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Original Article

ACTIVITY OF AVOCADO SEED PROTEIN AGAINST CANCER CELL LINES

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ABSTRACT

Objective: The purpose of this study was to determine the anticancer activity of avocado seed protein against HeLa, 4T1, MCF7, and Vero cells and the protein profile by SDS-PAGE.

Methods: The active protein fraction of avocado seeds was purified by column chromatography method with diethylaminoethyl ion exchange matrix (DEAE) and hydrophobic matrix such as butyl. Isolation was carried out on both matrices and the concentration of the active protein fraction of avocado seeds was read using Biodrop. Protein profile identification was obtained by SDS-PAGE. Cytotoxic tests were carried out using the MTT assay. IC_{50} was used to determine the cytotoxicity of the samples, while the amount of doubling time was measured to define the antiproliferative activity of the protein at hours 24, 48, and 72.

Results: Cytotoxic test results showed that the protein fraction of avocado seed purified with butyl matrix with showed has potent cytotoxic activity in MCF-7 cells with an IC_{50} of 12.21 µg/ml, and to 4T1 cells with IC_{50} 5.42 µg/ml, and did not have cytotoxic activity in HeLa cells, also did not have the antiproliferation activity of MCF-7 cells. The protein fraction concentration range tested was 7.125-57 µg/ml. The protein fraction from the DEAE matrix was not cytotoxic to HeLa, MCF-7, and 4T1 cells. The activity of avocado seed protein isolate using DEAE and butyl matrices against Vero cells was categorized as non-toxic because the average percentage of viable cells is more than 50%. The results of the doubling time value of the avocado seed fraction to 4T1 cells were 5.78 h while the control cells of Vero cells were 98.75 h. The analysis using SDS-PAGE showed the formation of protein bands in the fractionated avocado seeds that have a molecular weight between 25-30 kDa and this protein is included in category RIPs (Ribosom Inactivating Proteins) type 1.

Conclusion: The protein fraction of avocado seed from butyl matrix has cytotoxic activity on MCF7 and 4T1 cell lines, while from DEAE matrix has no cytotoxic activity on HeLa, MCF-7 and 4T1 cells. Protein fraction also has antiproliferative activity on 4T1 cell lines. The protein fraction of avocado seeds containing RIPs type 1. This protein fraction has the potential to be developed as an anticancer but require further purification regarding the activity of the active fraction on cancer cells to ensure the activity and selectivity.

Keywords: RIP, Avocado, Protein fraction, Anticancer, Cytotoxic, HeLa, 4T1, MCF7

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INTRODUCTION

Breast cancer is a disease caused by excessive cell growth and division. Cell division originates from glandular cells and breast-supporting tissues [1]. Research shows that there were cancer cases in Indonesia in 2020 reaching 396,914 cases and total deaths of 234, 511 cases. Breast cancer has the highest number of new cases in Indonesia at 65, 858 cases or 16.6% of the total 396, 914 cancer cases. Cervical cancer ranks second with 36,633 cases or 9.2% of total cancer cases [2]. HeLa cell culture is a continuous cell line obtained from epithelial cells of cervical cancer (cervix) from cervical cancer patients named Henrietta Lacks, these cells are immortal, productive, and are widely used in scientific research [3]. Proliferation in cancer cells is uncontrolled growth and indefinite division. Cancer prevention can be developed based on bioactive compounds from plants that can inhibit proliferation [4]. Various studies have shown that compounds from plants and other natural sources have various mechanisms of action in inhibiting cancer cell proliferation, inducing apoptosis, and inhibiting angiogenesis and metastasis. Therefore, further exploration of natural materials as alternative sources of anticancer therapy is very important to be developed as innovative solutions in the field of oncology.

Active compounds of plants are one of the alternatives in anticancer treatment [5] with minimal side effects that can be obtained from plant extracts or single bioactive compounds obtained from plant isolation results [6]. According to Ariantari *et al.* [7] *Ribosome Inactivating Proteins* (RIPs) are one of the bioactive compounds from plants that can be indicated to have anticancer activity. *Ribosome-Inactivating Proteins* (RIPs) are proteins that are widely distributed in tall plant species [8]. One of these compounds is avocado seeds (*Persea americana*). Avocado seeds contain a protein similar to RIPs. Based on research data on proteins, there is a plant of one family *Lauraceae* that is indicated to have RIPs activity, namely Cinnamomin. Cinnamomin is new type II RIPs isolated from mature seeds of camphor trees (*Cinnamomum champhora*). He *et al.* [9] showed that Cinnamomin has the inhibitory effect of IC_{50} on human hepatocarcinoma cells (7721) and M21 melanomaoma cells were 18.8 nmol, 7721, and 11.7 nmol, respectively.

Priya *et al.* [10] showed that avocado seed extract contains catechins and quercetin which are known to have anticancer activity. Quercetin and catechins have cytotoxic activity in neuroblastoma; HepG2 cells (Carcinoma); HCT15 cells (Colon); PC3 (Prostate) and MCF-7 cells (Breast). The avocado seed contain triterpenoids that can inhibit the proliferation of human breast cancer cells (MCF-7) and human hepatocellular, namely carcinoma cells (HepG2) while p there is research conducted by Nur *et al.* [11] explained that the content of triterpenoids in avocado seeds has anticancer activity or has activity as a cytotoxic cancer cell. Abubakar *et al.*, [12] reported that triterpenoids avocado seed extract can inhibit the proliferation of human breast cancer cells (MCF-7) and human hepatocellular carcinoma cells (HepG2) with IC_{50} values of 62 µg/ml and 12 µg/ml respectively and claimed to be safe for normal cells. In the same plant Alkhalaf *et al.*, [13] also reported inhibition of avocado fruit and seeds on the proliferation of HepG2 cells and colon cancer cells (HCT116). In addition, avocados were shown to inhibit the proliferation of HepG2 and HCT116 cells with IC_{50} values of 58 µg/ml and 14 µg/ml respectively, while avocado seeds significantly suppressed the proliferation of HepG2 and HCT116 cells with IC_{50} values of 12 µg/ml and 3 µg/ml respectively [14].

Fractionation and isolation of active protein purification of avocado seeds were carried out to obtain specific proteins by column

chromatography using DEAE ion exchange matrix and butyl hydrophobic interaction matrix. This study employs two matrices to identify the protein properties that most effectively inhibit cancer cells. This study aims to assess the cytotoxic and antiproliferative effects of avocado seed protein on HeLa cells as a cervical cancer model, as well as on 4T1 and MCF7 cells representing breast cancer models.

MATERIALS AND METHODS

The tools used in this study were glassware, *autoclaves, high speed refrigerated centrifuges*, electrophoresis (Mupid J), micro pipettes (Gilson), analytical balances (Libra-Shimadzu EB-330), pH meters (Electrofac merrohm), water baths, cuvettes, yellow tips, blue tips, ependorf tubes (Biorad), *incubator thermostats*, Ultra Violet lamps (Biorrad), oses, refrigerators, *magnetic strirers,* CO_2 incubators (Heraceus), nanodrop, 96 *well plate* (Nunc), *inverted* microscope (Zeiss), and ELISA *reader* (Bio-Rad) while the materials include: (1) for the extraction and purification of avocado seed protein is avocado seeds obtained from Pasar Gede Surakarta, NaCl, Ammonium sulfate, and columns with DEAE and *butyl* matrices; (2) for cytotoxic cell culture tests 4T1, HeLa, and MCF7 obtained from the stock of UMS Pharmaceutical Mammalian Cell Culture Laboratory, RPMI 1640 media, DMEM, sodium bicarbonate, and hepes; Cell culture media: RPMI 1640 media, Fetal bovine serum (Gibco) 10% v/v, penicillin-streptomycin (Gibco 1% v/v and fungizone (Gibco) 0.5% (v/v); DMSO (*Dimethyl sulfoxide*), MTT (3-(4,5 *dimethylthiazole-2-yl*)-2,5 *diphenyl tetrazolium bromide)* (Sigma); stopper reagent (sodium dodecyl sulfate 10% v/v in HC; 0.01 N), Doxorubicin.

Plant determination

Plant determination is carried out by matching plant morphological states based on the key determination using literature to ensure plant identity and avoid errors in crop retrieval.

Protein extraction of avocado seeds (*Persea americana*)

Avocado seeds are washed thoroughly, then a total of 50 g weighed and then cut into small pieces, mashed, after which extracted with 0.14 M sodium chloride at 4 °C in 5 mmol pH 7.2 sodium phosphate buffer as much as 100 ml. Next, the extract is squeezed using a small screen-printing filter, the liquid obtained is centrifuged at a speed of 7,000 rpm for 5 min. The supernatant obtained is sapwood extract then stored at 4 °C [15].

Isolation of avocado seed protein with deae matrix

The DEAE matrix is prepared by stroking it first using sterile aquades as much as 5 x volume or about 50 ml. After that, proceed to using sodium phosphate buffer 5 mmol, pH 7.2 as much as 10 ml. The sample in the form of a supernatant is then inserted as much as 10 ml in the column. Eluents in the form of NaCl solutions are prepared with increased molarity, starting from 0.2 M; 0.4 M; 0.6 M; 0.8 M; 1.0 M. NaCl solutions each starting from the lowest molarity are inserted into a column of 10 ml and their eluents are accommodated in marked test tubes. The results of each reservoir are protein fractions which are then measured protein levels. The active fraction is selected from the protein fraction that has the highest absorbance which is then deposited at 4 °C [7].

Isolation of avocado seeds protein with butyl matrix

Columns with butyl matrix are prepared by first stroking them using sterile aquades of 5 x volume or about 50 ml. After that, ammonium sulfate buffer 1 M, pH 7.2 was added as much as 10 ml, then elution was carried out. Samples in the form of supernatants were taken 100 ml then ammonium sulfate 20% w/v was added and then mixed using a stirrer. Eluents in the form of ammonium sulfate solution are prepared with decreased molarity, 1.0 M; 0.8 M; 0.6 M; 0.4 M, and 0.2 M respectively as much as 10 ml. Samples containing 20% ammonium sulfate were inserted into a column of 10 ml. Furthermore, ammonium sulfate eluents are added sequentially from 1.0 M to 0.2 M as much as 10 ml each and the eluents are accommodated using marked test tubes. The results of each reservoir are protein fractions which are then measured in protein concentrations [7].

Protein level measurement and SDS page profiling

Measurement of total protein content was carried out by means of sapwood extract taken as much as 2 µl, protein fraction isolated with DEAE and butyl columns, then absorption measured by nanodrop at a wavelength of 260/280 nm using blanks buffer sodium phosphate 5 mmol pH 6.5 [7]. Measurements were made at wavelengths of 260 nm and 280 nm so that the purity and concentration values of isolated DNA were obtained. The wavelength of 260 nm is the maximum absorption for nucleic acids, while the wavelength of 280 nm is the maximum absorption for proteins [16]. The protein fractions were also examined for molecular weight using SDS PAGE. The concentration of SDS PAGE used was 10%.

Harvesting and cell counting of cell lines

Cell cultures that have confluent 80% are harvested. Media in the flask culture was removed, and cells were washed using 5 ml PBS, then cells were poured with 450 µL trypsin-EDTA 0.25% and incubated in a CO_2 incubator for 5 min. Cells that have been incubated, plus 5 ml MK and resuspend to release cells from the flask culture. The 5 ml cell suspension obtained is transferred into a conical tube, and 10 µL is taken to be counted in a hemocytometer consisting of 4 counting chambers [17].

Cytotoxic assay on 4T1, MCF7, HeLa and vero cells

Cytotoxicity test using MTT assay. Each cell (4T1, HeLa, MCF7, Vero) was put into 96 well plates (Nunc) with a total of 10,000 cells per well and incubated together with test samples (avocado seed protein isolate) with 5 concentration series (71.2; 35.6; 17.8; 8.9; and 4.45 µg/ml) for 24 h in a CO_2 incubator. Cell control (media+cancer cells), media control (media only), solvent control (media+buffer cells), and positive control (media+cancer cells+doxorubicin) were used in this cytotoxic assay. At the end of incubation, 100 µL MTT is added in RPMI medium for 4T1 cells, and DMEM medium for HeLa, MCF7, and Vero cells, into each suction. Then, the plate is incubated again for 4 h at 37 °C until formazan crystals form (observation under an inverted microscope). Viable cells will react with MTT forming a purple color. After 4 h, the MTT reaction was stopped by adding a 10% SDS stopper reagent, 100 µL to each well, then incubated overnight at room temperature covered by aluminum foil. The absorption is read with an ELISA reader at a wavelength of 550 nm [18].

Proliferation inhibition test

Proliferation inhibition assay by active protein isolates against 4T1 and MCF7 cells was performed by the MTT method. The concentration of the sample used is below the IC_{50} value, i. e. a half of IC_{50} for one cell. The same MTT test was performed at 0, 24, 48, and 72 h [17].

Data analysis

Calculating IC_{50}

The cytotoxic test results are obtained by calculating the percentage of viable cells based on cell absorbance data, then a concentration log graph is made with the percentage of viable cells and calculated IC_{50}. IC_{50} calculation obtained from absorbance data using replications of controls and sample protein fraction. Next, a log graph of concentration vs percentage of viable cells is created. Then obtained a linear regression equation from the graph, so that the IC_{50} value is obtained [19].

Antiproliferative test

Antiproliferative test is performed using the MTT assay test. To assess the results of the anti-proliferative test, it is calculated by the following formula:

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

Y = log (2x the number of initial live cells); A = intercept; B = Slope [20]. The linear equation obtained from the curve between incubation time *vs*. log number of live cells can be used to calculate

the doubling time value. Doubling time is an antiproliferative parameter of a test material, if the doubling time is longer than the negative control, then it is said that the test material has antiproliferative activity [21].

RESULTS AND DISCUSSION

This study was conducted to determine the antiproliferative activity of avocado seed protein isolated on the DEAE and butyl matrix against 4T1 cells. The process carried out were isolating the active protein of avocado seeds using DEAE and butyl matrix then testing its cytotoxic ability and mechanism in inhibiting proliferation of 4T1 cancer cells.

Protein isolation of avocado seeds

Protein isolation aims to obtain proteins with a high level of purity. The principle of this isolation technique is to separate proteins from other macromolecules or separate proteins with certain properties from other unwanted proteins in the analysis [22]. The protein isolation used in this study is the DEAE and butyl matrix. In the DEAE matrix, the principle uses ion exchange chromatography, which is a purification technique using a stationary phase that can exchange cations or anions with mobile phases. Its stationary phase is a strong (rigid) matrix, whose surface is positively charged. The separation mechanism is based on electrostatic attraction [23]. The results of protein isolation in this study obtained the concentration of DEAE and butyl matrix protein isolation results (table 1).

In table 1, protein isolation data is presented which is conditioned at a temperature of 4 ℃ and has been checked for levels then put into a-20 ℃ freezer. Storage in this freezer aims to stop enzyme reactions from being damaged. Protein isolation is carried out at a very low temperature (4 ℃) so that the protein is not denatured [24]. Protein isolates of avocado seeds that have been conditioned at room temperature are checked for levels at nanodrop and this gives a constant concentration result. However, if checked directly at cold temperatures directly after coming out of the freezer, the protein concentration decreases so it must be conditioned at room temperature first.

Table 1: Protein concentration on matrices deae and butyl using biodrop

Sample deae	Protein A260/A280	Concentration (µg/ml)	Sample butyl	Protein A260/A280	Concentration (µg/ml)
0.4 M[1]	2.560	**197.8**	**0.8 M**[1]	3.188	**279.2**
0.4 M[2]	4.750	164.8	0.8 M[2]	4.750	164.8

Based on the results of reading table 1 using nanodrop, the highest concentration value (hereinafter referred to as the active fraction) is taken which is eluted in the eluent. The highest fraction in isolation results with the DEAE matrix was at an eluent concentration of 0.4 M (1) NaCl which was 197.8 ug/ml and in the butyl matrix of 0.8 M (1) ammonium sulphate which was 279.2 ug/ml. This data showed that the protein concentration from butyl matrix has a higher concentration than DEAE matrix. Furthermore, dilution was carried out on DEAE samples 0.4 M (1) and butyl matrix samples 0.8 M (1). Dilution is carried out on the protein fraction of avocado seeds, to obtain a smaller concentration of the active protein fraction of avocado seeds that will be used as a cytotoxic test concentration series.

Cytotoxic test on 4T1, HeLa, MCF7, and vero cells

A cytotoxicity test was conducted to determine the potential toxicity of avocado seed protein against cancer cells expressed with IC_{50} parameters. Based on the absorbance value obtained, the percent of viable cells and IC_{50} was determined. The IC_{50} value indicates a concentration value that can produce 50% cell proliferation inhibition as well as the potential toxicity of a compound to cells. This value is a benchmark for conducting observational tests of cell proliferation kinetics [25]. The toxicity potential of a sample is described by the small IC_{50} value. In this study, doxorubicin was used as a positive control. Doxorubicin has cytotoxin k activity by inhibiting DNA and RNA synthesis via topoisomerase II. Clinically doxorubicin is effective for the treatment of breast cancer [26, 27].

This cytotoxic testing method uses an MTT assay. This test is widely used to measure cytotoxic effects against drugs of varying concentrations *in vitro*. Measurement of the uptake of formazan salts can be used to detect increases and decreases in the number of cells present. MTT assay results can be read with an ELISA reader at a wavelength of 550 nm. The biochemical mechanism of the MTT assay involves NAD(P)-H-a cellular-dependent oxyreductase enzyme that converts yellow tetrazolium MTT to formazan (insoluble). The intensity of the purple color produced is directly proportional to the number of viable cells [28]. The intensity of purple color is directly proportional to the number of viable cells.

Based on the calculations of the log concentration and the % of average viable cells to obtain an IC_{50} value. The linear regression equation obtained is y =-272.59x+355.38 for the active fraction of proteins using the butyl matrix.

Based on the results of cytotoxic tests (table 2), avocado seed protein was obtained in the DEAE matrix NA (not available) which means it cannot show an IC_{50} value because it provides a high percentage value of viable cells (exceeding 100%) because the absorbance value of the sample shows the number of cells exceeding the absorbance value of cell control. From the results obtained avocado seed protein fraction in the DEAE matrix is not cytotoxic to 4T1 cells, while the butyl matrix shows an IC_{50} value of 5.42 µg/ml. Based on the National Cancer Institute, a fraction is declared active if it has an IC_{50} value<30 µg/ml, moderately active if it has a value of 30<IC_{50}<100 µg/ml and inactive if the IC_{50} value is>100 µg/ml [29].

Table 2: Results of cytotoxic test of avocado seed protein on cancer cells lines

Sampel avocado seed protein	IC_{50} (µg/ml)±SD	
	Sample deae	Sample butyl
4T1 cells	NA (not available)	4.35±2.69
Doxorubicin	5.067±5.477	5.067±5.477
HeLa cells	NA (not available)	NA (not Available)
MCF7 cells	NA (not available)	12,21±8,07
Doxorubicin	3.92±5.66	12.88±12.32

The results obtained on the butyl matrix have an IC_{50} value of<30 µg/ml which means that the active avocado seed protein fraction has potent anticancer activity against 4T1 cells. Meanwhile, the results of cytotoxic tests of avocado seed protein fraction with DEAE matrix did not have cytotoxic activity against Hela cells and MCF-7 cells. The butyl matrix has no toxic activity in HeLa cells and has an IC_{50} value of 12.21 µg/ml in MCF-7 cells. Research on cytotoxic test results on avocado seed protein fraction with DEAE and butyl matrix against Vero cells with a wavelength reading of 550 nm has a high percentage value of viable cells, exceeding 100%. Based on calculations carried out avocado seed protein fraction is not toxic to cause cell death in Vero cells (table 3).

Antiproliferative test on 4T1, MCF7 and vero cells

Antiproliferation tests are carried out by the MTT assay method. The test was conducted to observe the effect of avocado seed protein fraction on 4T1 cell multiplication. The same MTT tests were performed at hours 0, 24, 48 and 72 [17]. The concentration of the test compound used is a concentration below the IC_{50} value, so that too many cells do not die on observation for 72 h caused by the cytotoxic effects of the test compound [30]. In the antiproliferation test, the concentration used is below the IC_{50} value, which is 0.875 µg/ml. Absorbance readings on ELISA readers at 550 nm wavelength.

Table 3: Protein fraction from DEAE matrix and citotoxicity test with vero cell line

Sample	Concentration (µg/ml)	% Viable cell
Protein fraction	57.0	137.912
deae	28.5	127.193
	14.25	145.058
	7.125	154.188
Doxorubicin	25.0	28.345
	12.5	48.293
	6.25	53.355
	3.125	68.837

Table 4: Antiproliferation result of avocado seed protein (Butyl matrix) against 4T1 cells

Group	Concentration (µg/ml)	Viable cell				Equation between incubation time vs number of viable cells	Doubling time
		0 h	24 h	48 h	72 h		
Cell Control	-	10000	7760	13260	13492.27	y = 0.005x+3.8073	98.75
Sample (A5)	0.875	10000	73.50	39.63	32.24	y =-0.0467x+4.5711	5.78

Table 5: Antiproliferation results of avocado seed protein (Butyl matrix) against MCF-7 cells

Group	Concentration (µg/ml)	Viable cell				Equation between incubation time vs number of viable cells	Doubling time
		0 h	24 h	48 h	72 h		
Cell Control	-	10000	7702.5	11178.18	12706.91	Y = 0.0045x+3.7956	112.32
Sample (A5)	0.875	10000	104.83	201.56	159.64	Y = 0.0083x+3.789	53.54

The concentration obtained with the percentage of proliferation inhibition as shown in table 4 and 5. The line equation obtained is y = 0.005x+3.8073 in the control cell and y =-0.0467x+4.5711 in the protein fraction of avocado seeds (A5). The calculation results in this study obtained that the 4T1 cell control had a doubling time of 98.75 h, while the avocado seed fraction with a concentration of 0.875 µg/ml gave a doubling time value of 5.78 h. If the doubling time is longer than cell control, then it is said that the test material has antiproliferative activity. Based on the results obtained, the doubling time value of the avocado seed fraction shows a shorter time, which means that the speed of 4T1 cancer cell proliferation cannot be slowed down by giving avocado seed protein fraction. This suggests that the avocado seed protein fraction is unable to inhibit 4T1 cell proliferation.

The doubling time value can describe the volume of cancer cell growth, so it is often used to evaluate the efficacy of various models of anti-cancer therapy [31]. Doubling time, or the time it takes a tumor to double in size, is important in cancer proliferation because it indicates the rate of tumor growth, with a shorter doubling time indicating faster growth. Meanwhile, the results showed that the doubling time value on the active protein fraction of avocado seeds against MCF-7 cells was 53.54 h, while the control cells were 112.32 h. The results of this antiproliferative test showed that the avocado seed fraction was unable to inhibit proliferation against MCF-7 cells.

Active protein profile analysis of avocado seeds (Persea Americana mill.) with SDS-PAGE

SDS-PAGE is one of the methods used for protein profile analysis contained in certain samples so that the molecular weight of a protein can be known by separating proteins in the sample based on their molecular weight. This method works with the principle of the protein denaturation process caused by the ability of sodium dedosil sulfate to disrupt the stability of the three-dimensional structure of the protein, the process then continues with the electrophoresis method using polyacrylamide gel to separate molecules based on molecular weight [32]. Polyacrylamide gels can be modified to vary in size based on the total concentration of acrylamide gel to be added. The pores in the gel have a smaller size in line with the increasing concentration of the gel so that only protein molecules that have a small molecular weight can pass through it [33]. The results of measuring the active protein fraction levels of avocado seeds with the DEAE matrix and butyl matrix were tested using the SDS-PAGE method to determine the protein band of avocado seeds (fig. 1).

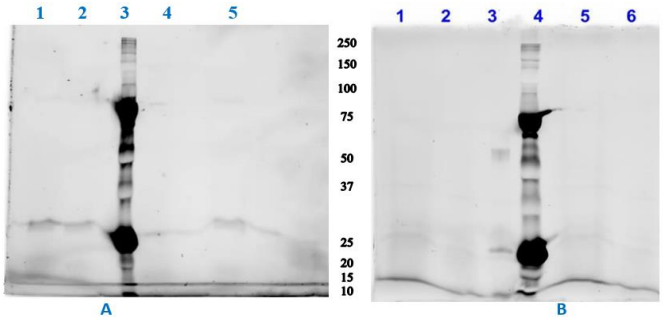

Fig. 1: Profile of the results of the SDS-PAGE analysis of protein fraction from DEAE and butyl matrices. A thin protein band appear at around 25 – 30 kDa

A

1 = Active Protein Isolate of Avocado Seeds using butyl Matrix (14.700 µg)

2 = Active Protein Isolate of Avocado Seeds using DEAE Matrix (4.100 µg)

3 = 250 kDa BIO-RAD Markers

4 = Active Protein Isolate of Avocado Seeds using butyl Matrix (8.640 µg)

5 = Active Protein Isolate of Avocado Seeds using DEAE Matrix (5.600 µg)

B

1 = Active Protein Isolate of Avocado Seeds using butyl Matrix (20 µg)

2 = Active Protein Isolate of Avocado Seeds using DEAE Matrix (20 µg)

3 = Other samples

4 = 250 kDa BIO-RAD Markers

5 = Active Protein Isolate of Avocado Seeds using butyl Matrix (20 µg)

6 = Active Protein Isolate of Avocado Seeds using DEAE Matrix (20 µg)

The thickness or thinning of protein bands produced in polyacrylamide gel can be interpreted by the amount of protein content in the fraction contained in the molecular weight of a protein [34]. The active protein fraction of avocado seeds (*Persea americana* Mill.) separated using SDS-PAGE in fig. 1-A shows the formation of thin protein bands in avocado seed proteins of 14.700 µg and 8.640 µg isolated by butyl matrix with molecular weights of 30 kDa and 25 kDa, while in the DEAE matrix isolation results avocado seed proteins 4.100 µg and 5.600 µg have protein bands with molecular weights of 25 kDa. Meanwhile, the SDS-PAGE results in fig. 1-B did not show any visible protein bands in either the 20 µg protein fraction results using the DEAE matrix or the butyl matrix probably some degradation. The absence of protein band results in the SDS-PAGE test is related to the quantity of protein content in the avocado seed fraction so that it is not detected in the analysis method. Very low protein levels in the testing process are one of the causes of failure to form protein bands. Protein denaturation is possible during the sample preparation process which can cause functional damage to the protein. The relatively long analysis process can affect the quality of the protein fraction. This can cause protein degradation so that the band cannot be detected [35].

RIPs can be classified into two types, the first type is a single chain with a molecular weight of 30 kDa, while the second type is a combined double chain between type 1 RIPs (chain A) and lectin chain (chain B) with a molecular weight of 60 kDa [36]. Based on the results of analysis using SDS-PAGE, the protein fraction of avocado seed matrix DEAE and butyl shows protein bands which are molecular weight measurement analysis of a protein containing RIPs with molecular weights ranging from 25-30 kDa, these results show that avocado seed protein (*Persea americana* Mill.) is predicted to contain type 1 RIPs which are single polypeptides with cytotoxic activity, thus the results of avocado protein fraction can be categorized as having certain types of RIPs and can be tested for activity contained in these RIPs. Plant proteins or peptides that may prevent cancer, such as breast cancer have been identified in various legume proteins, including soybean, chickpea, lentil, and black soybean. These plant peptides can impede the proliferation, adhesion, and migration of breast cancer cells, promote apoptosis, induce G0/G1 phase cell cycle arrest, and result in the overexpression of apoptosis-related proteins such as caspase 3, caspase-7, and caspase-8, thereby obstructing the advancement of breast cancer [37, 38]. Further study can be conducted by trials on in vivo or test animals, also in silico [39] focusing on the characterization and structure of active proteins. Thus, it may be advanced to clinical trials in the future.

CONCLUSION

Based on the result and discussion the following conclusions where are:

The concentration of active protein in avocado (*Persea americana* Mill.) seeds fractionated using the DEAE matrix has a higher-level value than the butyl matrix.

The protein fraction of avocado seeds with DEAE matrix does not have cytotoxic activity against HeLa and MCF-7 cells, as well as the results of protein isolate with butyl matrix in HeLa cells. The protein fraction of avocado seeds with butyl matrix has cytotoxic activity with an IC_{50} value of 12.21 µg/ml.

The result of doubling time value of the active protein fraction of avocado seeds is 5.78 h while the cell control is 98.75 h for 4T1 cell proliferation. The avocado seed protein fraction also was unable to inhibit proliferation against MCF-7 cells. The protein fraction of avocado seeds against MCF-7 cells has a doubling time value of 30.36 and 53.54 h against cell control which is 112.32 h.

The results of protein profile analysis using SDS-PAGE on avocado seed protein fractions of DEAE and butyl matrix showed protein bands with molecular weights ranging from 25-30 kDa, these results showed that avocado seed protein is predicted to contain type 1 RIPs.

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AUTHORS CONTRIBUTIONS

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution and data acquisition, analysis and interpretation, drafting, revising and reviewing the article.

CONFLICT OF INTERESTS

Declared none

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