Research Article **Open Access**

In vitro assessment of thrombolytic potential of red and white ginger (*Zingiber officinale*)

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Submission Date: October 18th, 2023**; Acceptance Date:** January 8th**, 2024; Publication Date:** January 12th, 2024

Please cite this article as: Pinontoan R., Leke P. A. I., Lesmana J. A., Purnomo J. S., Dikson., Samantha A. In vitro assessment of the thrombolytic potential of red and white ginger (Zingiber officinale). *Functional Foods in Health and Disease* 2024; 14(1): 62-73. DOI: https://www.doi.org/10.31989/ffhd.v14i1.1245

ABSTRACT

Background: Cardiovascular diseases (CVDs) are a leading cause of death, and their pathogenesis is commonly attributed to thrombosis. Although existing medications are effective and fast-acting for thrombosis management, they tend to be expensive and cause severe side effects. Plant-based thrombolytic agents are actively sought after as inexpensive and safe alternatives for the treatment and prevention of thrombosis. Red ginger (*Zingiber officinale* var. *rubrum*) and white ginger (*Zingiber officinale* var. *officinale*) are widely used in foods and beverages and are believed to confer a wide variety of health benefits.

Objective: This study aims to elucidate the thrombolytic and fibrinolytic potential of red and white ginger extracts in vitro.

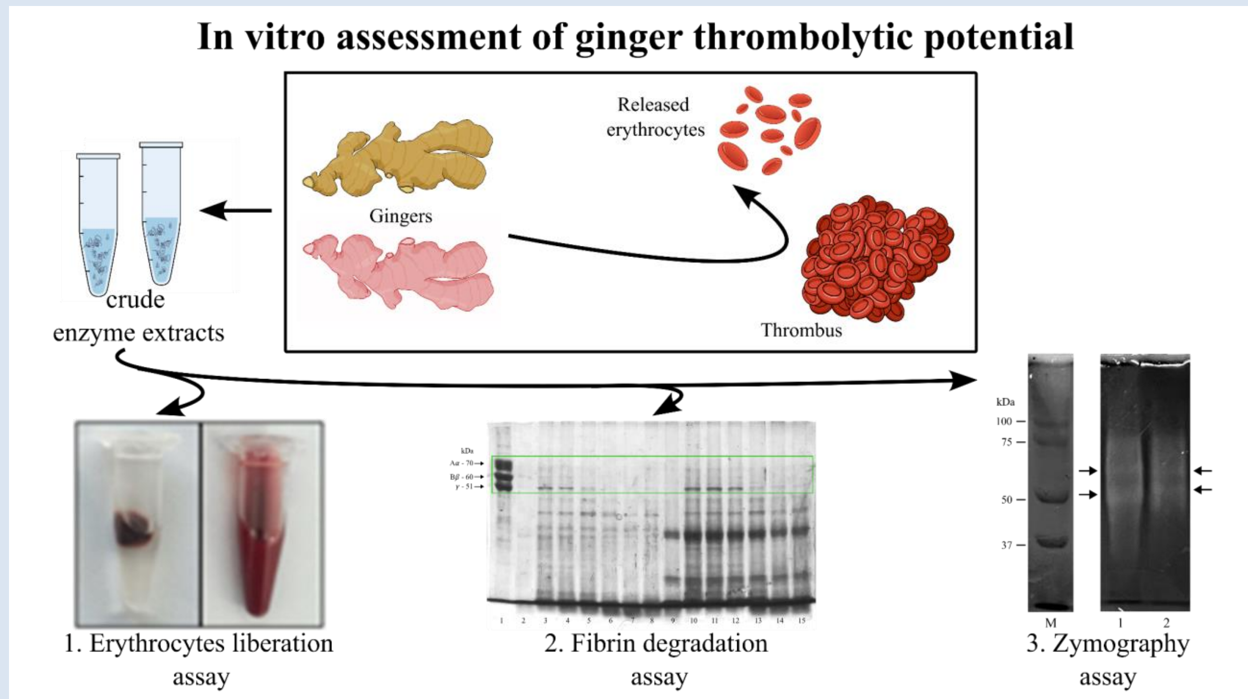
Methods: In this study, in vitro analyses were performed using erythrocyte liberation, euglobulin degradation, fibrin degradation, and fibrin zymography assays. The ability of crude enzyme extracts from both red (rgEx) and white ginger (wgEx) to degrade blood clots was analyzed using the erythrocytes liberation assay. Then, the thrombolytic and fibrinolytic activities of rgEx and wgEx were evaluated using euglobulin and fibrin degradation assays, both of which were visualized using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Finally, fibrinolytic enzymes were identified using a fibrin zymography assay.

Results: Red and white ginger extracts were found to have strong thrombolytic properties via high total liberated erythrocyte count from the erythrocyte liberation assay. The ginger extract proteases can rapidly degrade euglobulin

and fibrin, with their priority order beginning with Aα and Bβ, then γ chains. Fibrin zymography confirmed the presence of several proteases in the red and white ginger extracts.

Conclusion: Overall, while red and white ginger crude enzyme extracts have been reported to possess a strong thrombolytic and fibrinolytic potential and are therefore suggested to be good candidates for managing and preventing CVD, further studies such as full enzyme identification and in vivo studies are needed.

Keywords: fibrinolytic, ginger extract, in vitro assessment, protease, thrombolytic



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INTRODUCTION

Cardiovascular diseases (CVDs) are a leading cause of death within the population and are exacerbated by various risk factors, such as an unbalanced diet and a poor lifestyle. The World Health Organization reported an approximate annual mortality rate of 17.9 million which contributed to 32% of the global mortality in 2019 alone [1]. The common pathology shared between these CVDs is thrombosis caused by unwanted thrombus formation consisting of fibrin, platelets, erythrocytes, leukocytes, and neutrophil extracellular traps [2]. While thrombi may form in the healthy human body as a normal response to injury and damage to blood vessels to stop bleeding, thrombi may also form when an imbalance in hemostasias occurs [3-4]. These thrombi may reduce blood flow in the vessel lumen and cause tissue ischemia, leading to life-threatening CVDs, such as coronary heart disease, lung embolism, and stroke [5]. Thrombosis treatment, management, and prevention normally employ various medications such as anticoagulants, thrombolytics, and antiplatelets. Each has a different mechanism of action targeting the thrombus and its formation. Although effective and fast-acting, these medications tend to be expensive and have potentially

fatal side effects [6-7]. This prompts the search for safer and cheaper thrombolytic agents, preferably from natural sources, as preventative measures.

Functional foods are defined as natural or processed foods containing biologically active compounds in effective, non-toxic amounts, with clinically proven and documented health benefits through specific biomarkers such as promoting health, reducing the risk of chronic/viral diseases, and managing their symptoms [8]. Numerous functional foods have been extensively studied for their health benefits, particularly in the prevention and management of CVDs. These include fermented foods that contain fermenting bacteria that produce thrombolytic enzymes as well as plants that are traditionally used in foods and beverages that contain thrombolytic enzymes. These plants contain metabolites and enzymes and are used in certain diets to confer health benefits and prevent chronic diseases [9]. Ginger (*Zingiber officinale Rosc.*), in particular, has been extensively used by a wide range of ethnic groups across generations as an ingredient for foods, beverages, and medications [10-11]. Ginger can be categorized into three varieties based on rhizome size and color. These varieties include red ginger (*Zingiber officinale* var. *rubrum*), white ginger or giant ginger (*Zingiber officinale Rosc.* var. *officinale*), and emprit ginger or small white ginger (*Zingiber officinale* var. *amarum*) [12]. White ginger is commonly used as a spice, whereas emprit ginger is used as an essential oil [13-14]. Red ginger, on the other hand, is more often used as a traditional medication to treat diseases and is believed to confer a wide variety of health benefits via oral tradition. Contemporary studies have elucidated the beneficial properties of red ginger such as its antioxidant, anti-inflammatory, antiemetic, anti-atherosclerotic, antihypertensive, antihyperlipidemic, cytotoxic, antimicrobial, and immunomodulatory properties. These benefits are attributed to its 169 active phytocompounds, grouped into flavonoids, monoterpenes, diterpenes, sesquiterpenes, vanilloids, phenols, and enzymes [12, 15-19]. Ginger's cysteine protease, identified as Zingibain, has been used in various applications such as meat tenderization [19], milk curdling in cheese production [20], and as a potential bioinsecticide for controlling armyworm pests [12]. Although plant-based proteases show potential in a wide variety of biotechnological applications, particularly pharmaceutical and medical applications [21], the proteases in ginger have yet to be studied for their fibrinolytic effects, which would be useful for resolving thrombosis with the goal of preventing and treating CVDs at a low cost and with minimal adverse effects. Therefore, this study aimed to elucidate the fibrinolytic and thrombolytic potential of red and white ginger as alternative natural products for the treatment and prevention of CVDs, as established in our previous studies [22-24].

MATERIALS AND METHODS

Materials: Red and white ginger rhizomes were purchased from a local supermarket, cleaned, and peeled upon purchase. The gingers were simply identified based on rhizome size and colour. Chicken blood samples were collected from a local abattoir. Fibrin was produced by mixing human fibrinogen acquired from Sigma-Aldrich (USA) and thrombin acquired from Merck (Rahway, NJ, USA).

METHODS

This study was divided into four stages: (1) extraction of crude enzymes from red and white ginger, (2) evaluation of the thrombolytic activity of isolated bacteria, (3) investigation of the fibrinolytic activity of isolated bacteria, and (4) characterization of ginger proteases that have thrombolytic and fibrinolytic activity.

Crude enzyme extraction: A total of 10 gr of each ginger sample was homogenized using a multipurpose blender set to speed 4 out of 5 selectable speeds for 30 seconds

and filtered. Each filtrate (15 mL) was centrifuged at 13,000 × g for 5 minutes. The supernatant was mixed with cold acetone at a sample-to-acetone ratio 1:4 to precipitate the proteins. The mixture was cooled to -20°C overnight and centrifuged at 13,000 × g for 15 minutes. The supernatant was discarded, and the pellet was dried. The protein pellet was resuspended in phosphate-buffered saline (PBS) solution as a crude enzyme extract.

Hartree-Lowry protein quantification: The protein concentration of the crude enzyme extract was measured using the Hartree-Lowry protein quantification method. The crude enzyme extract was first incubated with the first reagent containing sodium carbonate at 50°C for 10 minutes, then with the second reagent containing copper sulfate at room temperature for 10 minutes, and finally with the Folin-Ciocalteu phenol reagent (Merck, Germany) at 50°C for 10 minutes. The Bovine Serum Albumin (BSA) concentration calibration curve was established with the BSA concentrations of 0.2 to 1.0 mg/mL with 0.2 mg/mL increments. The protein concentration of the sample was measured by plotting the Abs^{650} value against a BSA calibration curve. From this measurement, the protein concentration for both extracts was determined to be 2.8 ± 0.1 µg/mL and used for further analyses.

Erythrocyte liberation assay: Chicken whole-blood clots (approximately 0.15 gr) were prepared and washed three times with a saline solution (0.9% NaCl) [23]. The pieces were then inserted into three microtubes, each assigned as a positive control, negative control, and sample. Whole blood clots were incubated with 1 mL of 50 Fibrinolytic Units (50FU/mL) of Nattokinase enzymes (Doctor's Best, USA) (as a positive control), 1 mL of PBS (as a negative control), and 1 mL ginger crude enzyme extract as a sample. The microtubes were incubated at 37°C for up to 6 hours. The number of erythrocytes released from each treatment was calculated using a hemocytometer.

Euglobulin clot lysis test: Whole chicken blood (15 mL) was mixed with 200 µL of an anticoagulant solution containing 10% EDTA. Then, 1.5 µL of the mixture was centrifuged at 570 × g for 15 minutes, and the supernatant containing blood plasma was retrieved. One mL of the plasma was diluted with 9 mL of cold distilled water. By adding 100 µL of 1% acetic acid, the pH of the plasma solution decreased to 4.5. The plasma mixture (1.5 mL) was transferred to each microtube and centrifuged at 820 × g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 20 µL of PBS solution. A volume of 40 µL of 20 mM $CaCl_2$ was added to the suspension to precipitate the euglobulin clot, followed by the addition of 1 mL of PBS solution and incubated at 37°C for 60 minutes [24]. The euglobulin mixture was then transferred into PCR tubes, mixed with ginger crude enzyme extract sample at a 1:1 ratio, and incubated at 37°C for 1, 5, 15, 30, and 60 minutes. For the positive control, the euglobulin mixture was mixed with nattokinase (50 FU/mL) and incubated for 30 minutes. An euglobulin mixture was used as the negative control. Following incubation, the samples along with positive and negative controls, were analyzed using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Fibrin clot lysis test: One milligram of human fibrinogen was weighed and dissolved in 1 mL of PBS. The fibrinogen solution was mixed with 10 µL of 100 NIH units/mL thrombin and incubated at 37°C for 60 minutes to form non-crosslinked fibrin chains clot due to the absence of Factor XIII. The fibrin solution was then transferred into PCR tubes, mixed with ginger crude enzyme extract samples at a 1:1 ratio, and incubated at 37°C for 15, 30, 60, 120, and 180 and 240 seconds [24]. For the positive control, the fibrin mixture was mixed with 50 FU/mL of

nattokinase and incubated for 240 seconds. Fibrin mixture was used as the negative control. Following incubation, the samples along with the positive and negative controls, were analyzed using SDS-PAGE.

SDS-PAGE profiling: Analysis of fibrin and euglobulin degradation was performed by SDS-PAGE using a 12% polyacrylamide resolving gel. The gel was composed of 2 mL of 30% bis-acrylamide (Bio-Rad, USA), 0.05 mL of 10% SDS (Bio-Rad, USA), buffered with 1.3 mL of Tris HCl (1.5 M, pH 8.8), and dissolved in a 1.6 mL deionized water. Polymerization of acrylamide was performed by adding 50 µL of 10% APS (Bio-Rad, USA) and 5 µL of tetramethylethylenediamine (TEMED) (Bio-Rad, USA). The gel was submerged in a running buffer consisting of 0.03 gr Tris, 14.41 gr glycine, 1.00 gr SDS and 1 L deionized water. Each sample (10 µL) was then loaded into the gel and electrophoresis was performed at 150 V until the end of the run [23]. Upon completion, the gels were stained with Coomassie Brilliant Blue R250 (Bio-Rad, USA). The stained gels were visualized and analyzed using Gel Analyzer 19.1.

Zymography profiling: Fibrin zymography profiling was performed by loading the 10% polyacrylamide gel with 0.0006% (w/v) of fibrinogen mixed with 50 µL of 100 NIH units/mL of thrombin. The gel was polymerized and submerged in a running buffer, and all the samples were loaded in each well. Electrophoresis was performed at 150 V until the end of the experiment. The gel was submerged in a renaturation solution for 30 min at 25°C and then incubated in a developing solution overnight at 37°C [24]. Finally, the gels were stained with Coomassie Brilliant Blue R250 and analyzed using Gel Analyzer 19.1.

RESULTS

The thrombolytic potential of red and white ginger was analyzed in multiple steps. First, their capability to degrade blood clots was assessed using the erythrocytes liberation assay (ELA). Then to ascertain whether the blood clot degradation of both samples was due to protease activity, euglobulin and fibrin degradation assays were performed and visualized using SDS-PAGE. Finally, the proteases in both samples were identified using a fibrin zymography assay.

Thrombolytic activity of ginger extracts by erythrocytes liberation assay: ELA was performed to assess the fibrinolytic potential of red and white ginger against coagulated chicken blood. The assay was performed by incubating red ginger (rgEx) and white ginger (wgEx) crude enzyme extracts individually with coagulated chicken blood for 6 hours, and then the amount of released erythrocytes was observed using a hemocytometer. The results are shown in Figure 1.

There were observable and significant increases in the number of erythrocytes released during the 6-hour incubation period for both rgEx and wgEx (Figure 1). Both samples, as well as nattokinase, showed significant increases ($p < 0.05$) in released blood cells from the 2nd to the 6th-hour mark. Additionally, significant increases ($p < 0.05$) were observed from the 4th to the 6th-hour mark for rgEx, and wgEx, respectively. At the 6th hour mark, 5.4, 7.4 and 5.3 billion erythrocytes were liberated from the clot by nattokinase, rgEx, and wgEx respectively. In contrast, through natural processes (negative control), the total number of erythrocytes released from the whole blood clot was estimated to be only 134 million (Figure 1).

Thrombolytic activity of ginger extracts by euglobulin degradation assay: Euglobulin degradation assay was performed to confirm the results from the previous ELA, as the euglobulin fraction of the plasma contains only minute levels of anti-fibrinolytic agents [25]. The results are shown in Figure 2.

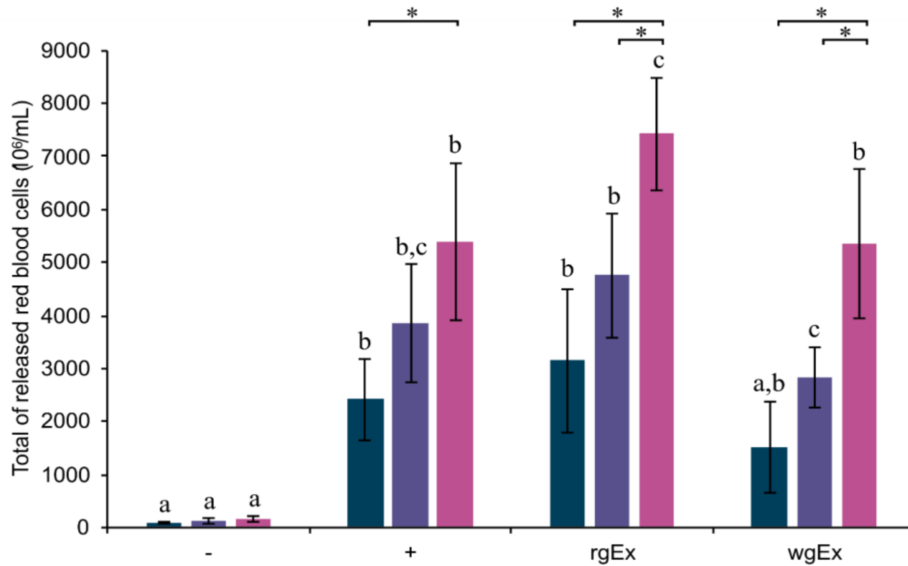

Figure 1. Erythrocyte liberation assay using red ginger (rgEx) and white ginger (wgEx) crude enzyme extracts. The whole blood clots and crude enzyme extracts were incubated in a microtube for 6 hours at 37°C. The total number of released erythrocytes was calculated using a hemocytometer at 2 ( ), 4 ( ) and 6 ( ) hour marks. Nattokinase (50FU/mL) was used as the positive control (+) whereas PBS was used as the negative control (-). * indicates a significant difference ($p < 0.05$) during the 6 hours of incubation for each sample, while the letters (a,b,c) indicate significant differences ($p < 0.05$) between samples with equivalent incubation periods.

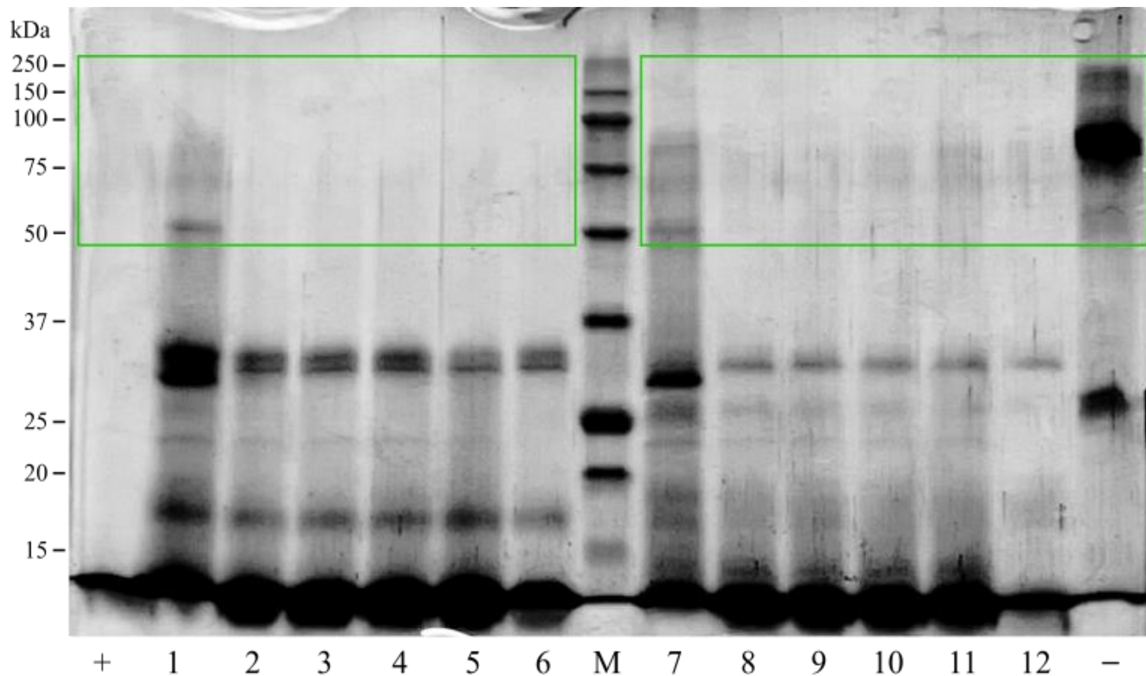

Figure 2. Euglobulin degradation pattern of ginger crude enzyme extract on SDS-PAGE. The green box highlights the euglobulin fraction degraded by ginger crude enzyme extracts. The content of each lane was as follows: (M) protein marker, (+) euglobulin incubated with nattokinase (50 FU/mL) for 30 minutes, (-) euglobulin in PBS, (1) red ginger crude enzyme extract (rgEx) in PBS, (2) euglobulin + rgEx incubated at 37°C for 1 minute, (3) 5 minutes, (4) 15 minutes, (5) 30 minutes, and (6) 60 minutes, while lane (7) was loaded with white ginger crude enzyme extract (wgEx) in PBS, (8) euglobulin + wgEx incubated at 37°C for 1 minute, (9) 5 minutes, (10) 15 minutes, (11) 30 minutes, and (12) 60 minutes.

The results showed that the euglobulin fraction consisted of multiple crosslinked fibrin chains and non-crosslinked fibrin chains, as well as plasmin and plasminogen (Figure 2, Lane –). Some of the detected fibrin chains bands were identified as non-crosslinked Aα (60 kDa) and Bβ (54 kDa) chains, crosslinked α-α (134 kDa) and γ-γ (84-89 kDa) chains, and tissue plasminogen activator (t-Pa) (31 kDa) and plasmin (25 kDa), as previously reported in our early study [23]. In contrast, multiple bands were profiled in the rgEx and wgEx lanes (Figure 2, Lanes 1 and 7), corresponding to a variety of proteins in the ginger extracts. The molecular weights of the rgEx protein bands were estimated to be 18, 22, 27, 30, 54, and 71 kDa, whereas those of wgEx were estimated to be 22, 24, 27, 53, and 83 kDa. Both rgEx (Figure 2, Lanes 2-6) and wgEx (Figure 2, Lane 7-12) were determined to have high thrombolytic activity, comparable to that of 50FU/mL nattokinase (Figure 2, Lane +) based on the euglobulin degradation time course assay. Fibrin chains were also degraded by both extracts, suggesting that fibrin degradation is one of the possible mechanisms that may play a role in the liberation of erythrocyte cells in ELA. Thus, a fibrin degradation assay was performed to ascertain the fibrinolytic activity of both the samples.

Fibrinolytic activity of ginger extracts by fibrin degradation assay: Having confirmed the euglobulin clot lysis activities of the ginger extracts, the direct fibrinolytic activity of both samples was analyzed by observing the degradation pattern of fibrin in an SDS-PAGE gel. To generate fibrin proteins, a mixture of human fibrinogen, bovine thrombin, and crude enzyme extract from each sample was incubated in PBS. Because the results from euglobulin fraction degradation displayed immediate degradation, even when it was incubated for only 60 seconds with the crude enzyme, the fibrin degradation assay was performed at a shorter duration, ranging between 15 and 240 seconds. The results are shown in Figure 3.

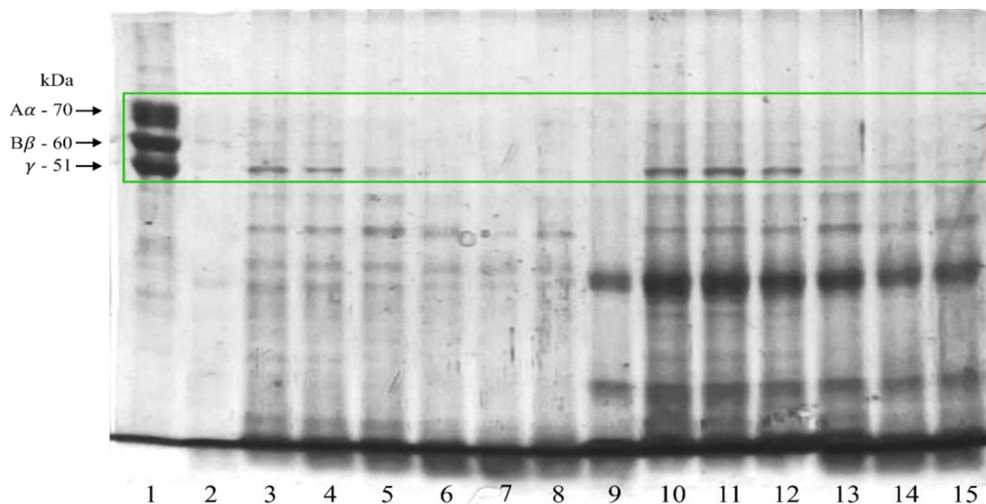

Figure 3. Fibrin degradation patterns of crude ginger enzyme extracts determined by SDS-PAGE. The green box highlights the fibrin subunit chains degraded by ginger crude enzyme extracts. The content of each lane was as follows: (1) fibrin, (2) red ginger crude enzyme extract (rgEx) in PBS, (3) Fibrin + rgEx incubated at 37°C for 15 seconds, (4) 30 seconds, (5) 60 seconds, (6) 120 seconds, (7) 180 seconds and (8) 240 seconds, while lane (9) was loaded with white ginger crude enzyme extract (wgEx) in PBS, (10) fibrin + wgEx incubated at 37°C for 15 seconds, (11) 30 seconds, (12) 60 seconds, (13) 120 seconds, (14) 180 seconds and (15) 240 seconds.

In this assay, fibrin formation can be observed in the forms of the three non-crosslinked fibrin chains: Aα (70 kDa), Bβ (60 kDa), and γ (51 kDa) as shown in Figure 3, lane 1. The time course of fibrin degradation showed gradual degradation of the fibrin chains. On close inspection, sample rgEx was able to degrade majority of the fibrin chains shown by the reduction of band intensity for all fibrin chains, with thin bands of Aα and Bβ chains still observable after 30 seconds of incubation while thicker band of γ chain was still observable after 60 seconds of incubation. In contrast, although the majority of fibrin was degraded by wgEx, its fibrinolytic activity was lower than that of rgEx. This was seen from the degradation pattern of Aα and Bβ chains, which were still observable even after 60 seconds of incubation, as well as the existing γ chain observed throughout the 240 seconds of incubation period.

Fibrinolytic zymography of ginger extracts: To identify the fibrinolysis-capable proteases, a fibrin zymography assay was performed for both rgEx and wgEx. The results are shown in Figure 4.

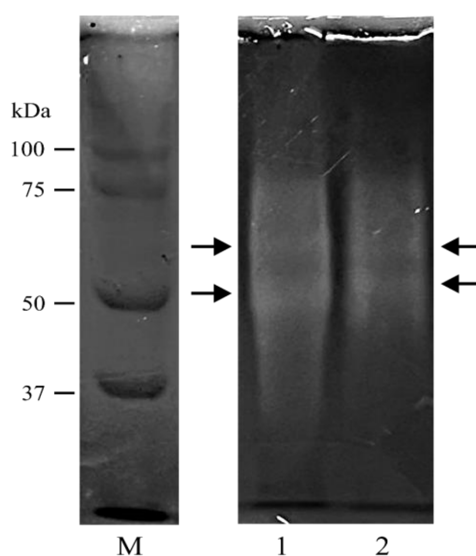

Figure 4. Fibrin zymography of both red ginger and white ginger extract. Each lane was loaded with (M) protein marker, (1) red ginger crude enzyme extract (rgEx), (2) white ginger crude enzyme extract (wgEx).

The clear zones seen as white bands in Figure 4 indicate the absence of fibrin protein owing to the fibrinolytic activity of the ginger proteases in the extract. The two identified proteases of wgEx with fibrinolytic activity were determined to have molecular weights of approximately 47 and 58 kDa, whereas the other two proteases of rgEx had molecular weights of approximately 50 and 57 kDa.

DISCUSSION

Ginger and its extracts have been widely used in traditional medicine to improve cardiovascular health. The efficacy of ginger for cardiovascular health has been attributed to its bioactive compounds, which act as antioxidant, anti-inflammatory, anti-obesity, antidiabetic, and antiplatelet aggregators, in controlling blood lipid profile and blood pressure [26]. While most studies have identified the beneficial bioactive compounds in ginger as phytochemicals [27], there is no comprehensive study on ginger protease as one of the mechanisms that confer health benefits. This study also elucidated the proteolytic activity of ginger extract for the management of blood clot formation in cardiovascular diseases using ELA, euglobulin and fibrin degradation and fibrin zymography assays.

In ELA, erythrocyte cells were successfully liberated from the blood clots after incubation with rgEx and wgEx (Figure 1). After 6 hours of incubation, significant

increases in the number of liberated blood cells were detected, indicating that the enzymes in the ginger crude extracts were able to degrade blood clots even after 6 hours. Remarkably, through comparison between samples, rgEx outperformed 50FU/mL nattokinase ability to degrade blood clots, while wgEx performed similarly to nattokinase. Nattokinase, an enzyme produced by *Bacillus subtilis* and commonly found in the Japanese fermented food Natto, was used as the positive control because of its beneficial effects on blood circulation and general cardiovascular health [28-29]. Given the limited information on the fibrinolytic activity of the crude extract, it is unclear whether the enzyme activity in red ginger surpasses that of nattokinase, or if the high degradation performance is solely due to the high concentration of the crude enzyme extract.

A closer examination of the mechanism underlying the thrombolytic capability of both rgEx and wgEx through the euglobulin degradation assay suggested that the degradation of blood clots was a result of proteolytic activity. The proteases inside the crude extract for both rgEx and wgEx were fast acting, as they were able to degrade euglobulin fractions within one minute of incubation (Figure 2). As fibrinogen, a precursor of the blood-clotting agent fibrin, is a major component of the euglobulin fractions that mediate blood clots, a more in-depth analysis of fibrin degradation time was performed.

The fibrin degradation results indicated that fibrin was readily degradable by both rgEx and wgEx, with a degradation time of less than a minute (Figure 3). This rate of fibrin degradation is comparable, if not better, than that of most reported plant fibrinolytic enzymes. The leaves of Leucas indica and Clerodendrum colebrookianum were reported to contain serine proteases namely lunathrombase and clerofibrase, that can degrade fibrin Aα chains within 30 minutes [30-31]. The ficin enzyme from Ficus and papain enzyme from Carica papaya, with sufficiently high enzyme concentrations, were able to degrade all fibrin chains within 30 minutes [32-33]. Additionally, the degradation pattern of fibrin by both extracts suggests that there are preferences for enzymatic actions on different fibrin chains. Both rgEx and wgEx degrade Aα and Bβ chains faster than γ chain. This suggests that the fibrin degradation mechanism of these crude enzyme extracts may not be concurrently progressing at the same rate. These chain preferences have also been reported in other studies. This result is also in line with the degradation pattern of other reported plants with fibrinolytic activity such as Ficus and Carica papaya which also have encountered the same delayed γ chain degradation [32-33]. Whereas both Leucas indica and Clerodendrum colebrookianum showed no degradation of both Bβ and γ chains [30-31]. This indicated that γ chain might be generally more difficult to degrade compared to Aα and Bβ chains. Therefore, the euglobulin and fibrin degradation assays reinforced the finding that both rgEx and wgEx have thrombolytic abilities.

Table 1. Putative ginger proteases with fibrinolytic properties

List of accession numbers	Types of enzymes	Calculated molecular weight (kDa)
XP_042441491	Zinc metalloprotease	59
XP_042460560	Serine protease	58
XP_042408871, XP_042381191, XP_042470689, XP_042374065, XP_042414219, XP_042418911	Aspartic protease	47
XP_042396153, XP_042392260, XP_042392259, XP_042463349, XP_042375219	Aspartic protease	50
XP_042397565, XP_042392257	Aspartic protease	57

To further ascertain the involvement and predict the identity of ginger proteases with fibrinolytic activity, a fibrin zymography assay was performed. Two clear bands were detected in each lane of rgEx and wgEx, with molecular weights of approximately 50 and 57 kDa, and 47 and 58 kDa, respectively (Figure 4). Although the main protease of ginger, zingibain, was identified to have a molecular weight ranging from 29 to 31 kDa [34], it was not detected in the fibrin zymography assay. However, a study did report a 62 kDa dimeric protease, which consisted of two monomers with molecular weights of 29 and 31 kDa, capable of degrading casein [35]. Thus, the heavier bands from both rgEx and wgEx were speculated to be zingibain dimers.

While zingibain is a cysteine protease, other types of enzymes including serine protease, threonine protease, metalloprotease and aspartic protease, are also capable of digesting fibrin and thus might also be contained in the extract [36]. In the National Center for Biotechnology Information (NCBI) database, three types of ginger proteases have been identified: a zinc metalloprotease, a subtilisin-like protease (serine protease), and aspartic proteases. In addition to their molecular weight matching that of the zymography results, proteases identified contained in the ginger genome are listed in Table 1. Overall, ELA, euglobulin and fibrin degradation assays, as well as fibrin zymography assay have successfully identified two main proteases that mediate the thrombolytic activity of ginger extracts.

CONCLUSION

In summary, a detailed characterization of ginger proteases from red and white ginger with thrombolytic activity has been reported. The proteases can rapidly degrade euglobulin fraction and directly degrade fibrin, with high affinity toward Aα and Bβ chains, then γ chain. Through molecular analysis of both the crude enzyme extracts profile using SDS-PAGE and fibrin zymography, the protease with fibrinolytic activities of red ginger and white ginger was predicted to have molecular weights of 50 and 57, as well as 47 and 58, respectively. These results suggest that both red and white ginger are potential natural alternatives for the treatment and prevention of CVDs. The enzymes may be purified and delivered either orally or intravenously. These results may also raise general awareness regarding the health benefits by different types of ginger. Despite successfully reporting the potential use of ginger as an alternative natural product for the treatment and prevention of CVDs, several unresolved questions are yet to be clarified. These questions include the identification of proteases with fibrinolytic activity and *in vivo* studies of protease activity. Protease identification can be achieved using western blotting and/or LC-MS-MS for protein sequence identification. An in vivo study observing thrombus formation in the mouse tail after oral or intravenous injection of purified ginger enzyme may further corroborate the thrombolytic potential of both red and white ginger. Further studies on these aspects may support the discovery of effective, low-risk, and inexpensive alternatives for CVD treatment and prevention.

List of Abbreviations: BSA: bovine-serum albumin; CVD: ardiovascular diseases; ELA: erythrocytes liberation assay; FU: fibrinolytic unit; rgEx: red ginger extract; wgEx: white ginger extract.

Competing Interests: The authors declare that they have no competing interests.

Authors' contributions: [RP]: [Conceptualization], [Supervision], [Funding acquisition], [Written and Reviewed the manuscript]. [PAIL]: [Formal analysis and investigation], [Written and Reviewed the manuscript]. [JAL]: [Formal analysis and investigation], [Written and Reviewed the manuscript]. [JSP]: [Formal analysis and investigation], [Written and Reviewed the manuscript].

[D]: [Written and Reviewed the manuscript]. [AS]: [Methodology], [Written and Reviewed the manuscript].

Acknowledgements and Funding: We would like to thank the Indonesian Ministry of Education, Directorate General of Higher Education, Research, and Technology (DIKTIRISTEK) (1429/LL3/AL.04/2023) for the funding of this research.

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