DNA cleavage test activity of pBSKS was determined using the electrophoresis method and visualized with a UV transilluminator at a wavelength of 312 nm. The cytotoxic activity of melinjo seeds was tested through the MTT assay method and was read on an ELISA reader with a wavelength of 550 and 595 nm seen from the IC_{50} value. The protein fraction of melinjo seeds isolated with DEAE and BUTYL matrices had concentrations of 470.1 and 81.02 g/mL, respectively. The visualization results showed that the DEAE and BUTYL protein fractions resulted in depletion of supercoiled DNA bands of pBSKS. The concentration of active protein in the melinjo seeds fractionated using the DEAE-650M matrix was higher than that of the BUTYL-650M matrix. In this study, after several cytotoxic tests were carried out, various results were obtained. The activity of protein isolates from melinjo seeds fractionated using DEAE-650M and BUTYL-650M against 4T1 and T47D cells could be categorized as non-toxic because the IC_{50} value was > 1000 g/mL and the average percentage of viable cells was more than 50%, except for the seed protein fraction. Melinjo fractionated using DEAE-650M against T47D cells which had an IC_{50} value of 127.62 g/mL could be categorized as quite active and cytotoxic.

Keywords: *Gnetum gnemon* L., protein isolation, ribosome-inactivating proteins (RIPs), DNA cleavage, cytotoxic, T47D, 4T1

1. INTRODUCTION

Cancer or malignant tumors are uncontrolled cell/tissue growth, always growing or increasing, and immortal. According to Bray et al. [1] in the 2018 GLOBOCAN estimate stated that 9.6 million deaths in the world were caused by cancer, with the highest percentage of lung cancer (18.4%), breast cancer (11.6%) and colorectal cancer (9.2%) .

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In Indonesia, there was an increase in cancer cases by 1.3 times from 2013 (1.4‰) to 2018 (1.79‰) (2,3). The incidence and mortality can be reduced by the development of new drugs as anticancer.

Anticancer development can be derived from a plant protein. RIPs (Ribosome-Inactivating Proteins) is an active plant protein that has the ability to cut DNA, which is the initial screening of anticancer compounds. Supercoiled DNA cleavage by RIPs demonstrated the performance of rRNA N-β-glycosidase, leading to cleavage of the adenine-4324 residue of 28S rRNA. As a result, the relationship between the elongation factors of the ribosome is disrupted which causes inhibition of protein synthesis and the occurrence of cell apoptosis [4]. Anticancer drugs that have a mechanism of action such as RIPs are 5-fluorouracil, flavopiridol, cycloheximide, and homoharringtonine which are able to inhibit rRNA synthesis in cells. 5-fluorouracil inhibits the formation of the 5' end and flavopiridol inhibits the processing of the 3' end of 28S rRNA [5]. One of the plants that have the potential to contain RIPs is melinjo seeds.

Melinjo seeds are proven to have protein, namely Gg-AOP which is a protein isolated from seeds. Gg-AOP consists of two proteins with molecular weights of about 30 kDa (Gg-AOPI) and 12 kDa (Gg-AOPII). The two protein fractions showed significant antioxidant activity ($p<0.05$) against free radicals such as DPPH, ABTS, and superoxide anions and showed similar activity to glutathione and BHT in the linoleic acid emulsion test system [6]. The presence of significant antioxidant activity can protect cells from DNA damage mediated by ROS (Reactive Oxygen Species), so that mutations and carcinogenesis in cells do not occur [7]. However, the presence of RIPs induces mitochondrial stress and damage and reduces antioxidant activity in proliferating cells (cancer cells) causing increased levels of ROS that mediate DNA damage and cell apoptosis [8].

Based on these data, protein isolates from melinjo seeds (*Gnetum gnemon* L.) in addition to their potential as antioxidants can also be developed into anticancer compounds by detecting the presence of proteins that have RIPs activity in cutting double-stranded supercoiled DNA. The DNA cutting used pBSKS DNA modeling in vitro. Melinjo seed protein (*Gnetum gnemon* L.) was obtained from isolation using column chromatography with DEAE and BUTYL matrices.

2. METHOD

2.1. Extraction and Isolation of Melinjo Seeds Material

Melinjo seeds (*Gnetum gnemon* L.) were obtained from Purwoasri Village, Kebonagung, Pacitan. Buffer for protein extraction: 0.14 M NaCl (Merck) in 5 mM Sodium phosphate pH 7.2 (NaH2PO4 and Na2HPO4) (Merck), ammonium sulfate and column with DEAE and BUTYL matrix.

2.1.1. Competent Cell Making Materials and Transformation of Plasmid DNA

Liquid culture of Echerichia coli DH5α; Plasmid transformation material: 50 mM calcium chloride (Merck); Transformation buffer (MgCl2-CaCl2); CaCl2 0.1 N; Plasmid pBSKS-ARE-LacZ; Ice; Ampicillin; Luria Bertani (LB) solid media consisting of 5 g/L triptone, 10 g/L yeast extract, 5 g/L NaCl, and 15 g/L agar; Luria Bertani Media (LB) was solid containing ampicillin (100 g/mL) and Luria Bertani Media (LB) was liquid (without agar).

2.2. Plasmid DNA Isolation Material

Plasmid DNA of pBSKS, Escherichia coli strain DH5α, Luria Bertani Medium (LB), liquid-ampicillin LB consisted of: 1% (w/v) (Oxoid), yeast extract 0.5% (w/v) (Oxoid), sodium chloride 1% (w/v) (Merck), aquabidest to desired volume, ampicillin 100 g/ml (Boicheme). Solid LB- ampicillin was prepared by adding 1.5% (w/v) agar (oxoid) to liquid LB. Solution I lysis: glucose 50 mM (Merck), tris chloride 25 mM pH 8.0 (Sigma), EDTA 10 mM pH 8.0 (Sigma), aquadest. Solution of lysis II: NaOH 0.2 N (Merck), Na dodecyl sulfate 1% pH 7.0 (Sigma). Solution of lysis III: Potassium acetate 5 M, pH 4.8, 60 ml (Merck), glacial acetic acid 11.5 ml, aquabidest 28.5 ml. TE buffer solution, pH 8.0: Tris chloride 10 mM, pH 7.4 (Sigma) and EDTA (ethylenediamine tetraacetate) 1 mM (Sigma). Extraction solution: phenol, chloroform, 0.01 M Tris chloride pH 7.5. Precipitating and washing solution: 96% ethanol, 70% ethanol.

2.3. Agarose Gel Electrophoresis Material

Agarose 1% (w/v) (Sigma). TAE Buffer (50x/stock): Tris HCl 2.0 M (Sigma), 0.99 M Concentrated Acetic Acid, EDTA 50 mM, pH 8.0 (Merck), SYBR Green (Sigma), loading buffer . Buffers for testing supercoiled DNA cutting activity, TMN buffers: Tris chloride

50 mM pH 8.0 (Sigma), Magnesium chloride 10 mM (Merck) and Sodium chloride 100 mM (Merck).

2.4. Tools

Glassware, autoclave, high speed refrigerated centrifuge, electrophoresis (Mupid J), micro pipette (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, magnetic strirer, sheker incubator.

2.5. Plant Determination

Plant determination was carried out at the Plant Systematics Laboratory of Setia Budi University by matching the state of plant morphology based on the determination key using literature to ensure plant identity and avoid errors in plant collection.

2.6. Extraction of Melinjo Seed Protein (Gnetum gnemon L.)

Melinjo seeds were washed, cut, weighed, and mashed, then extracted with 0.14 M sodium chloride at 4°C in 100 mL of 5 mM sodium phosphate buffer pH 7.2. The resulting extract was squeezed using a small screen printing filter, the liquid obtained was cold centrifuged at 14,000 rpm for 2 minutes. The supernatant obtained was sapwood extract, then stored at -20°C .

2.7. Isolation of Melinjo Seed Protein with DEAE Matrix

The DEAE matrix was prepared by first elution using sterile distilled water as much as 5 x volume or about 25 mL. After that, the elution was continued using 10 mL of 5mM sodium phosphate buffer pH 7.2. The sample in the form of the supernatant was inserted as much as 10 mL in the column. The eluent is a NaCl solution with increasing molarity, starting from 0.2 M; 0.4 M; 0.6 M; 0.8 M; and 1.0 M was put into the column as much as 10 mL and the elution result was accommodated in a 5 mL test tube that had been tared. The results of each reservoir are protein fractions which are then measured for protein content. The active fraction was selected from the protein fraction that had

the highest absorbance which was then stored at -20°C or directly used as a sample in the pBSKS DNA cutting test treatment.

2.8. Melinjo Seed Protein Isolation with BUTYL Matrix

The BUTYL matrix was prepared by first elution using sterile distilled water as much as 5 x volume or about 25 mL. After that, the elution was continued using 10 mL of 1 M ammonium sulfate buffer pH 7.2. Samples in the form of supernatant were taken 100 mL then added 20% w/v ammonium sulfate and mixed using a stirrer. The eluent is a solution of ammonium sulfate with a decreasing molarity, 1.0 M; 0.8 M; 0.6 M; 0.4 M and 0.2 M were prepared as much as 10 mL each. The sample which already contained 20% ammonium sulfate was put into the column as much as 10 mL and ammonium sulfate eluent was added sequentially from 1.0 M to 0.2 M each as much as 10 mL, then the elution result was accommodated using a tempered test tube. of 5 mL. The results of each reservoir are protein fractions which are then measured for protein content. The active fraction was selected from the protein fraction that had the highest absorbance which was then stored at -20°C or directly used as a sample in the pBSKS DNA cutting test treatment.

2.9. Protein Level Measurement

Measurement of total protein content was carried out with a biodrop instrument at a wavelength of 260/280 nm using a 5 mM sodium phosphate buffer pH 7.2; of 2 L in each sapwood extract, the protein fraction isolated by DEAE and BUTYL columns.

2.10. Escherichia coli DH5 Competent Cell Development

Eschericia coli DH5α suspension as much as 100 L was grown in 5 mL of liquid LB medium and incubated in a shaker incubator overnight at 37°C. 1 mL of the culture sample was subcultured again in 50 mL of liquid LB medium and incubated at 37°C for 3-4 hours, ie when it reached the middle of the exponential phase (density 5x106-2x107 cells/mL (OD600 = 0.6)). The cultures obtained were centrifuged at 4,000 rpm, 4°C for 5 minutes and the supernatant was discarded. The pellet was suspended in 0.1 M cold CaCl2 as much as 0.2 x volume of the initial culture and placed on ice for 5 minutes, then centrifuged at 4,000 rpm, 4°C for 10 minutes. The supernatant was carefully removed

and the pellet obtained was washed with 0.1 M cold CaCl2 as much as 0.04 x volume of the initial culture and allowed to stand on ice for 30 minutes [9].

2.11. pBSKS DNA Transformation

A total of 200 L of competent Escherichia coli suspension in sterile Ependorf tubes was placed on ice for 5 minutes, added pBSKS DNA (amount of 50 ng in a volume of 10 L) to each tube, and gently shaken. The suspension obtained was allowed to stand on ice for 30 minutes and subjected to a heat shock at 42°C for 90 seconds and immediately put back into the ice for 1-2 minutes. The suspension resulting from the heat shock was added with 800 L of liquid LB medium, mixed slowly by inverting the tube 5-10 times and incubated at 37°C for 45 minutes. The 200 L suspension was spread evenly on solid LB medium containing the antibiotic ampicillin and incubated at 37°C for 24 hours [9].

2.12. Isolation of pBSKS DNA

Escherichia coli colonies containing pBSKS were grown in 50 mL of ampicillin-liquid LB medium at 37°C overnight in an incubator shaker. The culture was poured into sterile Ependorf, centrifuged at 14,000 rpm, 4°C for 10 minutes, the supernatant was discarded. The pellets were suspended in 100 L of cold lysis buffer I, vortexed and left on ice for 5 min. Then 200 L of lysis II solution was added, mixed by inverting the tube 5 times, and left on ice for 5 minutes. After that, added 150 L of cold neutralization solution (lysis III) and left on ice for 5 minutes. The suspension was centrifuged at 14,000 rpm, 4°C for 10 minutes, the supernatant was transferred to a new sterile Ependorf tube, extracted with phenol:chloroform (1:1), vortexed and centrifuged at 14,000 rpm, 4°C for 10 minutes. The upper phase was separated in a new and sterile Ependorf tube, then 3 M sodium acetate was added one-tenth the volume, absolute alcohol was twice the volume, and kept at -20°C for 1 hour.

The steps were followed by centrifugation at 14,000 rpm, 4°C for 10 minutes. The supernatant obtained was discarded, washed with 1 mL of 70% ethanol and centrifuged at 14,000 rpm for 15 minutes. The supernatant obtained was discarded, the pellet was dried at 37°C, dissolved in 20 L buffer TE and stored at -20°C [9].

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2.13. Supercoiled DNA Cleavage Activity Test

Five μL of pBSKS DNA was mixed with 1 L of TMN buffer and incubated with the protein fraction of melinjo seeds in 4 concentration series with dilution factors (1; 0.75; 0.50; and 0.25 times). A negative control in the form of a TMN buffer was used, a positive control in the form of a restriction enzyme (EcoR1). Then incubated at room temperature (30°C) for 1 hour, added 2 μL of loading buffer, and electrophoresis was performed on agarose gel containing SYBR Green dye, using 1x TAE buffer. Electrophoresis was carried out at 50 volts to approximately three-quarters the length of the gel. Identification was carried out with a UV transilluminator.

2.14. pBSKS DNA Cleavage Test Analysis

The data obtained from the activity test results of the protein fraction were analysed qualitatively. The cutting activity of supercoiled DNA was determined by observing 3 criteria, namely: the presence of supercoiled DNA thinning, nick circular DNA thickening, and the formation of linear DNA on the electrophoretic DNA band under a UV transilluminator. The test is said to be positive if the results of cutting protein extracts show a pattern of DNA supercoiled DNA thinning and nick circular DNA thickening at low levels, while at higher levels, a thicker linear DNA band will appear.

2.15. Cytotoxic test by MTT assay

Testing with the active fraction in the form of melinjo seed protein (*Gnetum gnemon* L.) fractionated which has the highest absorbance level after being measured by nanodrop. Cancer cells were first cultured using RPMI media and incubated using a CO_2 incubator. Cell harvesting was carried out when the cells were 80% confluent, after which the cells were washed using 5 mL of PBS. Then, 450 μL of trypsin EDTA was added and incubated for 5 minutes. Trypsin inactivation was carried out by adding 5 mL of RPMI media. The cells are then resuspended with the intention that the cells do not gather so that it can facilitate cell counting on a microscope. The cell count was done by adding 10 L of the mixture of media and cells into the hemacytometer which was then observed under a microscope, then the cells were counted using a counter. The harvested 4T1 and T47D cells were then distributed into 96 well microplates as much as 100 μL per well. Five series of concentrations of active protein samples were added to the wells starting from the lowest concentration with a sample dilution factor of 0.125 X; 0.25 X; 0.50

X; 0.75 X and 1.00 X. Added cell control, media control, positive control (Doxorubicin). Then it was incubated for 24 hours using a CO_2 incubator at 37°C. The incubation results on a 96-well microplate were given MTT reagent 5 mg/mL in 100 µL of PBS to each well including the control media, then the cells were incubated for 2 hours with a CO_2 incubator. An inverted microscope was used to see if formazan crystals had formed. Live cells will produce purple formazan crystals. Add 10% SDS stopper reagent in 0.01 N HCl and wrapped in aluminium foil or paper then incubated for 24 hours at room temperature in a place protected from light. The absorbance value is obtained by reading using an ELISA reader with a wavelength of =550-600 nm (595 nm). The absorbance results are then used to calculate the % of viable cells from these cells.

2.16. Data Analysis

Analysis to determine the cytotoxic activity of melinjo seeds using the IC_{50} parameter. The IC_{50} parameter is obtained by calculating the percentage of living cells using the formula:

% Viable cells = ((Absorbance of treatment-Absorbance of control media))/((Absorbance of control of cells-Absorbance of control of media)) × 100%

The data obtained in the form of % living cells using a formula that calculate from absorbance data. A graph was made between the concentration log vs. the percentage of viable cells, then the linear regression equation was searched (10).

3. RESULT AND DISCUSSION

3.1. Plant Determination

The results of the determination by the Plant Systematics Laboratory of Setia Budi University in letter number 106/DET/UPT-LAB/07.12.2020 stated that the sample used in this study was true *Gnetum gnemon* L. with details 1b-2b-3b-4b-6b-7b -9b-10b-11b-12b-13b-14b-16a. Group 10. Single leaf, opposite. 239b-243b-244b-248b-249a. Gnetaceae family. 1. Gnetum. 1. *Gnetum gnemon* L.

3.2. DNA isolation of pBSKS

The isolation of pBSKS DNA was initiated by plating *Escherichia coli* bacteria containing pBSKS DNA in liquid Luria Bertani (LB) media with 100 g/mL of ampicillin added. The

use of ampicillin (50-100 g/mL) in liquid culture is intended to determine the growth of cells that only store plasmids (11). The growth is known by looking at the presence of turbidity in the liquid LB media. So that ampicillin added in liquid LB media acts as a selection agent.

Cell cultures containing plasmids were harvested using centrifugation. Cell proteins were removed by alkaline lysis method. In this method, the bacterial precipitate resulting from the centrifugation is suspended in an alkaline solution containing detergent (NaOH/SDS). Detergents function to lyse cells and denature proteins. Alkaline/alkaline conditions cause chromosomal DNA, plasmid DNA, and proteins to be denatured. DNA concentration can be done by adding ethanol (12). The measurement of pBSKS DNA concentration was carried out with a biodrop instrument at a wavelength of 260/280 nm.

DNA and RNA have maximum absorption at 260 nm, whereas most proteins have strong absorption at 280 nm. However, nucleic acids also absorb significantly at 280 nm (50-55% of ab sorbance at 260 nm), and most proteins absorb strongly at 260 nm (absorbance varies depending on the protein type). Thus, it is difficult to accurately measure the concentrations of DNA, RNA and protein in complex mixtures. However, by looking at the value of the A260/A280 ratio, it can provide validation of the purity of nucleic acid samples, namely with values above 1.8 for DNA or 2.0 for RNA; lower ratio values indicate the presence of protein or other contaminants (13).

Isolation was carried out 3 times at different times, due to the low stability of the plasmid in storage. The average value of the A260/A280 ratio of pBSKS plasmid DNA isolation was 2.999. Based on the statement of (13) these values indicate that the resulting plasmid has a low purity because it is not included in the range of A260/A280 ratio values of 1.8 – 2.0; possibly contaminated with RNA.

3.3. Extraction of Melinjo Seed Protein

One of the ways to filter protein is by adding salt. Protein has unstable properties outside the original matrix, so it is necessary to make adjustments to the replacement of water by phosphate buffer in the manufacture of 0.14 M sodium chloride. Extraction is carried out by adding a 0.14 M sodium chloride in 5 mM phosphate buffer pH 7.2 at 4°C.

The measurement of protein concentration from the extraction was carried out using a biodrop instrument with the UV protein method. Extraction was carried out 3 times at different times, due to low protein stability both in processing and storage. The third

extraction was chosen to be continued in the isolation process because it has better stability in storage.

3.4. Isolation of Melinjo Seed Protein

Isolation of extracted melinjo seed protein was carried out using 2 different matrices, namely DEAE and BUTYL. The DEAE matrix has a stationary phase, namely diethyl-amine-ethyl-cellulose which is cationic, so that the fixed charge (stationary phase) is positive, and the transferable charge (protein) in the mobile phase is negative. Negatively charged proteins interact with opposite charges in the stationary phase (positive charge), leaving another protein with an identical charge to the charge in the stationary phase (14), so that the protein will be bound to the matrix (stationary phase) and have a longer retention time. longer than those of similarly charged proteins with the stationary phase.

The bound protein can then be eluted or removed from the stationary phase by a new counter ion (usually NaCl) with a greater affinity for the stationary phase charge than the protein (15), making the protein that was originally bound to the stationary phase, will switch bound in the mobile phase, namely NaCl and eluted out of the stationary phase to the reservoir.

The BUTYL matrix is a stationary phase consisting of a basic matrix and hydrophobic ligands. Proteins that have a dominant hydrophobicity will bind to hydrophobic ligands, compared to proteins that are hydrophilic (15). Protein solubility is highly dependent on the salt concentration (ionic strength) of the medium. Proteins usually have low solubility in pure water. The solubility increases with increasing ionic strength, because more and more inorganic ions are bound to the protein surface, thereby preventing molecular aggregation (salting in). Meanwhile, the high ionic strength will cause the salt to attract water from the protein resulting in aggregation and precipitation of molecules (salting out). The addition of salts such as ammonium sulfate (NH4)2SO4 can be used to separate proteins from the mixture according to the degree of solubility (fractionation), because a salting out process will occur.

The two matrixes differ in terms of the eluent used and their molarity gradient. In the DEAE matrix the eluent used is sodium chloride solution with increasing molarity, starting from 0.2 M; 0.4 M; 0.6 M; 0.8 M; and 1.0 M. While in the BUTYL matrix the eluent used was ammonium sulfate solution with a decreasing molarity, 1.0 M; 0.8 M; 0.6 M; 0.4 M and 0.2 M. However, they have one thing in common, namely the isolation process using the earth's gravity.

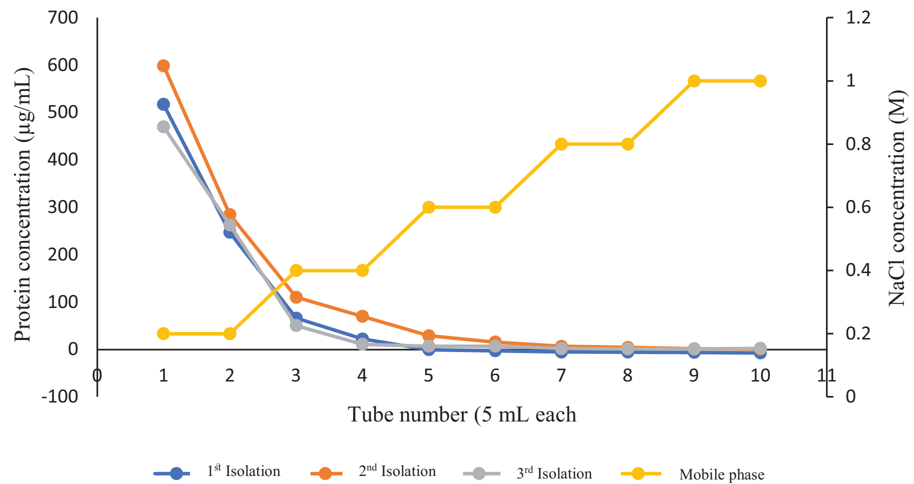

Figure 1: Graph of comparison protein isolation using DEAE matrix.

The highest average concentration in all isolations I, II, and III using the DEAE matrix is shown (Figure 3), namely at a concentration of 0.2 M sodium chloride in the first reservoir. This shows that the isolated protein fraction is stable at the same concentration, in addition, the nature of the protein fraction obtained can also be known. The high concentration in the first reservoir of 0.2 M sodium chloride 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. The average value of the third isolation concentration using the DEAE matrix was 470.1 µg/mL.

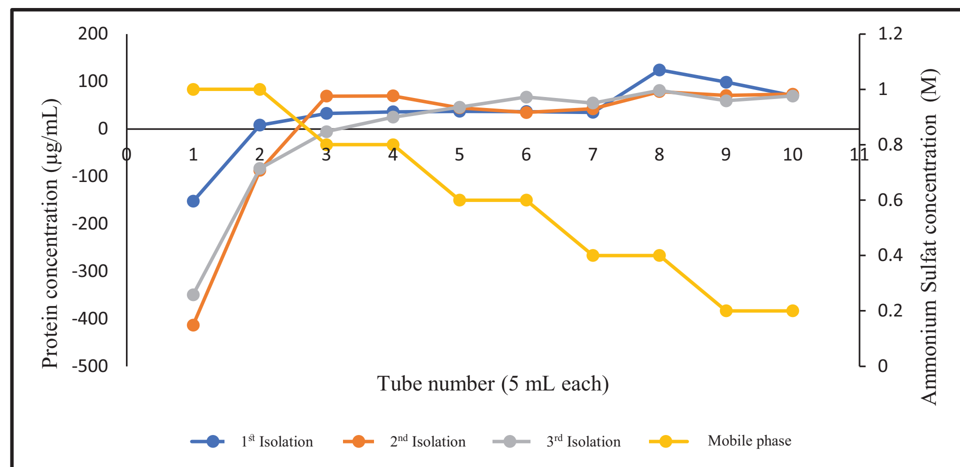

Figure 2. Graph of comparison protein isolation using BUTYL matrix

Figure 2: Graph of comparison protein isolation using BUTYL matrix.

The highest average concentration in all isolations I, II, and III using the BUTYL matrix is shown (Figure 2), namely at a concentration of 0.4 M sodium chloride in the second reservoir. This shows that the isolated protein fraction is stable at the same concentration. The resulting protein fraction was hydrophobic because the protein was retained longer in the stationary phase, indicated by a high concentration in the eighth reservoir. The average value of the third isolation concentration using the BUTYL matrix was 81.02 μg/mL.

The protein fraction used to test the pBSKS DNA cleavage activity was isolation III in each matrix, both DEAE and BUTYL. To determine the pBSKS DNA cleavage activity at a certain level, a concentration series of selected protein fractions was used (Table 1). Melinjo seed protein has the best concentration in the isolation with DEAE matrix with a concentration of 470.1 μg/mL.

Table 1: Protein consentrasi after dilution from isolation with DEAE dan BUTYL matrix

Protein Faction	Dilution factor	Protein concntration (μg/mL)
Protein fraction DEAE concentration 470,1 μg/mL	1, 00x	470,1
	0,75x	359,5
	0,50x	294,4
	0,25x	158,2
Protein fraction BUTYL concentration 81,02 μg/mL	1, 00x	82,02
	0,75x	64,72
	0,50x	48,78
	0,25x	27,70

3.5. pBSKS DNA DNA Cleavage Test

The isolated pBSKS plasmid DNA was checked using electrophoresis and visualized with a UV transilluminator to determine the presence of the plasmid. Electrophoresis was carried out using agarose gel with a concentration of 2% with 1X TAE buffer. The results of the visualization of the UV transilluminator are shown in Figure ??. The bright greenish band on the electrophoresis results indicates the presence of the pBSKS plasmid DNA.

The pBSKS DNA cleavage activity test was carried out by adding the protein fraction of melinjo seeds both isolated from the DEAE and BUTYL matrix in 4 concentration series with dilution factors (0; 0.75; 0.50; and 0.25 times) and protein crude extract in 5 μL of pBSKS DNA. A negative control in the form of TMN buffer was used, and a positive

control in the form of a restriction enzyme (EcoR1). Visualization (Figure ??) shows the depletion of supercoiled DNA bands for both the protein fraction using DEAE and BUTYL matrixes as well as the positive control (EcoR1) and protein crude extract.

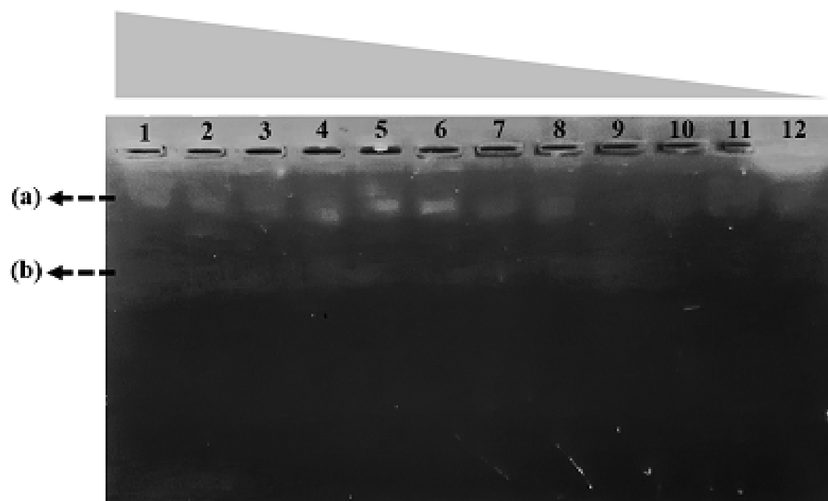

Figure 3: Visualisation electrophoresis of DNA cleavage test of pBSKS: [1] DEAE 1x; [2] DEAE 0,75x; [3] DEAE 0,50x; [4] DEAE 0,25x; [5] BUTYL 1x; [6] BUTYL 0,75x; [7] BUTYL 0,50x; [8]BUTYL 0,25x; [9] Marker; (10) Control (+); (11) Control (-); dan (12) Crude extract. (a) Depletion of DNA supercoiled ; (b) RNA band.

According to Indrayudha et al. (16)the cleavage activity of supercoiled DNA was determined by observing 3 criteria, namely: the presence of supercoiled DNA thinning, nick circular DNA thickening, and the formation of linear DNA in the electrophoretic DNA band under a UV transilluminator. The test is said to be positive if the results of cleavage the protein extract show a pattern of thinning of supercoiled DNA and thickening of nick circular DNA at low levels, while at higher levels, a linear DNA band will appear that is increasingly thickened. Based on these criteria, the protein fraction of melinjo seeds has pBSKS DNA cutting activity, as shown in wells number 1 - 4, DNA band thinning occurs along with the decrease in the concentration of protein fraction of melinjo seeds isolated using the given DEAE matrix. However, the protein fraction isolated using the BUTYL matrix showed that the lower the protein concentration, the more DNA band thinning occurred (Figure ?? lane 5 – 8). This is thought to indicate that the protein fraction isolated from BUTYL was actively cleaved at low levels.

The bands from the visualization of negative and positive controls (Figs. 3 lane 10 and 11) also showed a corresponding difference, namely that the positive control resulted in a higher intensity of supercoiled DNA depletion (pBSKS) compared to the negative control. In addition to this, the DNA band for the sapwood extract of the melinjo seed protein also showed supercoiled DNA depletion (pBSKS). The marker band does not appear on the visualization of the electrophoresis results, this is because

the electrophoresis on the marker is not optimal, so it is necessary to do electrophoresis at the optimal state of the marker which is carried out in 1% agarose gel with 0.5x TAE buffer. The results revealed that the active protein from melinjo seeds was protein isolated using the DEAE matrix without dilution with a concentration of 470.1 μg/mL.

3.6. Cytotoxic Test with MTT Assay

Cytotoxic test on the active protein of melinjo seeds (*Gnetum gnemon* L.) was carried out to determine the potential to inhibit the growth of breast cancer cells 4T1 and T47D with the MTT assay expressed in the IC_{50} parameter. The method used in the cytotoxic test is the MTT assay. The principle of the MTT assay method is the measurement of formazan crystals formed by the reduction of the yellow MTT tetrazolium salt (3-(4,5-dimethylthiazole-2-yl) diphenyl tetrazolium bromide) by the succinate tetrazolium reductase system present in the mitochondria of living cells (17). The reaction that takes place is an enzymatic reaction that takes place continuously, so a stopper solution is needed to stop the detergenic reaction and to dissolve the formazan salt so that the absorbance can be read with an ELISA reader.

This reaction resulted in the formation of purple formazan crystals whose absorbance was read with an ELISA reader. The intensity of the purple color formed is linear with the number of living cells, if the purple color produced is concentrated, the absorbance produced is greater because the number of living cancer cells is still large. The absorbance of formazan can be read with an ELISA reader spectrometrically at a wavelength of 550 nm (18).

This cytotoxic test uses breast cancer cells 4T1 and T47D. One hundred L of harvested cells were put into a 96-well plate and then incubated for 24 hours until the cells were 80% confluent and could be treated with samples.

From the test results, it can be concluded that 4T1 cells and T47D cells before being treated are elongated and irregular in shape similar to cells treated with melinjo seed fraction protein samples which indicate the cells are still growing or alive. This is different from the treatment of positive control doxorubicin which looks smaller and black and the cell density decreases which indicates that the cells have died.

Active protein activity of melinjo seeds fractionated using DEAE-650M in 4T1 cells with a wavelength reading of 595 nm has a percentage value of live cells 145.6% ± 2.8 of the cell population, while in T47D cells it has a percentage value of live cells 42.9%. ±3.3 of the cell population which had an IC_{50} value of 127.62 μg/mL

The active protein activity of melinjo seeds fractionated using BUTYL-650M in 4T1 cells with a wavelength reading of 595 nm has a percentage value of living cells 154.8% ± 1.9 of the cell population, while in T47D cells with a reading of a wavelength of 550 nm has a percentage value live cells 99.2% ± 3.7 of the cell population. It can be seen from the results obtained that the administration of the protein fraction of melinjo seeds was not sufficient to induce a high enough cell death, the percentage of live cells caused was quite fluctuating and non-linear because the increase in concentration was not always accompanied by a decrease in the number of living cells so that the IC_{50}value in the addition of the fraction protein cannot be counted. The positive control used was doxorubicin which is one of the anticancer drugs.

An active compound that has an IC_{50} value of < 100 μg/ml is categorized as having potential for cytotoxicity, if the value of 100 < IC_{50} > 1000 μg/ml is categorized as moderately active against cytotoxicity and if the IC_{50} value is > μ1000 g/ml it is categorized as non-toxic (19). The IC_{50} value describes the concentration in 50% of cells that can inhibit their growth activity. The higher the IC_{50} value, the lower the cytotoxic activity of the tested sample. In this study, after several cytotoxic tests were carried out, various results were obtained. The activity of the protein fraction of melinjo seeds fractionated using DEAE-650M and BUTYL-650M against 4T1 and T47D cells can be categorized as non-toxic because the IC_{50} value is > 1000 μg/mL and the average percentage of living cells is more than 50%, except for the seed protein fraction. melinjo fractionated using DEAE-650M against T47D cells which had an IC_{50} value of 127.62 μg/mL which could be categorized as quite active as cytotoxic. The diversity of results in this study with other studies can be caused by differences in the origin or source of obtaining melinjo seeds so that the number of fractions contained is also different.

4. CONCLUSION

Based on the results of the research that has been carried out, it can be concluded that the protein fraction of melinjo seeds has supercoiled DNA cleavage activity (pBSKS) and has the potential to be developed as an anticancer compound, but it is still necessary to test the pBSKS DNA cleavage activity with a higher concentration of protein fraction of melinjo seeds, so that it is expected that not only supercoiled DNA depletion appears but linear DNA bands appear.

The active protein concentration of melinjo seeds fractionated using DEAE-650M matrix was higher than that of BUTYL-650M matrix. The activity of the protein fraction of melinjo seeds fractionated using DEAE-650M and BUTYL-650M against 4T1 and

T47D cells can be categorized as non-toxic because the IC_{50}value is > 1000 μg/mL and the average percentage of living cells is more than 50%, except for the seed protein fraction. melinjo fractionated using DEAE-650M against T47D cells which had an IC_{50} value of 127.6 μg/mL which could be categorized as quite active as cytotoxic.

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