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Fibrinolytic Activity of Enzyme Extract of Pucuk Merah (*Syzygium myrtifolium* Walp.) Phosphate Buffer Solvent using Fibrin Plate and Clot Lysis Methods

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ABSTRACT: The fibrinolytic protease enzyme is an enzyme that has the ability to break down blood clots that cause cardiovascular disease. This enzyme can be found in pucuk merah plant (Syzygium myrtifolium Walp.), which is popular and easy to find. The aim was to determine the total protein content, protein molecular weight, and fibrinolytic activity of the enzyme extract from pucuk merah leaves tested using fibrin plate and clot lysis methods. The stages of the research included enzyme extraction using 0.1 M phosphate buffer pH 7, precipitation of crude enzyme extract with 80% ammonium sulphate, dialysis of enzyme extract using 12–14 kDa cut-off dialysis bags, enzyme extract characterisation using SDS-PAGE, total protein measurement using the Bradford method, and testing the fibrinolytic activity of enzyme extracts using the fibrin plate and clot lysis methods. Positive and negative controls are nattokinase and phosphate buffer. The fibrinolytic activity of the enzyme extract was analyzed by ANOVA using SPSS 26 software. The result showed that the highest total protein content was from the precipitate sample of the enzyme extract, measuring 1864 µg/ml. The molecular weight of the fibrinolytic enzyme extract of pucuk merah leaves ranges from 25 to 45 kDa. The precipitate sample of pucuk merah leaf enzyme extract had the highest fibrinolytic activity against fibrin plate testing (11.23 mm) and clot lysis testing (13.90%). Enzyme extract precipitate is the most effective sample because it has the highest fibrinolytic activity.

Keywords: clot lysis; fibrinolytic; fibrin plate; pucuk merah

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INTRODUCTION

Cardiovascular disease is caused by intravascular thrombosis, where, in this condition, the blood clots that accumulate in the blood vessels do not experience haemostatic balance from the anticoagulant factor, plasmin, so that it requires a fibrinolytic enzyme that can break down fibrin (Poernomo et al., 2015). According to data from the World Health Statistics (WHO, 2021), the total death rate from cardiovascular disease globally has grown by a quarter since 2000 to 17.9 million in 2019 with the largest percentage increase occurring in the Southeast Asia region, with double the number of deaths in 2019 compared to 2000. Based on Riskesdas Data (2018), it was found that the prevalence of cardiovascular disease in Indonesia reached 1.5%, which is equivalent to 15 out of 1000 people or around 4.2 million people currently. This finding is also reinforced by the report of the Institute for Health Metrics and Evaluation (2019) which states that stroke causes 25.9% of deaths in Indonesia, while ischaemic heart disease is 28.3%.

Fibrinolytic enzymes help dissolve blood clots that block coronary blood vessels, thereby restoring blood flow to the heart (Okafor and Gorog, 2015). Fibrinolytic enzymes have been widely studied in fermented foods and from microbes such as bacteria, actinomycetes and fungi, including natto from Japan, douchi from China, Chungkook Jang sauce from Korea, and tempeh from Indonesia (Yoon et al., 2002). Microbial fibrinolytic enzymes, especially those derived from food-grade microorganisms, have the potential for further development as functional food additives and drugs to prevent or cure thrombosis and other related diseases (Kotb, 2013). Besides fermented foods, Mazid and Al-Mamun (2012) also looked at the fibrinolytic activity in plants like *Tamarindus indica*, *Flemingia congesta*, *Lawsonia inermis*, *Mesua nagassarium*, and spices such as *Coriandrum sativum*, *Curcuma longa*, *Cinnamomum tamala*, *Nigella sativa*, and *Eugenia aromaticum*.

Research related to the fibrinolytic activity of pucuk merah leaves has never been conducted, but several studies related to plants of the genus Syzygium have shown activity as fibrinolytic agents. Mazid and Al-Mamun (2012) conducted a fibrinolytic activity test on several plants, one of which was *Eugenia aromaticum* or *Syzygium aromaticum*, showing fibrinolytic activity. Also, research on extracts from the bark and leaves of Syzygium cumini (Abedin et al., 2018), *Syzygium fruticosum* (Chadni et al., 2015), *Syzygium malaccense* (Patel et al., 2019), and *Syzygium operculatum* (Tarek et al., 2015) showed that they can break down fibrin, and this ability is not just due to secondary metabolites. Considering the possibility of the genus as an agent that can break down fibrin, the purpose of this study was to determine the total protein content, protein molecular weight, and fibrinolytic activity of the enzyme extract from pucuk merah leaves tested using the fibrin plate and clot lysis methods.

METHODS

The type of research in this study is experimental research, involving enzymes from pucuk merah leaves to carry out fibrinolytic activity tests.

Plant Determination

We conducted a plant determination based on literature on the morphological characteristics of pucuk merah (Wijiyadi, 2009). A pucuk merah plant tree, including leaves, stalks, stems and roots, which was taken from the Kemuning area of Karanganyar, Central Java, was subjected to determination detection at the Center for Research and Development of Traditional Medicinal Plants (B2P2TOOT) Tawangmangu, Karanganyar, Central Java.

Enzyme Extraction

Pucuk merah leaves were collected from Kemuning, Karanganyar, Central Java, in November 2022. A total of 50 grams of fresh leaves that had been measured using an analytical balance (Precisa) were cut into small pieces and homogenized in a mortar with 100 mL of 0.1 M phosphate buffer solvent at pH 7 (Merck). The homogenization results were put into a conical tube (Falcon) and then centrifuged using a centrifuge (5430-R). The homogenate was then centrifuged at a speed of 10,000 rpm for 30 minutes at a temperature of 4℃. The sediment was discarded, and the supernatant was used for further purification. The supernatant at this stage is referred to as the crude enzyme extract (modified from Matsubara et al., 2000).

Precipitation of Crude Enzyme Extract

The crude enzyme extract was precipitated with 80% ammonium sulphate (Merck). A total of 5.16 grams of ammonium sulphate was added to 10 mL of the crude enzyme extract and stirred with a magnetic stirrer (Nuova). The mixture is then stored overnight in the refrigerator (Sharp) for separation purposes. The precipitated protein was separated by centrifuge at 7500 rpm for 15 minutes at 4℃. The supernatant was discarded, and the precipitate was dissolved with phosphate buffer solution for further purification. The precipitate in the phosphate solution is known as the enzyme extract precipitate.

Enzyme Extract Dialysis

Dialysis is needed to remove ammonium sulphate salts and other ions that interfere with the stability of enzyme protein molecules when stored (Sajuthi et al., 2010). The enzyme extract precipitate is put into a 12-14 kDa cut-off dialysis bag and soaked in aquabides (PT.Brataco) and stored in a refrigerator while stirring with a magnetic stirrer (Nuova) at low speed for one. The dialysis result is known as enzyme dialysate.

Determination of protein molecular weight

Protein molecular weight was determined by SDS-PAGE using 12% gel under reducing conditions. The gel stack used contained 7% polyacrylamide (Sigma-Aldrich), while the separating gel contained 10% polyacrylamide, and then the gel was stained with Coomassie Brilliant Blue R-250 (Thermo Fisher Scientific), and the protein molecular weight was estimated using standard protein markers (Thermo Fisher Scientific) (Kim et al., 2015).

Determination of Total Protein Content

Protein content was determined according to the Bradford method by measuring absorbance at 595 nm (Kumaunang and Kamu, 2011). Samples of stock solutions and enzyme extracts were put into sterile cuvettes (OneMed) and measured using a UV-Vis spectrophotometer (Shimatzu 1800), and the results were displayed on the screen of the UV-Vis spectrophotometer. Bovine Serum Albumin (Thermo Fisher Scientific) was used as a protein standard.

Fibrinolytic Activity with Fibrin Plate

A fibrinolytic activity test using a fibrin plate was conducted by dissolving 14 grams of nutrient agar (Merck) in 500 mL of 0.01 M phosphate buffer solution at pH 7 and heating for 1 hour at 121℃. 8.3 mL of blood plasma (not heated) was mixed with nutrient agar. A total of 100 µl of sample was placed in each well made in a sterile Petri dish (OneMed) and incubated at 37℃ for 24 hours in an incubator (LEEC). Nattokinase (AbMole) was used as a positive control. The clear zone formed was analyzed visually.

Fibrinolytic Activity with Clot Lysis

The fibrinolytic activity test by clot lysis was carried out by adding 500 µl of fresh rabbit blood into an Eppendorf tube (Falcon) and then incubating it at 37℃ for 45 minutes in an incubator until a clot formed. Unclotted blood was removed. A total of 100 µl of sample

was put into an Eppendorf tube containing a clot and incubated for 24 hours at 37℃. After incubation, the remaining blood clot was carefully removed, and its weight was determined using an analytical balance. Nattokinase was used as a positive control. The percentage of blood clot lysis can be calculated using the formula:

$$\% \text{ clot lysis} = \frac{(initial\ blood\ clot\ weights - blood\ clot\ weight\ after\ lysis)}{(initial\ blood\ clot\ weights)} \text{ x } 100\%$$

RESULT AND DISCUSSION

Plant Determination

The results of the screening carried out on the pucuk merah plant samples confirmed that the plant identified was indeed the pucuk merah plant, species Syzygium myrtifolium Walp., with the synonym name Eugenia parva C.B.Rob. from the Myrtaceae family.

Determination of Protein Molecular Weight

The use of the SDS-PAGE electrophoresis method in protein analysis is to divide proteins based on their molecular weight using acrylamide gel as a support matrix. SDS-PAGE works by inhibiting hydrophobic interactions and breaking hydrogen bonds. At the same time, electrophoresis can determine the type of protein in the sample or material being tested (Wibowo, 2010).

Molecular weight measurements performed on crude enzyme extract samples, enzyme extract precipitates, and dialysates from red shoot leaf extracts resulted in the formation of thin bands. The measurements were carried out using marker proteins consisting of several enzyme components. Meanwhile, there were 10 wells formed, where the first well was filled with marker protein. There were three enzyme samples injected with different volumes, namely 7.5, 15, and 20 µl. The SDS-PAGE profile (Figure 1) showed three thin bands at BM 45, 30, and 25 kDa for the crude enzyme extract sample (A), enzyme extract precipitate (B), and enzyme extract dialysate (C).

Proteins with similar molecular weights do not indicate that they are similar proteins because each protein has variations in the type of amino acids, the number of amino acids, and the sequence of amino acids that make them up (Thomas et al., 2002). The larger the sample volume, the thicker the bands formed. The number of protein bands formed from SDS-PAGE electrophoresis indicates the number of protein subunits in the protein sample, but the bands produced are very thin for all samples. The protein band at the top is the largest subunit, while the protein band at the bottom reflects the smaller subunit. The size of a protein subunit can vary based on the number and type of amino acids that compose it (Garfin et al., 2003), which suggests that the number of amino acids in the pucuk merah leaf plant is tiny. The three bands from the sample align in a straight line with the band on the marker. This graph shows that the molecular weight of the crude enzyme extract sample, enzyme extract precipitate, and enzyme extract dialysate are identical because they come from the same plant, namely pucuk merah leaves. Molecules with equal mass move equal distances and form straight paths (Suranto, 2006).

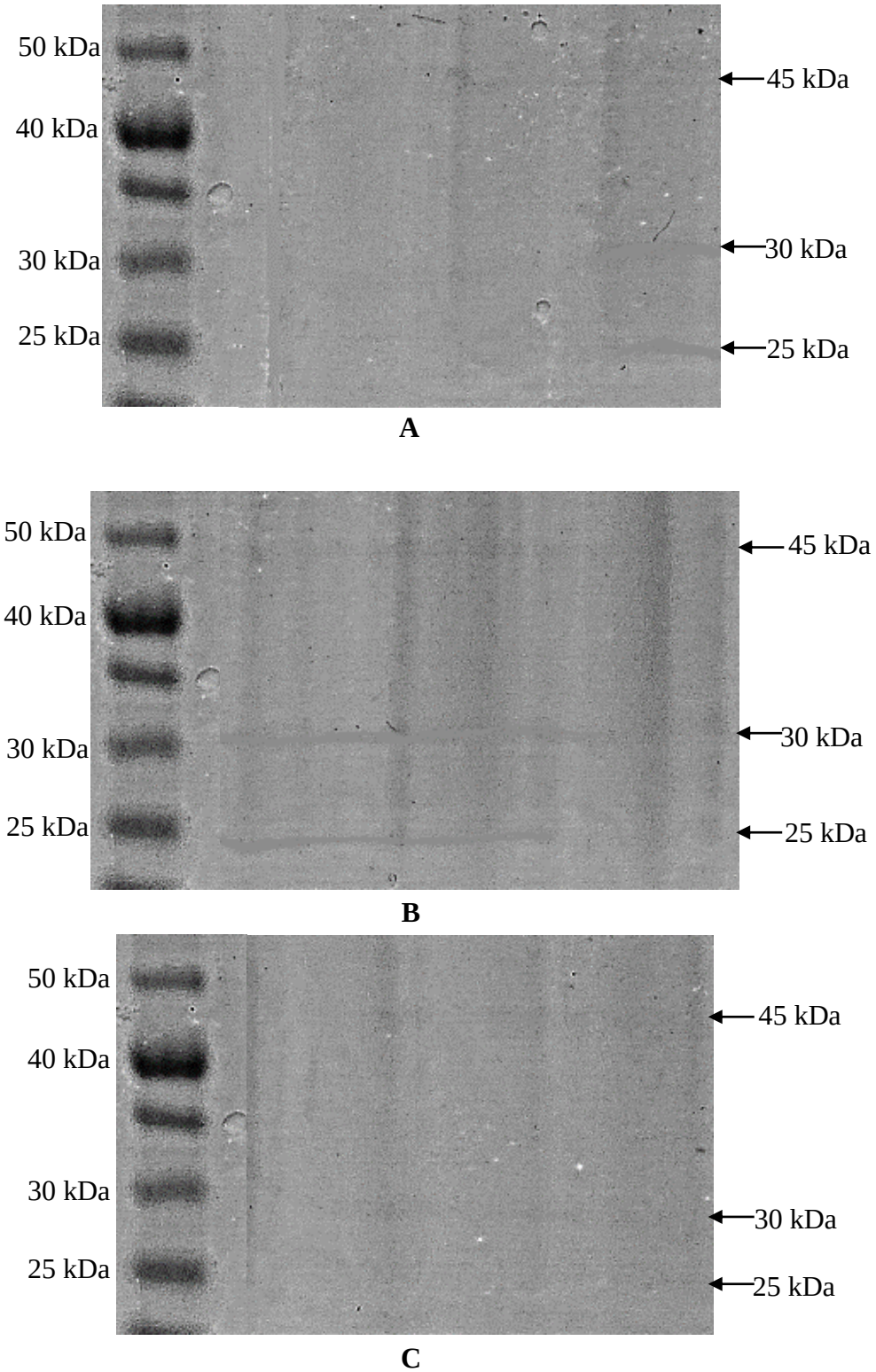

Figure 1. SDS-PAGE profile of enzyme extract samples. Crude enzyme extract (A), enzyme extract precipitate (B) and enzyme extract dialysate (C)

Research by Antao and Malcata (2005) found that the molecular weight of serine proteases isolated from plant sources ranged from 19 to 110 kDa, with the majority Putrivenn et al. reported that their findings included a molecular mass between 60 and 80 kDa. Research by Kim et al. (2015) on purifying fibrinolytic enzymes from Petasites japonicus found that the purified enzyme had a molecular mass lower than most serine proteases from the Euphorbiaceae family, which are usually between 43-74 kDa. The fibrinolytic enzyme purified from P. japonicus produced a clear and sharp band of 40 kDa on the fibrin zymogram, indicating that the enzyme has plasmin-like activity. Chung et al. (2010) in their research related to the purification of fibrinolytic enzymes against Allium tuberosum, revealed two active bands (90 and 55 kDa) which were analyzed using a zymogram gel. The comparison of molecular weight obtained from research with literature is almost the same, where in the experimental results, molecular weight ranges between 25 - 50 kDa, so it can be said that the molecular weight produced by pucuk merah leaf plants is generally almost the same as the molecular weight produced by other plants that have been studied.

Determination of Total Protein Content

Determination of total protein content is determined by the Bradford method, which aims to determine the total protein in the crude enzyme extract sample, enzyme extract precipitate and enzyme extract dialysate from 0.1 M pH 7 phosphate buffer solvent. The Bradford method is a colorimetric technique used to determine the total protein content in solution. This method utilizes a Coomassie brilliant blue (CBB) dye solution that will bind to protein in an acidic solution and produce a bluish color, because it produces color, the absorbance can be measured colorimetrically using a spectrophotometer at a wavelength of 595 nm, where CBB has a maximum absorbance value at that wavelength when it binds to protein (Kumaunang and Kamu, 2011).

The determination of protein levels was conducted using Bovine Serum Albumin (BSA) as a standard solution, with concentrations of 1, 2, 4, 8, 16, and 32 mg/ml. This experiment was carried out by dripping aquabidest into the BSA solution which acts as a solvent for the protein to be analyzed. The lower the absorbance value, the more protein is dissolved. The addition of biuret dye reacts with the protein-peptide bond in the sample. The presence of protein in the sample can be recognized by the purple color that appears in the sample which is formed due to the presence of a Cu+ ion complex that binds to the protein peptide (Khopkar, 2007). The preparation of standard solutions with various concentrations aims to determine the amount of protein in a sample using a straight line equation obtained from the standard solution graph, this is done to determine the protein content in the sample. BSA standard solution graph can be seen in Figure 2, and the result of protein content determination can be seen in Table 1.

The higher the absorbance value, the higher the concentration of protein detected (Rahmi et al., 2020). Table I above shows that the crude enzyme extract produces a low level compared to its precipitate. This result was because the presence of primary and secondary metabolites, along with other impurities, prevents the protein content from being measured in its entirety (Harahap, 2014). Chung et al. (2010) also found the measurement of the protein content in crude extracts from Allium tuberosum plants unreadable due to the large number of metabolite components in the crude enzyme extract

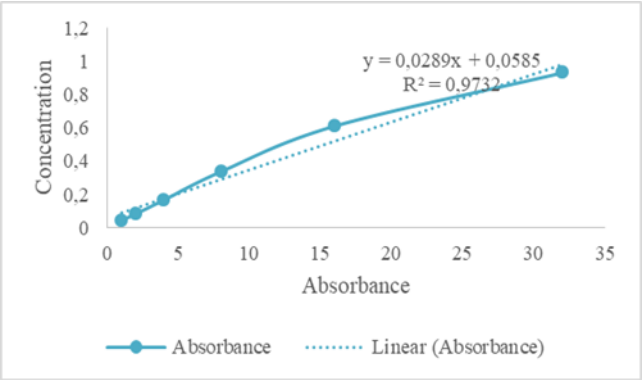

Figure 2. BSA standard solution graph

Table 1. Results of total protein content of samples using UV-VIS spectrophotometry

Sample	Total protein content (µg/ml)
Crude enzyme extract	784
Enzyme extract precipitate	1864
Enzyme extract dialysate	458

.Fibrinolytic Activity with Fibrin Plate

The fibrinolytic activity test using the fibrin plate method aims to see the ability of the enzyme extract to break down plasminogen into plasmin, which is seen through the formation of a clear zone around the well. The fibrin plate test uses the smallest concentration of 458 µg/ml. The result of the fibrinolytic activity with the fibrin plate can be seen in Table 2.

Table 2. Results of the fibrinolytic activity test of pucuk merah leaf enzyme extract with fibrin plate

Sample	Inhibitory diameter ± SD	Fibrinolytic index
Crude enzyme extract	7.23* ± 0.40	1.03
Enzyme extract precipitate	11.23* ± 0.50	1.60
Enzyme extract dialysate	5.00* ± 0.00	0.71
Nattokinase (+)	17.57* ± 0.51	2.51
Phosphate buffer (-)	0*	0

SD: Standard deviation
(+): Positive control
(-) : Negative control
(*) : Data for analysis

The formation of a clear zone is caused by the degradation of the fibrin substrate, which causes the degradation of plasminogen to plasmin by the fibrinolytic enzyme, so it can be said that the tested sample produces fibrinolytic enzymes. Fibrinolytic activity is then expressed by the fibrinolytic index, which is determined by comparing the diameter of the clear zone of the test sample with the diameter of the well. Based on the data listed in Table 2, it can be seen that all test samples showed the formation of a clear zone diameter. Based on the fibrinolytic index value, the enzyme extract precipitate sample showed fibrinolytic activity close to the nattokinase standard, with a ratio of 63.75% or an activity 0.64 times lower than nattokinase, which has a fibrinolytic index value of 1.60.

Research conducted by Nuraini et al. (2021) related to the results of the fibrinolytic index of Centella asiatica leaves fermented by Acetobacter tropicalis bacteria showed that the Acetobacter tropicalis starter inoculum had a fibrinolytic activity 0.98 times smaller and was closest to the nattokinase standard. When compared to the fibrinolytic index of the pucuk merah leaf enzyme extract, it is still smaller because protein production from plants is less than from microorganisms. Nattokinase works to increase t-PA activity so that it can bind to plasminogen to be converted into plasmin and degrade fibrin directly and efficiently (Chen et al., 2018).

The results of statistical analysis using the one-way ANOVA method from the data (*) in Table 2 showed that the significance value was 0.000, which was less than 0.05. Therefore, it can be concluded that there is a significant difference where the enzyme extract precipitate showed the greatest results compared to the crude enzyme extract and enzyme extract dialysate. Research conducted by Matsubara et al. (2000) related to the in vitro fibrinolytic activity test on marine green algae, *Codium divaricatum*, showed that in the fibrin plate test, 30 µl of pure enzyme sample with a concentration of 2 µg/ml produced a clear zone diameter of 95 mm2 (0.95 mm). This activity is not entirely in accordance with the previously observed algae extract which was larger than the test. Research by Kim et al. (2015) who conducted an in vitro fibrinolytic activity test on *Petasites japonicus* showed that in the fibrin plate test as much as 10 µl of pure enzyme concentration of 1 µg/ml produced the same clear zone diameter as the same plasmin concentration, where the fibrinolytic activity of the purified enzyme was higher than nattokinase. This shows that the purer the enzyme produced, the better its fibrinolytic activity.

In contrast to the results of the research on fibrinolytic enzymes of pucuk merah leaves, a decrease in levels and activity was found in the enzyme extract dialysate which is thought to be caused by the dialysis process where proteins below the pore size of 12 kDa which are thought to be able to provide fibrinolytic activity were also dialyzed together with ammonium sulfate salt, while the highest levels and activity were in the enzyme extract precipitate where in the precipitation process the ammonium sulfate salt was able to bind a lot of protein so that in the enzyme extract precipitate the protein levels increased so that its activity in breaking down plasminogen into plasmin and degrading fibrin was greater.

Fibrinolytic Activity with Clot Lysis

A clot lysis test is performed to evaluate the sample's ability to dissolve blood clots and measure its fibrinolytic activity in degrading fibrin. The fibrinolytic index reflects the activity of the fibrinolytic system. This examination is performed by observing and assessing the quantity of blood clots that are broken down after being given a test sample. The fibrinolytic index measures the strength of a fibrinolytic agent by comparing the percentage of sample lysis with the percentage of nattokinase lysis as a standard. The result of the fibrinolytic activity with clot lysis can be seen in Table 3.

Clot lysis testing uses the smallest concentration of 458 µg/ml. The percentage of lysis indicates the ability of the sample to degrade fibrin expressed in percent. The greater the percentage of lysis, the greater the ability of the sample to degrade fibrin. Fibrinolytic enzymes work by breaking down fibrin, which causes blood clots to become soluble substances and are removed from the bloodstream. This helps remove blood clots from blood vessels, as well as triggering the healing process in the blood vessel walls (Escobar et al., 2002).

Table 3. Results of the fibrinolytic activity test of pucuk merah leaf enzyme extract with clot lysis

Sample	% clot lysis ± SD	Fibrinolytic index
Crude enzyme extract	8.71* ± 0.38	21.97
Enzyme extract precipitate	13.90* ± 1.05	35.07
Enzyme extract dialysate	5.23* ± 0.67	13.19
Nattokinase (+)	39.65* ± 2.65	100
Phosphate buffer (-)	0*	0

SD: Standard deviation
(+): Positive control
(-) : Negative control
(*) : Data for analysis

The results of statistical analysis using the one-way ANOVA method from the data (*) in Table 3 showed that the significance value was 0.000 which was less than 0.05. Thus, it can be concluded that there is a significant difference that has an impact on the results of this analysis where the enzyme extract precipitate sample showed the greatest results compared to the crude enzyme extract sample and enzyme extract dialysate. The fibrinolytic index is obtained from the comparison between the sample and the positive control. The fibrinolytic index of the enzyme extract precipitate was 35.07. This value shows an increase compared to the fibrinolytic index of the crude enzyme extract which was only 21.97. This shows that the enzyme extract precipitate has a blood lysing activity 1.6 times greater than the crude enzyme extract.

However, in the enzyme extract dialysate, there was a decrease in the fibrinolytic index by 13.19, which also showed that the enzyme precipitate had a blood lysing activity 2.7 times greater than the enzyme extract dialysate. It is possible that the fibrinolytic enzyme contained in the enzyme extract precipitate is greater than in the enzyme extract dialysate so that the fibrinolytic activity of the enzyme extract precipitate produced is greater. Meanwhile, research conducted by Warninghiyun et al. (2023) regarding the clot lysis test on *Carica papaya* L. showed that the higher the level of enzyme purification, the greater the enzyme's lytic power on blood clots. The decrease in levels and activity in the enzyme extract dialysate can be caused by various factors. Factors that influence the decrease in levels and activity in the enzyme extract dialysate are likely to occur because changes in the environment have caused stress on the enzyme, resulting in a decrease in the number of active cells. Another factor that may play a role in the decrease in enzyme activity is the dialysis process, where it is possible that some of the enzymes are dialyzed with ammonium sulfate (Kusumo et al., 2021). Based on the description above, it can be explained that the extract of the shoot leaf enzyme has the potential as an agent that has fibrinolytic properties that work by breaking down plasminogen so that it can produce plasmin. Then, this plasmin will help in the process of breaking down clots that are rich in fibrin so that they produce fibrin degradation products (Chen et al., 2018).

CONCLUSION

Pucuk merah leaf enzyme extract has the potential to be a fibrinolytic agent with the highest fibrinolytic activity in the enzyme extract precipitate sample, namely 13.33 mm in the fibrin plate test and 27.81% in the clot lysis test, and a total protein content of 1864 µg/ml. The molecular weight of the pucuk merah leaf enzyme extract ranges from 25 to 50

kDa. It is necessary to first test the effect of substrate specificity, the effect of temperature and pH, the effect of metal ions and inhibitors, and the optimization of several solvents.

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AUTHOR CONTRIBUTION
YIP: Concepts or ideas; design; definition of intellectual content; literature search; experimental studies; data analysis; manuscript preparation.
AI: Concepts or ideas; definition of intellectual content; literature search; manuscript editing; manuscript review.
OS: Definition of intellectual content; manuscript editing; manuscript review.

CONFLICT OF INTEREST
"None to declare"

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