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RESEARCH ARTICLE

In vitro anticoagulant activity of crude fibrinolytic protease HSFT-2 as an alternative anticoagulant agent

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Thrombotic disorders are one of the leading causes of death in cardiovascular patients and contribute to the incidence of non-infectious diseases. The search for new anticoagulant agents to overcome the limitations associated with existing anticoagulant therapies is increasing, especially those derived from natural and microbial sources. *Staphylococcus hominis* HSFT-2 is a Gram-positive coccus isolated from rusip *Holothutia scabra* commonly known as sand sea cucumber. Fibrinolytic protease HSFT-2 has been reported to be able to degrading fibrin, which has shown several characteristics of fibrinolytic and clot-lysis activities. This study aims to evaluate the anticoagulant activity of crude fibrinolytic protease HSFT-2 in vitro as potential alternative anticoagulant agents. The research design was completely randomized, with a sample size of 21 participants calculated using the Federer formula. The material used was crude protease derived from *S. hominis* cultured in Skim Milk Broth. Various tests, including blood routine tests, platelet aggregation tests, and blood smear evaluations, were conducted to assess its effectiveness as an anticoagulant. Whole blood samples were divided into three groups: a positive control, a negative control, and a group treated directly with 100 µL of crude fibrinolytic protease. The normality of the data was evaluated using the Kruskal-Wallis test, while differences between groups were analyzed with one-way ANOVA. The one-way ANOVA results from the blood routine tests indicated a significant difference between the control and the fibrinolytic protease HSFT-2 ($p<0.05$). The platelet aggregation percentage decreased from 75.85% in the control group to 21.75% with the addition of crude protease HSFT-2. Furthermore, the blood smear evaluations revealed no abnormalities in cell formation when HSFT-2 protease was used, compared to samples without the addition of EDTA anticoagulant. In conclusion, the fibrinolytic protease HSFT-2 exhibits anticoagulant activity; however, it does not yet meet the standards required for use as an anticoagulant in routine blood tests. Future work should focus on the purification and characterization of the protease to enhance its anticoagulant potential for laboratory diagnostic applications.

1. INTRODUCTION

Thrombotic disorder is one of the global death threats and is included in the group of diseases related to the heart and blood vessels. According to the World Health Organization (WHO), this disease contributes to the world's death rate by 31%, and WHO predicts that in 2030, there will be an increase in cases to more than 23.6 million (1). Anticoagulant therapy plays a crucial role in preventing and treating thrombotic disorders by inhibiting the formation of blood clots and promoting their dissolution through the fibrinolysis process by plasmin. Anticoagulant agents are therefore essential in the treatment of disorders related to blood clotting. The development of diseases that are very massive and physiologically varied requires the right anticoagulant agents to support laboratory diagnosis (2). Using appropriate anticoagulants to treat blood clots is the right preventive step for handling and establishing a diagnosis. The existence of several types of anticoagulants does not guarantee success in blood clotting therapy. This is due to the need for the right anticoagulant agent regardless of the variation in pathophysiological conditions of the disease, composition of ingredients, dosage, and side effects, so innovation is needed in anticoagulant therapy to develop diagnostic reagents (3).

Anticoagulants are substances that function as inhibitors of the blood clotting process. Anticoagulants such as EDTA, Heparin, and Sodium Citrate have been widely used as laboratory examination materials for clinical samples (4). These anticoagulants, such as EDTA, Heparin, and Sodium Citrate, are used in routine blood tests to prevent clotting in blood specimens before the examination. In its application, fibrinolytic protease is known to have anticoagulant activity, namely preventing the blood clotting process by inhibiting the formation of thrombin and binding calcium ions. These calcium ions play a crucial role in the blood clotting process, and their binding by anticoagulants prevents the formation of clots (5).

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Bioactive compounds, such as fibrinolytic proteases, are of paramount in the pharmaceutical industry, serving as a crucial source of drugs, including anticoagulants. Fibrinolytic protease, an enzyme that can hydrolyze fibrin, is formed during the blood clotting process through a proteolysis mechanism in the presence of thrombin (6). The uncontrolled formation of blood clots can lead to disturbances in the hemostasis system, such as plasmin factors and anticoagulants, resulting in the blockage of blood vessels. The importance of fibrinolytic protease, sourced from various sources, including the fermentation of marine biota, in the pharmaceutical industry cannot be overstated, underscoring the urgency of our research in this field (7).

Microorganisms, plants, and animals produce fibrinolytic enzymes. Other sources that have the potential to produce fibrinolytic proteases include fungi, insects, marine animals, snakes, and earthworms (8). However, it is the microorganisms that are particularly valuable. They have the potential to produce economically significant enzymes, with optimal activity, and are easy to modify and manipulate genetically. This underscores the significance of your work. Fibrinolytic protease enzymes, including those from traditional fermentation processes, are easy to obtain. They can be produced by various types of bacteria, such as *Bacillus cereus*, *B. licheniformis*, *Stenotrophomonas* sp., and *B. amyloliquefaciens* RSB34 isolated from doenjang fermentation products, and *Bacillus pumilus* 2.g isolated from gembus fermentation products (9).

The fibrinolytic protease HSFT-2 is an enzyme produced extracellularly by *Staphylococcus hominis* HSFT-2 bacteria isolated from the fermented muscle tissue of the sand sea cucumber. Strain HSFT-2 produced fibrinolytic protease, antithrombotic, and antiplatelet activity, which are important characteristics of an anticoagulant agent. Our study, which builds on a previous one, revealed that the crude fibrinolytic protease HSFT-2 has activity as an anticoagulant, inhibiting blood clotting with a clot lysis percentage of 54.97% and prolonging the bleeding period or blood clotting time for 16 seconds (10). This study represents a novel exploration into the anticoagulant activity of fibrinolytic protease from Staphylococcus *hominis* HSFT-2 for laboratory diagnostic reagents, a field that has not yet been investigated. Our findings open up potential applications for protease-producing bacteria in vivo anticoagulant and toxicity assays, offering a promising direction for future research and development.

Fibrinolytic protease has been widely used in the pharmaceutical, cosmetic, and food industries. One of the applications of fibrinolytic protease in the pharmaceutical industry is as an alternative anticoagulant with almost the same function and activity. The blood clotting mechanism occurs due to fibrin accumulation, causing the blood to clot. Fibrin is the main protein of blood clots, which is derived from fibrinogen by thrombin (11). Several studies revealed protease-producing bacteria with anticoagulant activity, such as *B. tequilensis* strain HSFI-5, which showed anticoagulant activity in vitro but has not yet been tested for anticoagulant activity through in vivo assays (12). The anticoagulant activity of protease from *B. thuringiensis* strain HSFI-12 was reported to have anticoagulant and antiplatelet activities qualitatively by its ability to extend clotting time up to 12-21 mins (13).

This study introduces a novel anticoagulant reagent for laboratory examination of the fibrinolytic protease from *S. hominis* HSFT-2. The antithrombotic activity of fibrinolytic protease HSFT-2 has been revealed, showing the potential to break down the blood clot and prolong the clotting time. These characteristics support its application as an anticoagulant reagent to prevent clotting in blood specimens. The current anticoagulants, such as EDTA, heparin, and sodium citrate, have limitations, such as high costs, limited availability in some countries, side effects such as heparin-induced thrombocytopenia (HIT), and potential toxicity and instability during storage or long-term use, prompting the search for safer and more cost-effective alternatives (14). This study aims to test the anticoagulant activity of the fibrinolytic protease HSFT-2 as an alternative anticoagulant in routine blood tests for in-vitro blood cell counts, potentially offering significant advantages over current anticoagulants.

2. MATERIALS AND METHODS

This randomized research used three examination parameters: blood routine assays, platelet aggregation tests, and blood smear evaluations. The study was conducted at the Laboratory of Hematology of Universitas Muhammadiyah Semarang in November 2023. *Staphylococcus hominis* HSFT-2 (10) was previously isolated from rusip sand sea cucumber *Holothuria scabra* obtained from the Captivity of LIPI Lombok Marine Bio Industry Center and identified as the following criteria: 1-year-old *H. scabra*, with 144.01 grams weight, 4 cm width, 17 cm length, and 5 cm thickness. The intestine was removed and washed with sterile water, and clean muscle tissue was cut into pieces and made into rusip. Rusip fermentation uses a mixture of 2.84 grams coarse salt (local brand), 1.14 grams palm sugar, and 11.36 grams sea cucumber, then left for 7 days at a temperature of 25°C in a sterile closed container (anaerobic condition). 1 mL of the fermentation product was diluted at a concentration ranging from 10^{-1} – 10^{-5} and cultured on Nutrient Agar (NA) medium (Cat. No. M561, Himedia, India) at 37°C for 24 hours. The colonies with different appearances were purified individually. One type of bacterial colony that grew on the medium indicates that the colony is pure, then tested for proteolytic activity on Skim Milk Agar (SMA) medium (Cat. No. M763, Himedia, India) (10). The study's sample number was calculated using Federe's formula. The total number of treatments in this study was three groups, namely: 1) positive control; 2) negative control; and 3) 1 mL whole blood with the addition of 100 µL crude fibrinolytic protease. Each experiment group had a seventh replicate. The total number of study samples was 21. The blood samples used for the study were obtained from a healthy

volunteer willing to be a respondent. The research was conducted with the permission of the ethics committee of the Medical Faculty of Universitas Islam Sultan Agung Semarang, with the number 254/VII/2024/Bioethics Committee.

2.1. Preparation of Crude Fibrinolytic Protease HSFT-2

Bacterial colonies identified as proteolytic isolates were inoculated on Skim Milk Broth (SMB) medium (Cat. No. M609S, Himedia, India) containing 5 grams peptone, 1.5 grams beef extract, 35 grams NaCl, and 10 grams casein, and incubated for 72 hours at 37°C. SMB medium contains casein as a substrate that can stimulate bacteria to produce protease enzymes. Crude fibrinolytic protease HSFT-2 was extracted by centrifuging the bacterial culture at 4°C at 3,000 rpm for 15 mins. The supernatant was then subjected to a thorough test of anticoagulant activity (4).

2.2. Routine Blood Assays

The blood routine parameters used in the study, including hemoglobin (HGB), hematocrit (HCT), blood cell count (RBC, WBC, PLT), and erythrocyte index (MCV, MCH, MCHC), were analyzed using the Hematology Analyzer BC-2600 (Mindray, China). These parameters were crucial in examining of the anticoagulant activity of crude fibrinolytic protease from *S. hominis* HSFT-2. The findings of this study, which used EDTA 10% as a positive control, could potentially have significant implications for our understanding of hematology and biochemistry, opening new avenues for research and clinical applications. Whole blood was mixed with crude fibrinolytic protease HSFT-2, then homogenized and measured using a hematology analyzer.

2.3. Platelet Aggregation Test

The platelet aggregation test was performed to examine the antiplatelet activity of crude fibrinolytic protease HSFT-2 as the anticoagulant agent. A blood mixture was prepared with fibrinolytic protease extract 4:1, and a blood smear was immediately made to read the 0-minute aggregation. The remaining blood mixture was added with ADP (Cat. No. 101312, BIO/DATA, USA) and left for 3 mins. Another blood smear was made to read the 3 mins aggregation. The percentage of platelet aggregation between 0 min and 3 mins was then calculated, expressing the antiplatelet activity. The results of this test are of immense significance as they provide crucial insights into the antiplatelet activity of the fibrinolytic protease, potentially shaping the future of antiplatelet research. The determination of platelet aggregation percentage was carried out using the following formula (15):

$$\text{Aggregation (\%)} = \frac{\text{Platelet aggregation}}{\text{Platelet total (free}-\text{aggregation)}} \times 100\% \quad \text{..... (1)}$$

2.4. Evaluation of Blood Smear

The blood smear evaluations were performed to evaluate the anticoagulant activity of fibrinolytic protease HSFT-2 by looking at the distribution of blood cells that are separated from each other. A total of 10 µL of blood sample was dropped on a glass slide, then meticulously shifted with a spreader, then dried, and then the blood smear preparation was painted with 10% Giemsa (Cat. No. 109204, MERCK, Germany). The blood smear was then observed under a microscope with 100X objective magnification (16).

2.5. Data Analysis

Data were analyzed using SPSS 25 software (IBM, USA). Data normality test using Kruskal Wallis. The One-way ANOVA test followed normal data. A significant value of less than 0.05 was expressed as the result with a significant difference.

3. RESULTS AND DISCUSSION

3.1. Routine Blood Assays

The bacterial isolate of *Staphylococcus hominis* HSFT-2 used in this study was from a previous study originally deposited in glycerol stock. 50 mL of crude fibrinolytic protease was obtained as a supernatant after subculturing and centrifugation in the SMB medium. This sample underwent routine blood tests, platelet aggregation tests, and blood smear evaluations. Blood routine results (Figure 1) showed a significant difference in the result both in positive and negative control compared to crude fibrinolytic protease HSFT-2. The p-value was 0.000 ($p<0.05$).

Anticoagulants function as inhibitors of the blood clotting process. Currently, anticoagulants such as Ethylenediaminetetraacetic acid (EDTA), Heparin, and Natrium Citrate have been widely used as examination materials for laboratory clinical samples (17). Routine blood tests, such as EDTA, are needed to prevent clotting in blood specimens before the examination (18). Previous research showed that fibrinolytic protease HSFT-2 has the potential to be an anticoagulant that can inhibit blood clotting and break down blood clots. This characteristic is important in being an

anticoagulant agent (10). In its application, fibrinolytic protease is known to have anticoagulant activity, namely preventing the blood clotting process by hydrolyzing fibrin. The blood clotting mechanism occurs due to the fibrin accumulation, causing the blood to clot. Fibrin is the main protein of blood clots, and it is derived from fibrinogen by thrombin (19).

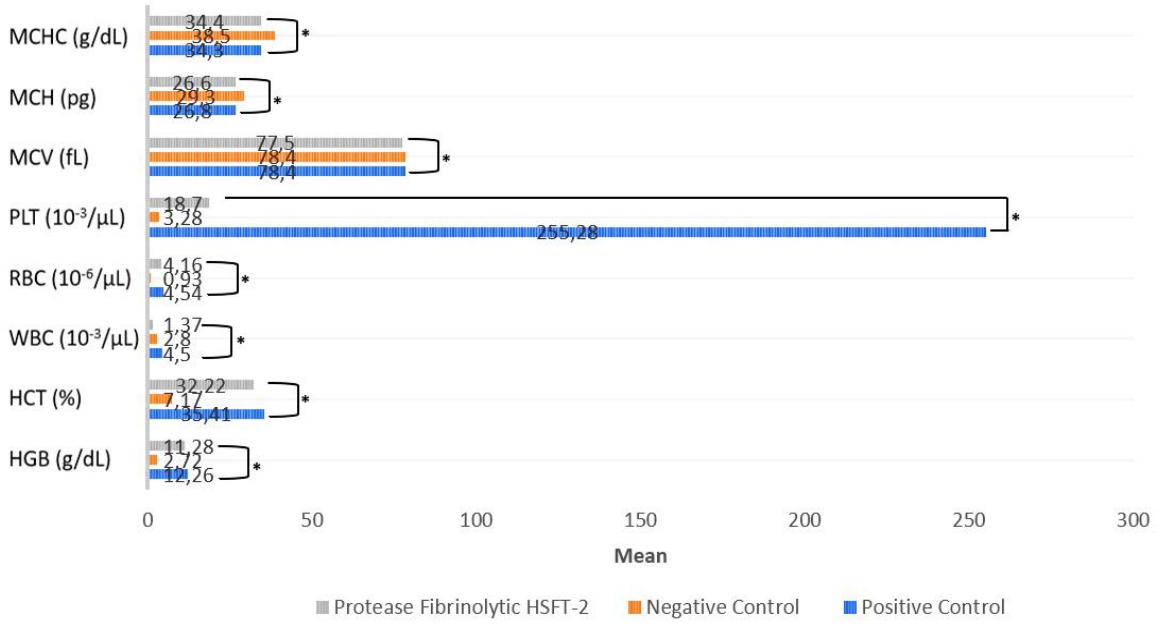

Figure 1. Routine blood test results between groups. *Significant

Using fibrinolytic protease HSFT-2 as an alternative anticoagulant agent shows the potential to prevent clotting in blood specimens for routine blood laboratory tests. The results of routine blood tests (Figure 1) indicate that HSFT-2 fibrinolytic protease can be an alternative to EDTA anticoagulant. However, it needs to be further developed to maximize its potential as an alternative anticoagulant agent in laboratory tests. A comparison between the results of blood specimens using EDTA anticoagulant (positive control) and fibrinolytic protease HSFT-2 shows a difference in the results of routine blood tests based on statistical analysis.

In contrast, the results of routine blood tests of specimens without anticoagulant (negative control) show a significant difference in blood specimens with the addition of fibrinolytic protease HSFT-2. These results indicate that fibrinolytic protease HSFT-2 has anticoagulant activity as a clotting inhibitor in blood specimens for routine blood laboratory tests. Although the results indicate that fibrinolytic protease HSFT-2 can prevent blood clotting, this study has not directly compared its effectiveness with conventional anticoagulants such as heparin and sodium citrate. Further comparisons are necessary to evaluate whether fibrinolytic protease HSFT-2 could serve as a replacement or adjunct to existing anticoagulant agents in clinical laboratory settings.

3.2. Platelet Aggregation Test

The results of the platelet aggregation test in the control group using ADP and the treatment group with the addition of 100µL of crude fibrinolytic protease HSFT-2 are shown in Table 1. Based on Table 1, there was a significant difference in the mean value of platelet aggregation between the control group and the treatment group. This finding suggests that the administration of the HSFT-2 protease enzyme can prevent the formation of platelet cell aggregation to prevent blood clot formation (20). Platelets play a role in primary hemostasis and blood clotting. Platelet aggregation tests aim to see the function of platelet cells, especially in the blood clotting mechanism. Some bleeding disorders result in excessive blood loss, prompting platelets to form clots as a hemostatic response (21).

The use of anticoagulants prevents the formation of platelet clots, ensuring smooth blood circulation. Once platelet clots bind to fibrin, they form complex clots that become more difficult to break down (22). Our results demonstrated a decrease in the percentage of platelet aggregation using a mixture of blood and fibrinolytic protease HSFT-2. A similar study was conducted using fibrinolytic enzymes produced by *Bacillus thuringiensis* HSFI-12 (13) and *Bacillus cereus* (16), which prevent blood clotting and cause platelets not to aggregate. Fibrinolytic protease, an enzyme that can hydrolyze fibrin, is a key in the breakdown of fibrin that forms when clots occur, causing complex clots to break down. This mechanism is interpreted as anticoagulant and/or antiplatelet activity (23).

The presence of platelets in the blood affects blood formation. The formation of platelet aggregation triggers blood clotting (thrombus) regardless of pathological conditions such as coagulation factor disorders (24). A study shows that an individual with blood type O has a higher risk of thrombus formation than an individual with non-O blood type. This is

related to the von Willebrand coagulation factor (vWF). Individuals with blood type O have vWF levels around 30% higher than individuals with non-O blood type (25). In this study, the protease enzyme produced from bacteria has the same clot lysis activity regardless of different blood types. Enzyme purification techniques such as chromatography-based methods can maximize protease activity and clot lysis specifically (26).

Table 1. Platelet aggregation test result

Group	Aggregation (%)
No anticoagulants	75.85
Fibrinolytic protease HSFT-2	21.75

3.3. Blood Smear Evaluations

Blood smear evaluations were conducted to observe erythrocyte distribution and assess the presence of blood clots. The condition of blood cells when clotting occurs is described as blood cells that stick together (27). This condition resembles several blood disorders, such as agglutination and rouleaux formation. Clinically, these abnormalities arise due to a deficiency in blood clotting factors and allergies to anticoagulant administration (28). The use of protease enzymes can be an appropriate alternative anticoagulant agent to overcome this problem.

The blood smear evaluation, observed in Figure 2, provides critical insights into the effects of anticoagulant absence and protease treatment. In samples A0, B0, and C0—prepared without anticoagulants—erythrocytes in zones IV and V appear aggregated, forming clusters indicative of spontaneous clotting due to the lack of anticoagulant agents. This cellular aggregation emphasizes the natural coagulation process when blood is left untreated. In contrast, samples A1, B1, and C1—treated with the fibrinolytic protease HSFT-2—exhibit a more uniform distribution of erythrocytes, with cells appearing separated rather than clumped. This suggests that the addition of fibrinolytic protease HSFT-2 effectively inhibits clot formation, functioning similarly to traditional anticoagulants by preserving the integrity of the sample for microscopic analysis (29).

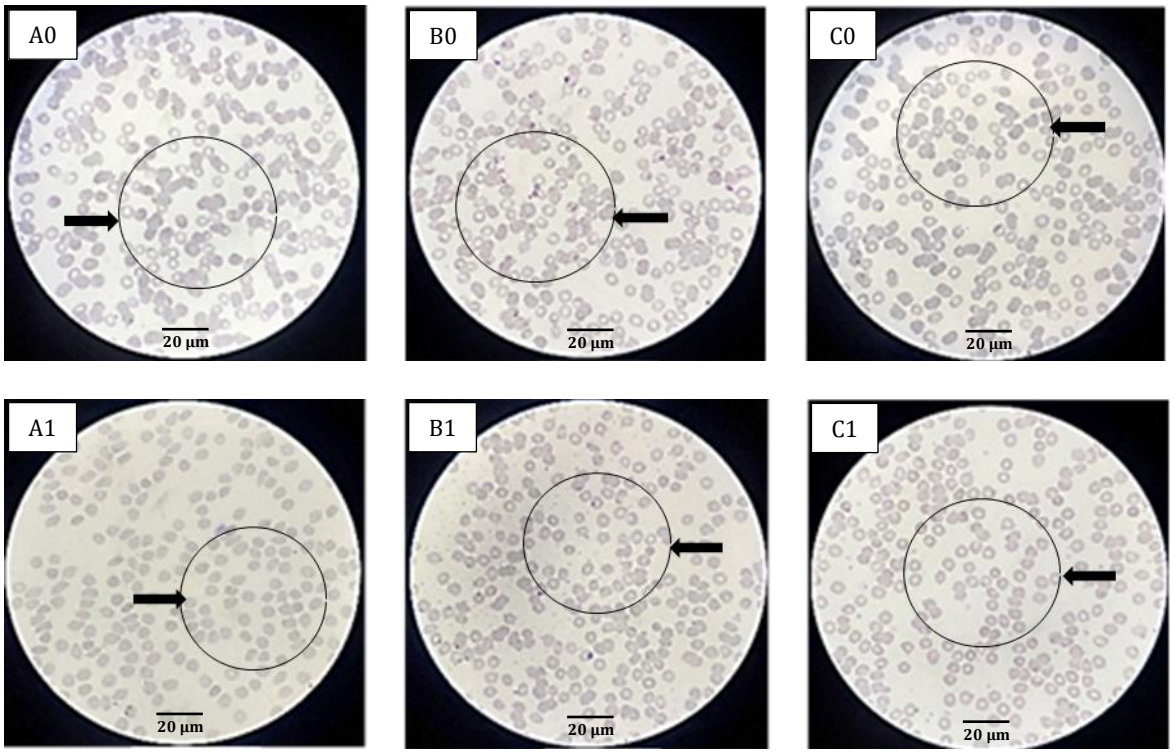

Figure 2. Blood smear evaluation results. A0, B0, and C0 without anticoagulant; A1, B1, and C1 with the addition of crude fibrinolytic protease HSFT-2

A similar study utilized protease from *Bacillus thuringiensis* HSFI-12 (13) and *Bacillus cereus* HSFI-10 (16) in blood specimens, with protease treatment resulting in evenly distributed and non-aggregating normal erythrocyte cells. These results are in line with this study, blood samples with protease treatment showed an even distribution of erythrocyte cells and showed a decrease in platelet aggregation. On the contrary, blood samples with anticoagulant treatment showed erythrocyte cells attached and overlapping with each other and a high percentage of platelet aggregation

(Figure 1, Figure 2). This shows the potential of bacterial protease as an anticoagulant reagent, even though it is still in the form of a crude enzyme or not yet in the form of a pure enzyme (dialysate).

Anticoagulants in laboratory examinations, especially hematology, can prevent blood from clotting, so they can be used more for examination and diagnosis purposes (30). Anticoagulants are substances that can prevent blood clotting. The hematological examination can be useful for clinical purposes. Therefore, to the preparation, specimen type, anticoagulants (anti-clotting substances), and quality control must be paid attention. Quality control is a critical aspect of using anticoagulants in laboratory examinations. It ensures that the anticoagulant prevents clotting without interfering with the examination results. The addition of anticoagulant functions to prevent clotting (31). Anticoagulants prevent blood clotting by binding (chelation) or precipitating (precipitation) calcium ions in the blood or by inhibiting the formation of thrombin, which is needed to convert fibrinogen into fibrin in the clotting process (32).

Crude fibrinolytic protease was added to determine whether it could be used as an alternative anticoagulant. Qualitatively, protease enzymes can be applied as anticoagulants using the in vitro blood clot degradation method. The principle of the method in vitro blood clot degradation is to determine the ability of the fibrinolytic protease enzyme to degrade blood clots contained in the tube (33). A related study on using protease enzymes leads to antithrombosis agents for cardiovascular disease therapy. Previous studies reported that crude proteases from several species, such as *Bacillus tequilensis* (7) and *Bacillus thuringiensis* (8), have potential activity as antithrombosis agents. This study underscores the potential of crude protease as an anticoagulant, a promising development that could lead to significant advancements in the field. This finding can be a reference for developing anticoagulant reagents in laboratory examinations, especially hematology.

Using fibrinolytic protease enzymes as alternative anticoagulants for laboratory examinations is a breakthrough in the diagnostic reagent industry. Fibrinolytic proteases are known to have the exat mechanism as anticoagulants by preventing blood specimens from clotting before they are examined. The characteristics of fibrinolytic protease enzymes in degrading fibrin, the main component of blood coagulation, make them suitable for use as anticoagulant agents. However, in their application, more in-depth testing is needed (34). In vivo tests on various clinical conditions using human blood specimens are an important benchmark in assessing complex anticoagulant activity. The diversity of physiological blood conditions and examination parameters causes variations in the anticoagulant types. Moreover, in patients with anticoagulant allergies, they cause rejection of several types of anticoagulants, such as heparin. The use of heparin in patients with heparin anticoagulant allergies can cause a decrease in platelet count, requiring the replacement of the appropriate type of anticoagulant (35). Fibrinolytic protease enzymes can be an alternative in this case but require comprehensive testing if used as an alternative anticoagulant.

This study can be an initial reference for testing the activity of fibrinolytic protease as an anticoagulant agent for developing of diagnostic reagents to support laboratory examination and diagnosis. A series of further tests, such as the use of variations in blood specimens from various clinical conditions, can be carried out to determine the flexibility of fibrinolytic protease HSFT-2 as an alternative anticoagulant agent. In addition, fibrinolytic protease in its application as an anticoagulant agent in the therapy of blood clot-related diseases such as antithrombotic agents, and diverse applications as enzybiotics, digestive-aids, anti-inflammatory, fibrinolytic agents, anti-cancer agents, and biofuels (35). Previous studies have shown that fibrinolytic protease HSFT-2 has antithrombosis and fibrinolysis activities. This can support the development of anticoagulant agents for laboratory diagnostic purposes and blood clotting therapy agents.

The limitation of this study is that it does not use varying controls of anticoagulants and blood specimens. Therefore, in the future, it is necessary to purify and characterize the fibrinolytic protease and compare it with various anticoagulants such as heparin and sodium citrate. in addition, in-vivo testing and clinical trials using blood specimens with various clinical conditions can be done to expand the reference as an alternative anticoagulant reagent for laboratory diagnostic purposes.

This study focuses on effectiveness but does not discuss safety issues, because the research is directed at developing protease enzymes produced from bacteria to develop of anticoagulant reagents to prevent clotting in blood specimens for in vitro-based laboratory examinations. Anticoagulant effectiveness testing is carried out to determine the feasibility of being used as an anticoagulant agent. In addition, safety concerns are needed as intravenous applications so that testing such as in vivo toxicity and dose regulation is required (36).

4. CONCLUSIONS

The crude fibrinolytic protease from *Staphylococcus hominis* HSFT-2, with its intriguing anticoagulant activity demonstrated in platelet aggregation assays and blood smear evaluations, requires further research. The HSFT-2 anticoagulant activity test on routine blood tests showed differences in routine blood parameter values compared to controls using EDTA. However, the purification and characterization of crude fibrinolytic protease HSFT-2 are necessary to fully understand and optimize its potential as an alternative anticoagulant for laboratory diagnostic reagents.

Author contributions: MAA: Conceptualization, writing original draft, collecting and analyzing the samples. TR: interpreted the result and worked on the manuscript.

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Ethics statement: Since the study did not involve animals or human beings, it did not require ethics approval.

Conflict of interest: There is no conflict of interest in the process of carrying out the research.

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