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ORIGINAL ARTICLE

In Vivo Antithrombotic Potential of Protease From *Bacillus Thuringiensis* HSFI-12

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Abstract

Background/Aim: Cardiovascular diseases (CVDs) are the primary non-communicable disease at the global level due to abnormal platelet aggregation by fibrin forming clots in blood vessels called thrombus. The search for thrombolytic drugs is largely carried out to treat thrombosis. Crude extract and dialysate protease of *Bacillus thuringiensis* HSFI-12 is known to have thrombolytic activity *in vitro*. The *in vivo* thrombolytic activity evaluation of concentrated protease of the bacterium is yet to be done. This study aimed to evaluate *in vivo* thrombolytic activity of concentrated protease produced by ultrafiltration of crude *B thuringiensis* HSFI-12 protease using *Rattus norvegicus* as animal model.
Methods: Carrageenan was used as thrombosis induction agent in rats. Intravenous injection of *B thuringiensis* HSFI-12 concentrated protease doses of 75, 150, 300, 600 µg/kg body weight (BW) was administered to rats, then induction of carrageenan was given intravenously to the rats' tails 30 min after injection of *B thuringiensis* HSFI-12 protease concentrate. The average length of the infarct area in the tail of the rat was shorter in the rats that were given various doses of *B thuringiensis* HSFI-12 protease concentrate compared to the negative control (rats induced by carrageenan 20 mg/kg BW).
Results: The PT examination results showed a prolonged PT time at 300 µg/kg BW dose, while there was at risk of bleeding at 600 µg/kg BW dose. The activated partial thromboplastin time (aPTT) examination results showed that time elongation beyond the normal range did not occur in rats after treatment. The amount leukocytes (WBC) and erythrocytes (RBC) after treatment were within the normal range indicating that they did not affect the haemostasis mechanism, while the platelet count (PLT) assay showed decrease in the number of platelets (thrombocytopenia). However, after treatment the number of platelets (PLT) showed a positive response as seen from an increase in values close to normal range. As conclusion, induction of carrageenan conducted had successfully caused thrombosis in *R norvegicus*' tail used as the thrombosis model.
Conclusion: Concentrated protease of *B thuringiensis* HSFI-12 showed *in vivo* antithrombotic potential with an effective dose of based on PT, aPTT and blood count evaluation at 150 µg/kg BW.

Key words: *Bacillus thuringiensis* HSFI-12; Cardiovascular diseases; Carrageenan induction; *In vivo* thrombolytic test; Thrombolytic agent.

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Introduction

Cardiovascular diseases (CVDs) are a non-communicable diseases with mortality rate reaching 35 % in Indonesia.[1-3] CVDs are mainly caused by the formation of thrombus in the blood vessels (thrombosis) due to an imbalance in the formation of fibrin and fibrinolysis resulting in

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platelet aggregation by fibrin. Such aggregation may continue to forming clots that can interfere with blood flow.[4-6] Fibrin can be hydrolysed by fibrinolytic enzymes such as plasmin.[7, 8] Plasmin acts in the breakdown of fibrin in blood clots, but when plasmin cannot work normally, enzymatic thrombolytic agent therapy is required.[9-11]

Commercial thrombolytic agents such as tissue plasminogen activators (tPAs), urokinase (UK), streptokinase (Sk) and nattokinase (Nk) have the disadvantages of causing gastrointestinal bleeding and allergic reactions, are expensive, low specificity and also has short half-life. Therefore, it is necessary to develop new thrombolytic agents. The expected thrombolytic agent is cheaper and safer as they do not show antigenicity and bleeding.[11-13] Thrombolytic agents sourced from bacteria are the most widely produced because they have great biotechnological potential with several advantages including fast growth, high substrate specificity, abundant availability in nature and low production costs. Thrombolytic agents can be protease enzymes, which is known to be safe to use, one of which produce by *Bacillus spp* isolated from fermented products of the digestive organs of sea cucumbers (*Holothuria scabra*).[9] The protease produced by the strain of *Bacillus spp* HSFI-12 was then partially purified through ammonium sulphate precipitation followed by dialysis. It was found that *in vitro* blood lysis activity of the dialysate protease of strain *Bacillus spp* HSFI-12 was higher than its crude protease and the activity was higher than the commercial Nk as a positive control. This shows that higher purity of the protease enzyme, can cause the higher activity in lysing blood clots. Based molecular and bioinformatics analysis results, *Bacillus spp* isolate HSFI-12 was later identified as *Bacillus thuringiensis*.[14]

In vivo activity and the antithrombotic mechanism of action of the *Bacillus thuringiensis* HSFI-12 protease was yet to be known.[11, 15] *In vivo* antithrombotic assays of protease enzymes in experimental animals can be done on the thrombosis model, for example thrombosis model carried out by carrageenan induction in rat's tails.[16, 17]

This study aimed to evaluate the thrombolytic *in vivo* activity of protease concentrates from the crude enzyme *B thuringiensis* HSFI-12. Evaluation of the antithrombotic activity *in vivo* includes anticoagulant activity tests, namely prothrombin time (PT) and activated partial thromboplastin time (aPTT) examinations, thrombolytic effects (through examination of % thrombolysis by measuring the tail length of thrombotic rats). Other haematology assays that also evaluated were leukocytes as white blood cells (WBC) count, erythrocytes as red blood cells (RBC) count and platelets (PLT) count.[15]

Methods

The materials used in this study included *Bacillus thuringiensis* HSFI-12 bacterial isolate originated from the fermented product of the digestive organ of the sea cucumber (*Holothuria scabra*). The isolate was obtained from previous studies,[10, 14] which had been stored in glycerol at -20 °C.

Animal and ethics statement

Eighteen male rats (*Rattus norvegicus*), stock Wistar, aged 2-4 months (8-16 weeks), tail length ≥ 13 cm weighing 200 g were used in study. These rats were breed by Animal Test Laboratory, Universitas Muhammadiyah Semarang, Central Java, Indonesia. Selected animals were housed in stainless steel cages in a ventilated room. Light/dark regime was 12/12 h and living temperature was (22 ± 2) °C with relative humidity of (55 ± 10) %. Standard compressed rat feed BR-1 and drinking water were supplied *ad libitum*. Food was given as much as 10 % body weight, which is around 10-15 g/head/day. Ethical clearance was approved by the Health Research Ethics Commission (KEPK) Faculty of Public Health, University of Muhammadiyah Semarang (No 634/KEPK-FKM/UNIMUS/2022). The rats were allowed 7-days quarantine and acclimation period prior to start of the study.

Bacterial subculture

Subculture of isolates was carried out as confirmation test of *Bacillus thuringiensis* HSFI-12 sample used. The media used were *Nutrient Agar* (NA, Oxoid, UK), *Blood Agar Plate* (BAP, Oxoid, UK). The next step included Gram staining test and the protease production on *Skim Milk Agar* (SMA, Oxoid, UK) media.[14]

Enzyme production

Starter media used was *Minimal Synthetic Medium* (MSM) with the addition of 1 % skim milk. Pure

isolates were grown in *Luria Bertani Agar* (LBA, Oxoid, UK) media containing 2 % skim milk. The clear zone formed around the colony was the parameter of the isolate's ability to produce proteases. A loop-full of bacterial isolate was put into 300 mL of starter medium and incubated on a shaker incubator at 37 °C for 24 h. A total of 10 % starter was inoculated into 300 mL of liquid production medium and incubated for 48 h under the same conditions as the starter. After 48 h, the samples were then centrifuged at 6000 rpm for 30 min at 4 °C to separate bacterial cells. Supernatant regarded as crude protease was separated and then concentrated by ultrafiltration.[18, 19]

Enzyme activity and concentration assay

Enzyme activity was measured using the Takami method (1989) with modifications.[20] Samples were prepared by mixing 250 mL of 0.05 M citrate buffer at pH 6, 250 mL of 2 % casein and 50 mL of enzyme solution, then incubated at 60 °C for 10 min. A total of 0.5 mL of 10 % 0.4 M TCA was added to it, then centrifuged at 4 °C at 10,000 rpm for 10 min. The supernatant was separated and 0.5 mL of it was reacted with 2.5 mL 0.4 M Na_2CO_3 and 0.25 mL Folin reagent, then incubated for 30 min at room temperature. The absorbance was measured at λ = 660 nm. Blanks were made using an inactive enzyme with the same reaction. One unit of protease enzyme activity is defined as the amount of enzyme that can produce 1 mmol of tyrosine per minute under measurement conditions.[18-20] Extract resulted from ultrafiltration of crude protease was regarded as protease concentrate was subjected to concentration analysis by the modified Bradford method.[20, 21]

In vivo experiment

The *in vivo* thrombosis induction on rat tail was carried out using carrageenan (20 mg/kg BW) (Sigma-Aldrich, St. Louis, USA). Rats were with simple random sampling divided into 6 groups. Group 1 (No Treatment/NT), those with no enzyme supply and without carrageenan induction. Group 2 (Carrageenan Induction/CI/Negative Control), rats induced by carrageenan at their dorsal tail vein as a negative control. Protease concentrate of *B thuringiensis* HSFI-12 in graded doses of 75, 150, 300 and 600 mg/kg body weight (BW) in PBS 1x were intravenously injected to the lateral tail vein of the rats (sequentially Group 3, 4, 5 and 6). After 30 min of intravenous injection of the protease concentrate *B thuringiensis* HSFI -12, the rat tail was ligated, where carrageenan was then induced by intravenous injection in the rat tail. Ligation was removed after 15 min of induction. These experiments were performed under ether anaesthesia. The length of the burgundy-coloured infarct area was measured after 24 h of treatment by the protease thrombolytic agent of *B thuringiensis* HSFI-12 as schemed in Figure 1.[22]

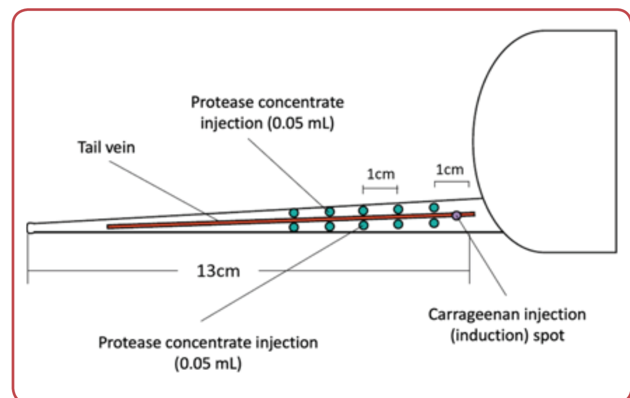

Figure 1: Schematic of the injection sites in the rat tail for in vivo thrombosis evaluation after bacterial protease treatment[22]

The infarct area will increase over time and stabilise at 24 h after carrageenan induction.[22] Thus, 24 h was the point in time at which a successful thrombosis model was created. The thrombolytic activity of the *B thuringiensis* HSFI-12 protease enzyme was measured using the following formula (eq.1):[8]

$$\%\ \text{Thrombolysis} = \frac{\text{Thrombus length on control rat's tail} - \text{Thrombus length on treated rat's tail}}{\text{Thrombus length on control rats tail}} \times 100\%$$

At the end of the study period, the rats were euthanised. Organs and tails were harvested and stored for subsequent research histopathological studies targeting thrombus to develop a concentrated protease effect generated by crude *Bacillus thuringiensis* HSFI-12 protease ultrafiltration.

Anticoagulation assay

PT and aPTT (TECO) coagulation tests were performed after 2 h of carrageenan induction. Blood samples were taken from the orbital sinus of the eye of rats using micro haematocrit. The blood that came out was accommodated in *Eppendorf* tube containing 3.2 % sodium citrate (9:1 v/v). Platelet-poor plasma (PPP) was obtained by centrifugation of blood at 3000 rpm for 10 min. A 25 mL of PPP was used in each examination. The stopwatch was terminated when a fibrin clot was formed.[4, 22]

Red blood cells, white blood cells and platelet count assays

Whole blood samples were taken after 24 h of carrageenan induction by etching micro haematocrit into the orbital sinus of the rat's eye. The blood that came out past the end of the micro haematocrit was accommodated in an EDTA vacuum tube microtainer that already contained the anticoagulant K_2-EDTA.[25, 26] The recommended amount of K_2-EDTA should be around 1.5-2.2 mg/mL of blood. The whole blood sample was then homogenised manually or using a special mixer. Determination of the RBC, WBC and PLT using a Haematology Analyzer as previously reported was performed.[27, 28]

Statistics

IBM SPSS Statistic 26 programme was used to analyse parameters. Normality data test was calculated using the Shapiro-Wilk test. The results of the normality test in each group were normally distributed by showing a significant value greater than 0.05 ($p > 0.05$). The Levene test was used to test homogeneity as a condition before using the One-way analysis of variance (ANOVA) test. A significance value greater than 0.05 ($p > 0.05$) was indicated that the data were homogeneous between groups. ANOVA test was conducted to see whether there was a difference of the test parameter (% thrombolysis and haematology count assays (PT, aPTT, RBC, WBC, PLT) in the treatment group ($p < 0.05$).[29] Then the *post-hoc* LSD was carried out to see if there was a significant difference in the treatment compared to the treatment control (NT or CI) ($p < 0.05$).[30]

Results

Bacterial subculture

B thuringiensis HSFI-12 colonies were sub-cultured and the result showed that the colonies grew in uniform with no signs of contamination. Observations on NA media showed colonies with a circular shape with a diameter of 4 mm, entire edge, convex elevation, smooth consistency and milky-white in colour. Bacterial growth on BAP media showed β-haemolysis, characterised by a clear or colourless zone around the colony because bacteria could dissolve all red blood cells. Microscopic characterisation showed Gram-positive characteristics with bacilli-shaped purple cells (Figure 2).

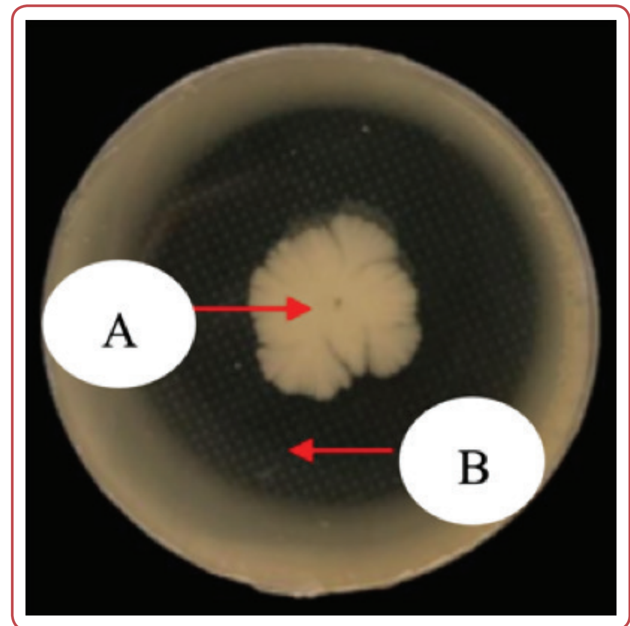

Figure 2: Result of the protease production capacity test of Bacillus thuringiensis HSFI-12 on solid media Skim Milk Agar (SMA), on the 7th day of observation. A. Bacterial colony B. Clear bacterial proteolytic zone.

Activity and concentration of obtained concentrate protease

Results of activity test on *B thuringiensis* protease from crude protease before and after ultrafiltration process is summarised in Table 1.

Table 1: Activity (U/mL) and concentration (μg/mL) of crude enzyme and ultrafiltration concentrate protease of Bacillus thuringiensis HSFI-12

Bacterial protease sample	Enzym activity (U/mL)	Protein Concentration (μg/mL)
Crude	4.6709 ± 0.0023	100.5521 ± 0.0041
Concentrate	9.3717 ± 0.0034	268.4285 ± 0.0014

In vivo thrombolysis assays of protease concentrate

The results of carrageenan induction as swelling and redness (of thrombus) seen as burgundy in the tails of the rats in the CI/negative control group (Figure 3 A). The results of administration of the protease concentrate prior induction to prevent thrombosis are shown in Figure 3 B-E.

Determination of effective dose of protease concentrate to prevent thrombosis

The effective dose of bacterial protease to prevent thrombosis was determined based on percentage of reduction (%) in thrombus length (mm) of *R norvegicus*'s tails. The results are shown in Table 2.

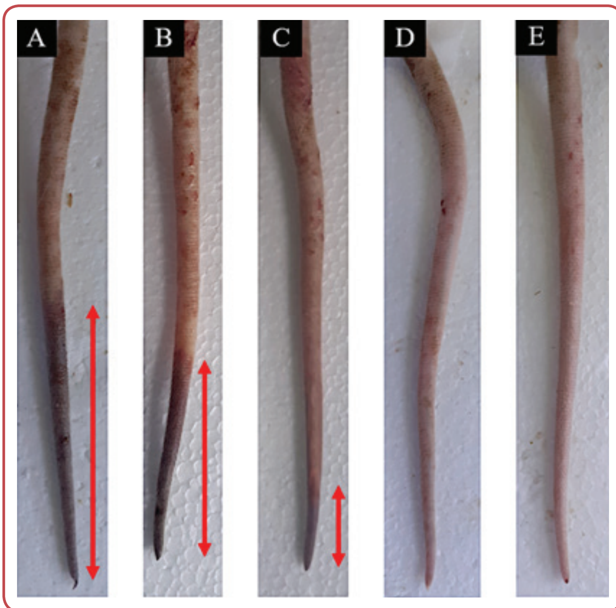

Figure 3: Representation of different thrombus length (seen as swelling and redness indicated by red arrows) in Rattus norvegicus' tails in a carrageenan-induced (20 mg/kg BW) thrombosis model. Prior to carrageenan induction, the tails were treated with varied dose of Bacillus thuringiensis HSFI-12 concentrated protease obtained by ultrafiltration. (A) Negative control.

Table 2: Percentage of reduction in thrombus length of Rattus norvegicus's tails after the administration of varied dose of concentrated protease of Bacillus thuringiensis HSFI-12 as thrombosis prevention agent followed by carrageenan induction as thrombosis model

N	Treatment Group	Thrombus length (mm)	Thrombolysis percentage (%)
3	Negative control (CI)	9.0 ± 0.35	0 ± 0.18
3	CI + CP 75	3.8 ± 0.36	58 ± 0.22[a]
3	CI + CP 150	1.9 ± 0.15	79 ± 0.21[a]
3	CI + CP 300	1.0 ± 0.10	89 ± 0.15[a]
3	CI + CP 600	0.5 ± 0.05	94 ± 0.17[a]

N: number of rats; CI = Carrageenan induction (20 mg/kg BW); CP = Concentrate Protease (µg/kg BW); [a] significance difference of % thrombolysis with negative control ($p < 0.05$);

Anticoagulant and blood count assay

The coagulation cascade was tested based on two parameters, PT and aPTT. The safest dose of protease without affecting the aPTT of the treated rats was also determined by comparing difference between aPTT values of the untreated and treated rats (NT) group (Table 3).

Table 3: Haematology count results as part of antithrombosis in vivo assays in control and treatment groups of Rattus norvegicus induced by carrageenan as thrombosis model

N	Group	PT (s)	aPTT (s)	WBC (10^3/µL)	RBC (10^6/µL)	PLT (10^3/µL)
3	NT (no treatment)	30 ± 3	285 ± 2	8.7 ± 0.3	6.32 ± 0.04	1022 ± 5
3	Negative control (CI)	23 ± 2	115 ± 3	18.4 ± 0.2	4.61 ± 0.05	707 ± 6
3	CI + CP 75	24 ± 1[b]	310 ± 1[a]	18.3 ± 0.4[b]	4.87 ± 0.04[b]	561 ± 2[b]
3	CI + CP 150	33 ± 1[a]	349 ± 2[a, b]	15.8 ± 0.7[b]	4.96 ± 0.03[b]	658 ± 2[b]
3	CI + CP 300	38 ± 3[a, b]	381 ± 3[a, b]	11.8 ± 0.2[a]	5.20 ± 0.02[a, b]	798 ± 1
3	CI + CP 600	41 ± 2[a, b]	403 ± 2[a, b]	9.3 ± 0.6[a]	5.72 ± 0.04[a]	927 ± 3

CI = Carrageenan induction (20 mg/kg BW); CP = Concentrate protease (µg/kg BW); PT = Prothrombin time; aPTT = Activated partial thromboplastin time; WBC = White blood cells; RBC = Red blood cells; PLT = Platelet/thrombocyte; a = significance difference with CI ($p < 0.05$); b = significance difference with NT ($p < 0.05$);

Discussion

B thuringiensis HSFI-12 bacterium as living object in this study was confirmed to produce protease enzymes as indicated by the formation of a clear zone around the colonies on SMA media. The results were in line to those previously reported.[9, 14] Based on this, activity assays on protease produced by *B thuringiensis* HSFI-12 could be done.

For bacterial protease activity assays, the 5M tyrosine standard with volume ranged from 0 - 5.4 µL was used to create the tyrosine standard curve. The equation obtained from the tyrosine standard curve, $y = 0.1404x + 0.0411$, was then used to calculate the enzyme activity in the sample solution. Results of activity test on *B thuringiensis* protease showed that there was a 2-fold increased value from crude protease before and after ultrafiltration process. BSA standard solution was used to determine crude protease and protease concentrate based on absorbance measurement of at 595 nm according to Bradford method.[18-20] The results showed that compared to the crude extract, the protein content of concentrate protease was higher more than twice by 268.4285 ± 0.0014 µg/mL.

As seen in Figure 3 after 24 h of 20 mg/kg BW carrageenan induction, swelling and redness (thrombus) were seen as burgundy in the tails of the rats in the CI/negative control group, whereas in the group given the protease concentrate a dose of 75, 150, 300 and 600 g/kg BW 1 x PBS there was decreased in swelling and redness. The results showed that higher protease dose of *B thuringiensis* HSFI-12, the greater thrombus size reduction. The percentage of thrombolysis increased following the increased dose of concentrated protease. All used doses caused significantly different thrombolysis levels within the CI/negative control group, but not within the doses of 300 μg/kg BW and 600 μg/kg BW. It means, the most effective dose of protease *B thuringiensis HSFI-12* to cause thrombolysis in carrageenan-induced rat tails is 300 μg/kb BW.

The results of coagulation cascade tests based on PT and aPTT showed that the concentrated protease of *B thuringiensis* HSFI-12 could prolong PT and aPTT. The results also showed that bacterial protease brought significant antithrombotic effect by regulating the intrinsic and extrinsic coagulation pathways. The PT value was prolonged with the increase in the protease dose, but the difference was significant with the negative control starting at the *B thuringiensis* HSFI-12 protease dose of 150 μg/kg BW. Furthermore, only the *B thuringiensis* HSFI-12 protease dose of 150 μg/kg BW had the same PT value as the untreated/normal rats (NT) group, therefore it can be concluded that the best dose without disturbing the PT time of the treated rats was 150 μg/kg BW. For other coagulation factors, aPTT values began to be significantly different from the negative control at a *B thuringiensis* HSFI-12 protease dose of 75 μg/kg BW and continued to increase with increasing doses. Based on Table 3, the safest dose of protease without affecting the aPTT value of the treated rats was 75. It is because the treatment produced aPTT value that was not significantly different from that of the untreated/normal rats (NT) group.

Haematology testes showed that induction of carrageenan causes an increase in WBC value. A significant decrease in WBC values only occurred at a *B thuringiensis* HSFI-12 protease dose of 300 μg/kg BW, reaching the same WBC values as untreated rats. Therefore, the minimum protease dose to restore WBC values in treated mice was 300 μg/kg BW. On the other hand, induction of carrageenan decreased RBC and PLT values. The effective dose of *B thuringiensis* HSFI-12 protease that can significantly increase the RBC value is 300 μg/kg BW, although the increase has not yet reached the RBC value in untreated/normal rats. The dose of *B thuringiensis* HSFI-12 protease that could restore PLT values was the same as in untreated rats starting with a dose of 300 μg/kg BW. It can be inferred however, that a dose of *B thuringiensis* HSFI-12 protease of 300 μg/kg BW was the minimum dose used to restore WBC, RBC and PLT values after rats were induced by carrageenan.

Overall results of this study demonstrated that the number of leukocytes and erythrocytes were in the normal range indicating that the provided treatment did not affect the homeostasis mechanism. Yet, the PLT caused a decrease in the number of platelets (thrombocytopenia tendency). The effective dose of *in vivo* antithrombotic protease concentrate *B thuringiensis* HSFI-12 based on the overall test results is 150 μg/kg body weight with consideration of the risk of prolongation of PT time at the least before bleeding. In other words, at least 150 μg/kg BW concentrated protease of *B thuringiensis* HSFI-12 obtained by ultrafiltration method could prevent thrombus caused by carrageenan induction in rat's model. The decrease in % thrombolysis reached 79 % with the result that aPTT parameter did not extended beyond the normal range. The WBC and RBC were in the normal range despite thrombocytopenia but the PLT after treatment gave a positive response as seen from the increase in the PLT approaching normal rat.[20]

Routine blood tests were carried out in this study to measure the health status of rats after being treated with the protease concentrate *B thuringiensis* HSFI-12 with various doses. Blood parameters are relevant in evaluating the risk of changes in the haematological system to predict the safety of test animals.[21, 22] For that reason, such examination was performed at the end of our experiment with haematological data fell within normal values for *R norvegicus*.[21]

Presented results showed that crude bacterial protease can be concentrated to increase its catalytic activity to improve its ability to prevent thrombosis. An easy-to-use purification method as a way to concentrate bacterial protease is the membrane ultrafiltration method.[23-25] Ultrafiltration is a pressure-driven separation process that functions to increase enzyme activity. During en-

zyme purification, the basic consideration is to produce a safe, cost-effective and high-value end product using economical techniques.[24]

A number of studies have reported that microbial enzymes can be directly used as drugs, one of which is as a lytic agent therapy to treat thrombosis. The genus *Bacillus* in particular, is the most dominant and prominent source of protease production. *Bacilli* and their proteases are receiving renewed attention due to the increasing demand in medical and industrial commodities. Presented results along with the previous findings supported this by showing that intravenous injection proteases from the genus *Bacillus* on rat tails could inhibit thrombus formation *in vivo.*[16, 22, 26, 27] Toxicity tests, followed by pre-clinical and clinical tests should be done before the *B thuringiensis* HSFI-12 protease can be used as commercial antithrombosis agent.

Research limitations

The study only focused on reducing thrombus in the tail as a target for thrombosis and knowing the characteristics of antithrombotic activity of *Bacillus thuringiensis* HSFI-12 protease concentrate *in vivo* on coagulation parameters (PT, aPTT), WBC, RBC and PLT.

Conclusion

Based on the results of the study, it can be concluded that induction of carrageenan had successfully caused thrombosis in *R norvegicus* rat's tail used as thrombosis model. Overall, concentrated protease of *B thuringiensis* HSFI-12 showed *in vivo* antithrombotic potential based on PT, APTT and blood count evaluation assays.

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Conflict of interest

None.

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