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Thrombolytic Activity and Protein Characterization of Extract from White Oyster Mushroom (*Pleurotus ostreatus*)

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Abstract: Venous thromboembolism (VTE) is a condition resulting from the presence of thrombi within blood vessels. Globally, these illnesses are the leading causes of death. In 2022, the WHO estimates that 19.8 million fatalities or 32% of worldwide deaths were a result of cardiovascular disease, which is initiated by thromboembolism. The use of thrombolytic medicines is one treatment option for thromboembolic illness. The exploration revealed that numerous mushrooms contain protease enzymes with a thrombolytic effect. A common edible mushroom in Indonesia is the white oyster mushroom (*Pleurotus ostreatus*). By assessing thrombolytic activity using the Blood Clot Lysis Assay method and characterizing proteins using SDS-PAGE electrophoresis, this study aims to determine the thrombolytic activity of the extract and crude protein from the white oyster mushroom. The findings demonstrated the thrombolytic activity of the extract and crude protein of oyster mushroom at 29.89% and 37.89%, respectively. The extracts with volumes of 500 µl and the crude protein with a concentration of 0.05 g/mL exhibited the highest thrombolytic activity. The thrombolytic activity results of the extract (500 µl) and crude proteins (0.02 g/mL and 0.05 g/mL) of the white oyster mushroom were significantly higher than the positive control ($p<0.05$). According to protein characterization, the white oyster mushroom extract and crude protein had protein molecular weights of 45.50 kDa, 32.51 kDa, 21.09 kDa, and 15.1 kDa, respectively. White oyster mushroom extracts and crude protein have the potential to be developed as thrombolytic agents, serving as a safe, natural, and cost-effective alternative for thromboembolism disorders. While the results offer an important foundation, further studies are necessary to confirm and extend these findings.

Keywords: thrombolytics; blood clotting; cardiovascular; white oyster mushroom, *Pleurotus ostreatus*

1. INTRODUCTION

Thrombosis is the development of a blood clot in arterial or venous blood vessels that restricts the normal flow of blood. A complicated balance between blood cells, platelets, plasma proteins, coagulation factors, inflammatory factors, and the endothelium lining the arterial and venous lumens is necessary for blood to flow freely in vessels. An increased risk of thrombosis results from imbalances in this physiological process, which involves stopping bleeding, preventing blood flow, and clogging blood vessels. Thrombosis can lead the thromboembolism, where a piece of a blood clot breaks off and travels through the bloodstream. This triggers adhesion, as well as the activation of platelets, the formation of fibrin, and the entrapment of blood cells. The thrombus that forms in the veins can become detached and flow to other organs through the bloodstream, a condition referred to as venous thromboembolism (VTE). It potentially causes a blockage elsewhere that causes partial or complete blood flow obstruction and results in various diseases, which is the primary cause of death [1, 2, 3].

Recent global epidemiological reports show that cardiovascular diseases (CVDs) continue to be the leading cause of death worldwide. The World Health Organization reported that 19.8 million

deaths were attributable to CVDs in 2022, accounting for over 32% of global mortality [4]. Similarly, the *Global Burden of Disease (GBD) Study 2023* estimated that global CVD deaths reached approximately 19.2 million in 2023, accompanied by rising disease prevalence and increasing disability-adjusted life years (DALYs) [5]. Given this substantial and growing burden, the search for effective, safe, and affordable thrombolytic agents remains a critical area of research. Antithrombotic agents mostly function by directly suppressing platelet activity (e.g., aspirin, clopidogrel, and dipyridamole) or by obstructing thrombin, hence preventing platelet activation and fibrin synthesis (e.g., heparins, warfarin, and direct thrombin or factor Xa inhibitors) [6].

Thrombolysis agents include anticoagulants (such as vitamin K antagonists and heparins) and fibrinolytic agents (tissue plasminogen activator, streptokinase, and urokinase), which are commonly used in clinical practice for the treatment of this disease. Conversely, they have similar side effects, such as reperfusion arrhythmias, hemorrhage, hypotension, allergic responses, and angioedema. They also have a high production cost, a short half-life, and limited fibrin specificity [7,8]. Some natural resources have been explored as candidates for a thrombolytic agent with high therapeutic efficacy and low side effects.

Natural sources of drugs for thrombolytic agents can be derived from various plants, animals, microorganisms, recombinant proteins, and food. Mushrooms are alternative agents that can be investigated further as thrombolytics [9,10]. Several edible mushrooms, such as *Cordyceps militaris, Pleurotus ostreatus,* and *Schizophyllum commune*, are reported to contain protease enzymes with fibrinolytic, thrombolytic, and anti-atherosclerotic properties in the systemic circulation [7, 11, 12, 13].

Pleurotus ostreatus, or oyster white mushroom, is one of the most ubiquitously, easily cultivated, and consumed mushroom species in Indonesian society, both in urban areas and rural areas. Several studies have revealed that *Pleurotus ostreatus* possesses numerous disease-curing properties, including anti-cancer activity, immune-modulating effects, antiviral, antibiotic, and anti-inflammatory activities. Petraglia et al. reported that *Pleurotus ostreatus* has the highest fibrinogenolytic activity, followed by *Pleurotus eryngii, Lentinula edodes*, and *Agrocybe aegerita* [7]. Meanwhile, the benefits of white oyster mushrooms for inducing thrombolytic activity have not been widely reported.

This study performed the thrombolytic activity of the extract and crude protein from the oyster white mushroom (*Pleurotus ostreatus*) using the Blood Clot Lysis Assay method. Furthermore, this study employed SDS-PAGE electrophoresis for protein characterization. The purpose of this study is to evaluate the potential of white oyster mushroom extracts as a thrombolytic agent.

2. MATERIALS AND METHODS

2.1. Materials and tools

Some main materials, such as the white oyster mushrooms (*Pleurotus ostreatus*) from a local farmer in Jember Regency, the protein marker (Sigma Aldrich), and flour earthworm (*Lumbricus rubella).* The blood sample was collected from human volunteers (Ethical approval number 132/1125.1.11/KE/2012 by The Ethics Committee of The Medicine Faculty, Jember University). The other materials such as acetone, 30% acrylamide (acrylamide and bis acrylamide), 1.5 M tris HCl pH 8.8, 0.5 M tris HCl pH 6.8, 10 % Sodium Dodecyl Sulfate (SDS), distilled water, 10% Ammonium persulfate, TEMED (tetramethylethylenediamine), electrophoresis buffer (trisma base, glycine, SDS), sample buffer (glycerol, SDS 10%, β- mercaptoethanol, bromphenol blue, 0.5 M Tris HCL pH 6.8), staining (methanol, coomasie brilliant blue R-250, acid acetate glacial), destaining (acid acetate glacial and methanol) were acquaired from Brataco.

In this research, the following tools are used: Blender (National), Scales (Sartorius), Distillation Tools, Rotary Evaporator (Buchi), Rod Stirrer, Whatman 20 Paper Filter, Vial, Tube Microcentrifuge (Ohaus), Incubator (Memmert), Stopwatch, and Gel Electrophoresis Apparatus (Thermo Fisher).

2.2. Preparation of White Oyster Mushroom Extract and Crude Protein

The white oyster mushroom, 250 g, mixed with 900 ml of 50 mM phosphate buffer solution (pH 7.0, using a blender. White oyster mushroom extract was produced by centrifuging at 40°C with

a speed of 6,000 rpm for 15 minutes, resulting in a supernatant that was designated as the extract. Half of the extract (supernatant) was used to assess its thrombolytic activity.

The other half of the extract (supernatant) was separated and mixed with cold acetone (twice the volume of the supernatant) to precipitate the protein. The supernatant mixture was centrifuged at 40°C with a speed of 6,000 rpm for 15 minutes, until a sediment formed, which was referred to as the crude protein [14].

2.3. Thrombolytic Activity Testing in Extract and Crude Protein of White Oyster Mushroom

2.3.1. Preparation Solution of Earthworms (Lumbricus rubellus) Flour

As much as 1 g of earthworm *flour* mixed with 1% CMC Na is used to reach a volume of 100 ml. Suspension of earthworm flour is used as a control positive due to its effective fibrinogenolytic activity and thrombolytic properties [15].

2.3.2. Preparation of Crude Protein

The crude proteins, at concentrations of 100 mg, 200 mg, and 500 mg, were dissolved in 10 mL of distilled water. The mixture was stirred until homogeneous with a shaker for 30 minutes, then filtered with a 0.2 μm membrane filter. As the test solution, crude protein solutions were prepared at three concentrations: 0.05 g/mL, 0.02 g/mL, and 0.01 g/mL.

2.3.3. Human Blood Sample Preparation

Subjects must be healthy and not currently consuming anticoagulant or contraceptive drugs. A total of 500 μL of the blood sample was then transferred to a microcentrifuge that had been previously weighed [16].

2.3.4. Thrombolytic Activity Testing

A tube microcentrifuge was filled with the sample blood and incubated at 37° C for 60 minutes. After incubation, clotted or lump blood formed, and the blood serum part was separated. Every tube containing a blood clot was weighed to determine the weight of the clot [16].

The samples used in this testing were the extract and crude protein. The samples of extract (supernatants) with volumes of 100 μL, 200 μL, and 500 μL were added to each microcentrifuge tube containing a blood clot. At the same time, 100 μL solution samples of crude protein (0.05 g/mL, 0.02 g/mL, and 0.01 g/mL) were also placed in a tube microcentrifuge. The control positive is made by adding 100 μl of a suspension of flour earthworm at a concentration of 0.01 g/ml. The control negative is made by adding 100 μl of distilled water. The entire tube was then incubated at 37 °C and observed at 30, 60, and 90 minutes to inspect blood clot formation. After incubation was finished, the dissolved or lysed blood was removed by inverting the microcentrifuge tube until only a blood clot remained. Then, the microcentrifuge tubes were weighed again to determine the change in weight of the blood clot, expressed as a percentage of lysis. The percentage of lysis of the blood clot obtained with the method is calculated by the following formula [16]:

$$\%\ Blood\ Clot\ Lysis = \frac{weight\ before\ treatment - weight\ after\ treatment}{weight\ before\ treatment}\ x\ 100\%$$

2.4. Protein Characterization

Table 1. SDS-PAGE gel composition

Material	4% *Stacking Gel*	10% *Separating Gel*
30% Acrylamide	325 μl	3.333 ml
Tris HCl 0.5 M (pH 6.8)	625 μl	-
1.5 M Tris HCl (pH 8.4)	-	2.5 ml
Distilled water	3.050 ml	4.002 ml
10% SDS	25 μl	100 μl
10% Ammonium persulfate	15 μl	50 μl
TEMED	5 ml	15 μl

The 10% SDS-PAGE electrophoresis method was used for protein characterization. Protein characterization with a composition separating gel and a stacking gel used in SDS-PAGE is shown in Table 1. The component separating gel was mixed in a glass beaker and then poured into a mold, separating up to three-quarters of the volume. Distilled water filled the mold. After the separating gel hardened, distilled water was removed from the mold.

When the separating gel was formed, the material stacking gel, which had been mixed, was poured into the mold. Before the stacking gel hardened, the comb well was installed. Once the gel was solidified, remove the comb-well, and the gel was ready for use. A sample buffer was added to each sample (supernatant extract and crude protein) at a ratio of 5:1, and then heated for 5-10 minutes. Before electrophoresis started, the assembled SDS-PAGE tool was cleaned with 70% alcohol or SDS. A 15 μL sample of extract, a 10 μL sample of crude protein, and a 20 μL protein marker were added to the well of the gel. In a pH 8.3 electrophoresis buffer, running electrophoresis was performed for 2.5 hours at room temperature with a voltage of 125 volts. The gel was taken, then soaked overnight in a solution of Coomassie Brilliant Blue R-250 mixed with glacial acetic acid and methanol. Then, the gel was destained for one hour using a staining solution without dye, and the results were documented. The formation of a blue ribbon indicates the presence of protein. The size of molecules in each group was determined using an extrapolated curve standard, where the log of molecular weights was plotted against the migration distance of the protein marker.

2.5. Data analysis

The difference in lysis clot blood percentage by extracts and crude protein was replicated three times each and tested statistically using SPSS. The lysis clot blood percentage results will first be assessed for normality using the Kolmogorov-Smirnov Test, followed by an evaluation of variance homogeneity using Levene's Test. A parametric one-way ANOVA will be used for further analysis if the data satisfy the presumptions of homogenous variance and normal distribution. An LSD Post-Hoc test will be run if $p < 0.05$ is obtained. The data are presented as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Extraction and Production of White Oyster Mushroom Crude protein

The first stage of this study was to obtain extracts and crude protein from the oyster white mushroom. Extracts were obtained by centrifuging white oyster mushroom powder until 250 ml of supernatant was obtained, which is called the extract. Half of the extract (125 ml) was centrifuged again with twice the volume of acetone, resulting in 80 grams of crude protein sediment.

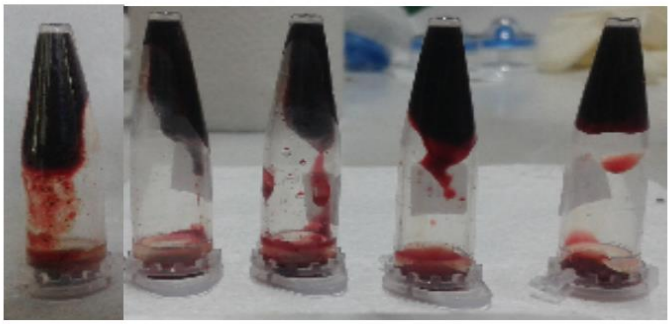
(a) (b)

Figure 1. (a) Extract of white oyster mushroom; (b) Crude protein of white oyster mushroom

3.2. Thrombolytic Activity Testing

The thrombolytic activity of the white oyster mushroom extract and crude protein was determined using the blood clot lysis percentage method. Extracts and crude protein in different concentrations were added to the blood sample. Suspension *Lumbricus rubellus* was added to the control positive, whereas distilled water was used as the control negative. Each test treatment was observed at 30, 60, and 90 minutes.

Figure 2 and Table 2 show the percentage results of blood clot lysis from oyster white mushroom extracts and crude protein. The graph shows the percentage results of blood clot lysis from each sample at 90 minutes, see Figure 3.



(**a**) (**b**) (**c**) (**d**) (**e**)

Figure 2. Lysis blood clot: (a) Control Positive; (b-d) Test Group; (e) Control Negative

Table 2. The results of blood clot lysis

Samples	Concentration	Blood clot lysis percentage (%)		
		30 minutes	60 minutes	90 minutes
Distilled water (negative control)	100 µL	0.34±0.58	1.23±1.17	0.00±0.00^{a}
Flour earthworm (positive control)	0.01 g/mL	14.48±6.31	19.29±7.13	27.03±5.70^{b}
WOM extract	100 µl (64 mg)	13.44±1.54	20.59±4.64	24.64±6.70^{bc}
	200 µl (128 mg)	10.74±2.99	18.87±4.14	22.02±1.85^{bd}
	500 µl (320 mg)	14.73±2.20	15.93±6.08	29.89±10.99^{e}
WOM crude protein	0.01 g/mL	5.33±1.52	1.71±0.47	8.33±0.42^{ab}
	0.02 g/mL	6.29±1.76	16.62±3.75	28.30±5.93^{f}
	0.05 g/mL	17.54±1.48	25.93±3.10	37.89±2.33^{g}

WOM: White oyster mushroom**;** The numbers in the table followed by different letters in the same column show significantly different results based on the smallest difference in real tests with a level of confidence α=5%

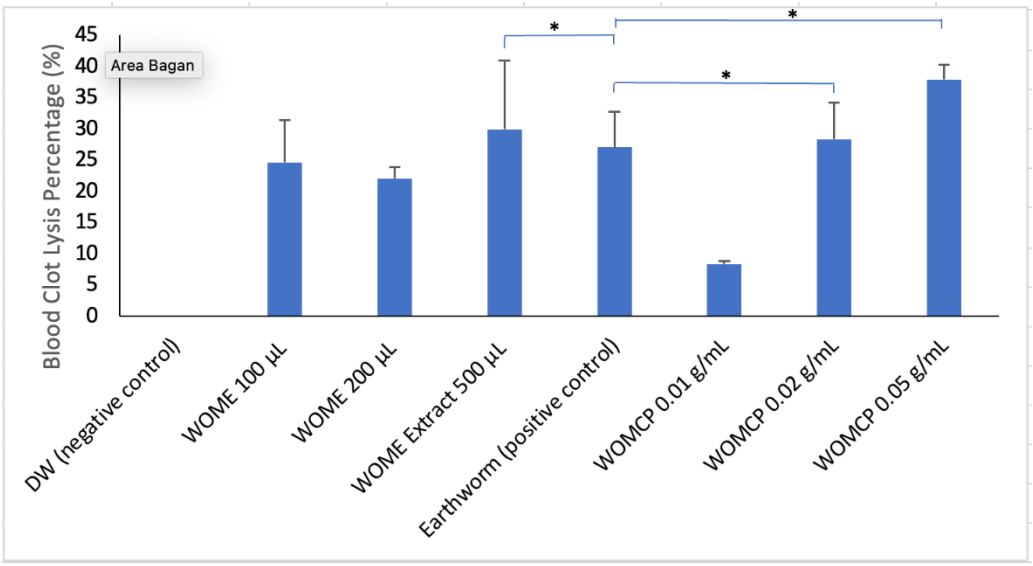


Figure 3. The blood clot lysis percentage of the thrombolytic activity testing (DW: distilled water; WOME: white oyster mushroom extract; WOMCP: white oyster mushroom crude protein; * = significant difference, $p<0.05$)

A suspension of *Lumbricus rubellus* earthworms was used as a positive control. Earthworms have been utilized extensively in recent years to treat various chronic illnesses in China, Japan, and Indonesia. Based on broader research, earthworms have been proven to be useful as antimicrobial agents [17], anti-inflammatory [18], and anticancer [19]. Trisina et al. showed that the blood clot lysis

volume could be measured as early as the first hour of the incubation period, and the volume continued to increase until the 12th hour. This indicates that *Lumbricus rubellus* possesses a fast and long-acting fibrinolytic activity [15]. *Lumbricus rubellus* can directly lyse fibrin and activate plasminogen. The active compound from *Lumbricus rubellus* contains fibrinolytic enzymes, which are a group of serine protease enzymes, consisting of several isoenzymes that are able to cross the intestinal epithelium into the blood [15,20]. This indicates that the enzyme exhibits both fibrinolytic and fibrinogenolytic properties, reducing blood viscosity and platelet aggregation, and enhancing thrombolysis in the blood [15, 21, 22].

The results of the thrombolytic activity test using the blood clot lysis percentage assay method show that the white oyster mushroom extract shown in Table 2 can dissolve blood clots. The results indicate that distilled water, as a negative control, exhibited no blood-lysing ability. The results in Table 2 indicate that white oyster mushroom extract and crude protein have thrombolytic activity, as demonstrated by their comparison to the negative control. The thrombolytic activity of white oyster mushroom extract was observed at each concentration after 30 to 90 minutes of incubation. Based on the incubation period, the longer the incubation time, the higher the blood clot lysis activity. The ability of white oyster mushroom extract and crude protein to dissolve blood clots was highest after 90 minutes. Sharif et al. also showed a similar result, where the absorbance of clot lysis from various mushroom extracts increases with increasing incubation time [23].

Table 2 and Figure 3 show the result of the thrombolytic activity of the samples. The 500 μL extract had the highest thrombolytic activity, at 29.89%, compared to the 100 μL (24.64%) and 200 μL extracts (22.02%). The highest thrombolytic activity of 500 μL extract (29.89%) was significantly different and higher than that of the positive control at 27.03% ($p<0.05$). The crude protein of the white oyster mushroom has also been shown to be capable of dissolving blood clots. After 90 minutes, the crude protein at concentrations of 0.01 g/mL, 0.02 g/mL, and 0.05 g/mL exhibited thrombolytic activities of 8.33%, 28.30%, and 37.89%, respectively. The thrombolytic activity of crude proteins at concentrations of 0.02 g/mL (28.30%) and 0.05 g/mL (37.89%) was significantly higher than that of the positive control at 27.03% ($p<0.05$). The higher the concentration given, the greater the thrombolytic activity. Similar results were observed in another study by Nasrullah et al., where an increase in the percentage of clot lysis was noted with increasing amounts of extract [21]. Islam & Uddin also reported that the mean clot lysis activities of *Pleurotus ostreatus, Ganoderma lucidum, and Lentinula edodes* were affected by both the dose of the sample and the incubation period [24].

Kunwar et al. defined a similar blood clot lysis percentage from methanolic extracts of *Moringa oleifera* leaves and flowers, ranging from 19.07 ± 2.36% to 41.40 ± 2.02% [25]. Other studies explored the thrombolytic activity from various natural resources, such as leaves of *Uncaria acida* (19.90%), *Antidesma cuspidatum* (21.64%), *Scaphium macropodum* (20.74%) [26], *Ficus cunia* (34.72%) [27], and *Leea indica* (32.58%) [28]. Petraglia et al. supported that the crude extracts from *Pleurotus ostreatus* had *the* highest fibrinogenolytic activity (39.14 ± 1.01 μg of PE/mg protein) than other species, such as *Agrocybe aegerita, Pleurotus eryngii,* and *Lentinula edodes* [7]. *Pleurotus ostreatus* also showed the highest clot lysis activity at 18.62%, followed by *Ganoderma lucidum* (17.01%) and *Lentinula edodes* (9.02%) in the identical incubation [23]. The white oyster mushroom (*Pleurotus ostreatus*) exhibits hydrolytic properties that affect its ability to dissolve blood clots [7].

The thrombolytic activity of the white oyster mushroom crude protein was higher than that of the extract (supernatant). It may have occurred because the crude protein was purer than the supernatant, resulting in lower interference. In the production of crude protein from white oyster mushrooms, acetone is used in the process to precipitate the protein contained in the white oyster mushrooms. Acetone was chosen as a solvent because it is easier to use and does not require a dialysis process to remove interfering chemical compounds from the precipitation [14]. Another study showed purified extract of *Styrax paralleloneurum* was chosen due to its better activity [29].

The thrombolytic activity of the white oyster mushroom was supported by Liu et al. Using submerged culture fermentation, an edible *Pleurotus ostreatus* mushroom was developed to produce a fibrinolytic enzyme. The enzyme demonstrated both fibrinogenolytic activity and fibrinogen

breakdown patterns. The enzyme degraded the α chain, β chain, and the γ chain, respectively. Liu et al. reported that the fibrinolytic enzyme from *Pleurotus ostreatus* is capable of dissolving fibrin directly (direct lysis), and can also activate plasminogen to plasmin (plasminogen activator type) [30].

3.3. *Protein Characterization using SDS-PAGE*

The molecular weight of each white oyster mushroom extract and crude protein was measured using 10% SDS-polyacrylamide gel electrophoresis (PAGE). The electrophoresis results of the crude protein protein samples and white oyster mushroom extract are shown in Figure 4. Smaller proteins passed through the gel faster than larger proteins. There is a linear relationship between molecular weight and molecular motion. Standard SDS-PAGE curves are shown in Table 3 and Figure 5.

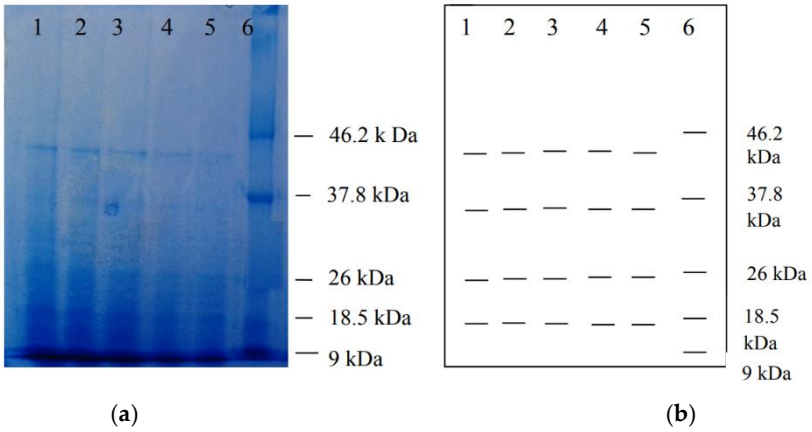


Figure 4. SDS-PAGE electrophoresis results: (a) SDS-PAGE electrophoresis; (b) Visualization of SDS-PAGE results. Note: (1-3) white oyster mushroom crude protein, (4-5) white oyster mushroom extract, (6) protein markers

Table 3. Extrapolation results distance displacement sample to curve standard

X (log BM)	Y (Migration standard)	Y1 (Migration sample)	BM Log	BM
1.665	2	2.5	1.658	45.5
1.578	2.9	3.2	1.512	32.51
1.415	4.1	4.1	1.324	21.09
1.267	4.8	4.8	1.179	15.1
0.954	5.5			

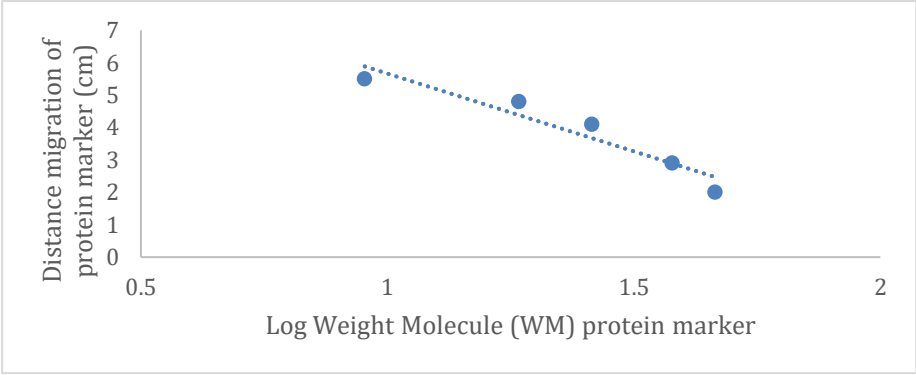


Figure 5. Standard curve of protein markers

This study employed the SDS-PAGE method, which enables the separation of proteins based on their electrophoretic migration properties. The separation of each charged component is achieved through the difference in migration speed or molecular weight (MW) in an electric field. Under the

influence of an electric current, acrylamide gel proteins are pulled down. Proteins with the lowest molecular weight move quickly, so they are attracted to the bottom of the gel, while proteins with the highest molecular weight are at the top of the gel. Molecular weight determination is done by extrapolating the distance of protein sample migration using a protein standard curve between the log of molecular weight and the distance of marker protein migration. Based on the SDS-PAGE results in Figure 4, the crude protein (Samples 1-3) and extracts (Samples 4-5) have four protein subunits with the same protein migration distance, so that by extrapolation the estimated molecular weights of the four protein subunits are respectively 45.50 kDa, 32.51 kDa, 21.09 kDa, and 15.1 kDa. This result is closely related to the molecular weight of the white oyster mushroom protein, namely 32 kDa, which has fibrinolytic activity. The number of protein bands in electrophoresis indicates the number of protein subunits the protein contains. The protein band indicates the largest protein subunits at the top, while smaller protein subunits are indicated by the protein band at the bottom [31]. The number or type of amino acids contained in a protein determines the size of a protein subunit. Generally, proteins that contain many amino acids have larger subunits.

4. CONCLUSION

In summary, this study provides valuable preliminary insights. The highest thrombolytic activity was observed in the *Pleurotus ostreatus* extract at 500 μL and the crude protein at 0.05 g/mL, with values of 29.89% and 37.89%, respectively. The thrombolytic activity of the white oyster mushroom crude protein was higher than the extract. The thrombolytic activity of the extract and crude protein of the white oyster mushroom was significantly higher than that of the earthworm powder suspension as a positive control. Protein characterization of the crude extract and enzyme by SDS-PAGE revealed four protein subunit bands with estimated molecular weights of 45.50 kDa, 32.51 kDa, 21.09 kDa, and 15.1 kDa. These results are almost in accordance with the molecular weight of the white oyster mushroom protein that has fibrinolytic activity. However, several limitations should be acknowledged, including the absence of purified enzyme preparations, the determination of enzyme concentration, the use of zymography assays, and the lack of in vivo validation. Therefore, while the results offer an important foundation, further studies are necessary to confirm and extend these observations.

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