Prospective *In Vivo* Assays on the Antithrombotic Potential of Protease Extracted from *Bacillus* sp. HSFI-12

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ABSTRACT
CVD (cardiovascular disease) is a group of non-communicable diseases and a global cause of cardiovascular death. The search for gents inhibiting blood clot formation (thrombus) and enhancing antithrombotic activity are important for the prevention and treatment of CVD. Previous study reported that bacteria isolated from fermented intestine of *Holothuria scabra* produced protease enzyme with high antithrombotic activity *in vitro*. Among bacterial strains producing thrombolytic proteases isolates from *H. scabra* is *Bacillus* sp. HSFI-12. This review study summarized in vivo antithrombotic assays conducted proteases developed as antithrombosis agent worldwide in the last decade. The study specifically targeting the potential of bacterial protease as antithrombosis agent tested on rats or mice as animal models. The aim was to oversee the trend of antithrombotic assays and their induction agents mainly used in the *in vivo* studies using rats of mice as testing animals. The results were then used to recommend the most suitable antithrombotic assays to evaluate *in vivo* antithrombotic activities of protease from *Bacillus sp.* HSFI-12 on rats of mice and the most suitable material to induce these testing animals with thrombosis. Based on the findings of this literature review, an in vivo study to develop an antithrombosis agent from *Bacillus sp.* protease is known to be feasible. Following the in vitro study of HSFI-12, that demonstrated competitive thrombolytic activity. The recommended assays to be conducted in the prospective *in vivo* study include the measurement of Thrombus Infarct Length (TIL), Bleeding Time (BT), Partial Thromboplastin Time (aPTT), and Prothrombin Time (PT). The recommended material for thrombosis induction is carrageenan, while the recommended administration of antithrombotic enzyme to animal model is by intravenous intervention on tail rather than oral treatment.

Keywords: *Animal model, Antithrombosis in vivo assay CVD, Bacillus sp. HSFI-12, Thrombosis.*

1. INTRODUCTION

CVD (cardiovascular disease) is a group of non-communicable diseases and a global cause of cardiovascular death. Based on the 2019 Global Burden of Disease (GBD), CVD was responsible for almost doubling from 217 million cases in 1990 to 532 million cases in 2019, with the number of CVD deaths continuing to increase from 12.1 million in 1990 to 18 ,6 million cases in 2019 [1-2]. WHO (World Health Organization) stated that (CVD) counts for 32% of deaths worldwide, of which more than three quarters are in low or middle income countries [3].

Cardiovascular disease (CVD) is caused by the occurrence of thrombosis blockage in blood vessels [4].

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Thrombosis occurs as a result of the accumulation of platelets, fibrin, and thrombin in the arterial vein or venous circulation, which leads to the formation of atherosclerotic plaques and the blockage of blood vessels [5, 6]. Imbalance between fibrin formation and fibrinolysis besides hyper-fibrinogemia, usually trigger thrombotic events, while also increase the myocardial risk through blood clotting [7].

Agents inhibiting blood clot formation (thrombus) and enhancing antithrombotic activity are important for the prevention and treatment of CVDs [8]. Available thrombolytic drugs such as urokinase (uPA), streptokinase (SK) and tissue plasminogen activator (t-PA) have limitations such as high cost, short half-life, allergic reactions and intravenous bleeding. Thrombolytic drugs that are safe, effective, cheaper and easy to find are expected to overcome the fundamental problems associated with thrombolytic drugs. To date, the administration of thrombolytic drugs to degrade blood clots is the primary alternative to remove the blockage of thrombus [9].

Several types of thrombolytic drugs have been developed for therapeutic purposes. Agents (enzymes) which are frequently used as drugs are urokinase (uPA), streptokinase (SK) and tissue plasminogen activator (t-PA), which work by activating and converting plasminogen to plasmin with the ultimate goal to degrade fibrin. However, the drugs developed so far have not reached the ideal outcome. A reasonable cost, no antigenicity, no effect on blood pressure, no procoagulant effect, selective fibrin, low re-occlusion rate, low bleeding incidence, and resistance to plasminogen activator inhibitors are all desirable characteristics of an ideal thrombolytic drug. In fact, agents such as urokinase (uPA) and tissue plasminogen activator (t-PA), have limitations such as higher cost, low fibrin specificity, short half-life, allergic reactions and tendency to cause systemic bleeding. Thus, the search for an ideal thrombolytic drug is a challenge in the management of thrombolysis [2] [6-8].

Most potential antithrombosis agents are proteases involved in total protein hydrolysis, which can be produced from all living cells, one of which is microorganisms [12]. Many bacterial proteases have been reported, yet not all of them have been tested and evaluated for their *in vivo* antithrombotic activities. The thrombolytic enzymes used in the treatment of CVD are predominantly bacterial originated from food or fermentation products, very rarely from plant or animal tissues. The group of bacteria producing proteases applied to CVD mostly are *Bacillus* sp [10].

Sea cucumbers are rich-protein marine organisms, which are potential as sources of proteases with high antithrombotic activity, particularly from bacterial isolates in their tissues [10-11, 13-14]. Among other sea cucumbers, *Holothuria scabra* has been known to have the highest protein content, so that it can be an ideal substrate for protease enzymes including fibrinolytic types with clot lysis properties. Previous study reported that bacteria isolated from fermented intestine of *H. scabra* produced protease enzyme with high antithrombotic activity *in vitro*. Among bacterial strains producing thrombolytic proteases isolates from *H. scabra* is Bacillus sp. HSFI-12. The *in vitro* thrombolytic activity of *Bacillus* sp. HSFI-12 was found to be competitive when compared to commercial protease Nattokinase, currently known as standard antithrombosis agent [12-14]. To achieve the targeted treatment of thrombosis, the *in vitro* antithrombotic thrombolytic agents including protease from isolate *Bacillus* sp. HSFI-12 should be followed by *in vivo* assays. By far, the reported *in vitro* results have not been confirmed by any *in vivo* assays.

Rats and mice have been the most popular testing animals used for various *in vivo* experimental purposes [9,15-16]. *In vivo* studies using testing animals are required for the evaluation of the potential of bacterial proteases to be developed as thrombolytic agents. The advantages of using rats and mice as animal model in experiments related with antithrombosis lie to the fact that they have similarities with humans in terms of physiological anatomy, accurate and continuous observation and measurement of thrombus [9,15-16].

The high morbidity and mortality of CVD requires appropriate *in vivo* assays following the previous *in vitro* assays to validate the potential of protease of HSFI-12 bacterial isolate as antithrombosis agent. As there have been numerous and diverse types of *in vivo* antithrombotic assays used and reported on various rats and mice as animal models, it is therefore important to determine what assays are suitable to be applied in the *in vivo* study on protease extract of HSFI-12 bacterium to validate its potential as an antithrombosis agent.

This review study summarized *in vivo* antithrombotic assays conducted on proteases developed as antithrombosis agent worldwide in the last decade.

The study specifically targeting the potential of bacterial protease as antithrombosis agent tested on rats or mice as animal models. The aim was to oversee the trend of antithrombotic assays and their induction agents mainly used in the *in vivo* studies using rats of mice as testing animals. The results were then used to recommend the most suitable antithrombotic assays to evaluate in vivo antithrombotic activities of protease from *Bacillus* sp. HSFI-12 on rats of mice and the most suitable material to induce these testing animals with thrombosis

2. METHODS

A literature review was carried out by following the previously reported steps of review article writing [17-

19]. The selected studies were sourced mainly from the PubMed database published between 2010-2021 discussing the parameters of *in vivo* antithrombosis assays with rats as animal model. Search articles used Medical Subject Title Headings (MeSH) with several combinations including “thrombolytic agent”, “platelet aggregation”, “*in vivo* study”, “in vivo assay”, “thrombolytic test”, “experimental animal models”, “testing animal”, “intravenous injections”, “thrombolytic activity”, “thrombolytic effect”, thrombotic induction and “induced thrombosis”.

2.1. Study Eligibility Criteria

A summary of the parameters of the antithrombotic test in rats and mice as well as the types of mice or experimental mice has been carried out, which can show the suitability of the parameters of the antithrombotic test as an evaluation of the development of thrombolytic drug discovery. The study selection was based on the inclusion criteria: [i] the evaluation study was limited to the type of antithrombotic assay parameters by animal modeling experiments. [ii] used rats or mice as experimental subjects related to agent injection in CVD therapy [iii] published in Indonesian and English. [iv] Conducted by observation. The publication year is limited to the last 10 years.

Literature review was done based on previously used procedures and guidelines [17-19]. The relevant literature search was carried out and grouped in 2 categories: 1. Types of parameters for antithrombotic testing in mice. 2. The types of materials injected into the tail of thrombotic mice. In the first category, the initial literature search was performed manually and non-manually through the PubMed and ScienceDirect databases. Manual searches regardless of publication date were performed using the set MeSH terms. Using this strategy, there were 70 relevant references but only 20 are retained.

The full text of all articles was extracted by electronic advanced search and manual search from Pubmed and ScienceDirect. The studies which are excluded from this study were: [i] studies conducted before 2010 [ii] studies on other types of antithrombotic assays other than mice [iii] reviews [iv] reviews. For the second category, computerized searches were performed via Google Scholar and Garuda Portal and manual searches regardless of publication date were performed using MeSH. The studies excluded from this review were: [i] studies conducted before 2010 [ii] agents injected other than mice [iii] agents injected other than tail [iv] reviews [v] reviews. The results of the identification are 12 papers with this method.

2.2. Study Selection

Three authors (OYD, SNE and MDR) independently identified studies that met the inclusion criteria in this review. Initially, both the title and abstract of the records generated by the search were evaluated to determine which inappropriate studies should have been excluded based on the exclusion criteria. Review articles were excluded, while articles (full text) of the remaining studies were taken to meet the inclusion criteria. Studies were screened and evaluated by 2 authors (AS and SD) using the STROBE checklist for observational studies [20].

2.3. Control of Bias

In this review, the following issues are included to control the bias or quality assessment: (i) completeness of reporting information regarding the type of antithrombotic test parameters in mice and humans (ii) selective outcome reporting (iii) selection of outcome measures (practical test of ability thrombotic agents using protease enzyme extracts were applied during the development of antithrombotic treatment in Indonesia (iv) study design and (v) conflict of interest in conducting the study. When overall criteria were met, a reasonable risk of overall bias can be regarded as low [20-21].

3. RESULTS AND DISCUSSION

The search for more effective and safer treatments continues. To achieve the aforementioned goals, it is necessary to test the antithrombotic effect of various thrombolytic agents in vivo. Bacillus sp. is one of several bacteria genera known to produce proteases. Bacteria are a potential source of more beneficial enzymes because they grow quickly, can grow on cheap substrates, and are easier to increase yields through growth condition regulation and genetic engineering [12]. One of the richest sources of protein from marine organisms is sea cucumbers. In Indonesia, the sea cucumber (*Holothuria scabra*) has the highest protein content of any sea cucumber [15]. *Bacillus sp.* HSFI-12 is derived from fermented sea cucumber *H. scabra* which is capable of producing protease enzymes with high antithrombotic activity.

The development of thrombolytic agents requires preclinical assessment of their biochemical effects in vivo. Selection of several animal models and examination methods is an important consideration in interpreting the results in order to provide safety and efficacy of new drugs developed. Testing of protease enzymes from the fermented sea cucumber *Holothuria scabra* is currently only done *in vitro*, *in vivo* testing has not been reported.

The initial literature search using the set strategy (see the method section) was resulted in the 32 study articles to review. The summarized data related with *in vivo* studies related with the antithrombotic agents showed that such agents were not only sourced from food bacteria or their fermentation products, but also from tissue extracts of plants or other organisms. However, the focus of this literature review is on enzymes of bacteria investigated for their potential as antithrombotic agents. Cardiovascular disease (CVD) is the leading cause of death in the world. To date, thrombolytic treatment is still being developed to reduce CVD-related mortality [22]. Of various sources of thrombolytic protease, the *in vivo* tests on protease from bacteria isolated from sea cucumber, has not been reported. This demonstrated that the in vivo assays on thrombolytic protease of isolate HSFI-12 are promising and need to be assessed.

Table 1 summarizes the types of *in vivo* antithrombotic tests on various potential antithrombotic materials worldwide using mice/rats in the last few decades (2010-2021). These Antithrombotic assays were performed to evaluate newly developed antithrombotic agents [23]. The data from **Table 1** shows that *in vivo* antithrombotic assays are advanced in order to support better the treatment of thrombosis cases. In the collection of various sources, the bacterial thrombolytic agent of sea cucumber has not been reported. The evaluation based on the last 10-year studies reviewed (**Table 1**) has also revealed that for the development of antithrombotic agents, the various *in vivo* antithrombotic assays used include: Partial Thromboplastin Time (aPTT), Prothro mbin Time (PT), Thrombus Infark Length (TIL), Bleeding Time (BT), Thrombin time (TT), International Normalized Ratio (INR), D-Dimer (DD), Clotting/ Coagulation Time (CT), Platelet Count (PC), Fibrinogen Levels (FL), D-Dimers and Fibrin Degradation Products (FDP) [23-24]. The four top most frequent of successful assays to conduct among them were Bleeding Time (BT), followed by TIL (Thrombus Infark Length), aPTT (Partial Thromboplastin Time), PT (Prothrombin Time). Based on this, the BT, TIL, aPTT, and PT could be recommended as assays to be conducted in the *in vivo* experiment for the thrombolytic protease of islate HSFI-12. **Table 1** shows the various antithrombotic assay parameters for the evaluation of various reported thrombolytic agents.

Parameters that are often used to assess antithrombotic agents include Partial Thromboplastin Time (aPTT), Prothrombin Time (PT), Thrombin time (TT), Bleeding Time, Clotting Time, thrombus infarct length, Platelet Count, Fibrinogen Level, D-Dimer and Products. Fibrin Degradation (FDP).

Partial Thromboplastin Time (aPTT) and **Prothrombin Time (PTT)** tests are routine laboratory tests used to assess the coagulation system's function. The PT test detects coagulation factor deficiencies in the extrinsic pathway (e.g., tissue factor and factor VII), whereas the APTT detects abnormalities in the intrinsic (contact) pathway (eg deficiency of factors VIII, IX, IX, and XII) [44-46].

Thrombin time (TT) is a simple coagulation test used as a screening measure to measure the ability of fibrinogen to be converted into fibrin, after the addition of thrombin reagent. The TT test is used to detect qualitative and quantitative fibrinogen abnormalities and to evaluate impaired fibrin formation [47][48].

Table 1. Studies reporting *in vivo* antithrombotic assays of antithrombotic agents on blood of rats and mice as animal models in the last decade

No.	aPTT	PT	TIL	BT	TT	INR	DD	PC	FL	CT	References	Country
1.	√	√	√	-	-	-	-	-	-	-	Frias *et al.,* 2021 [24]	Portugal
2.	√	√	-	√	-	-	-	-	-	-	Wu, *et al.,* 2020 [25]	China
3.	-	-	√	√	√	-	-	-	-	-	Lim, *et al.,* 2020 [26]	South Korea
4.	-	-	-	√	-	-	-	-	-	-	Pauran *et al.,* 2019 [27]	Indonesia
5.	-	-	-	√	-	-	-	-	-	-	Kim, *et al.,* 2019 [28]	South Korea
6.	-	-	-	√	-	√	√	√	√	-	Osunsanmi, *et al.,* 2018 [29]	Nigeria
7.	√	√	-	√	-	-	√	√	√	-	Golakiya *et al.,* 2017 [30]	India
8.	-	-	-	√	-	-	-	-	-	-	Fauzi, *et al.,* 2017 [31]	Indonesia
9.	√	√	√	-	√	-	-	-	√	√	Majumdar, *et al.,* 2016 [7]	India

Table 1. Studies reporting *in vivo* antithrombotic assays of antithrombotic agents on blood of rats and mice as animal models in the last decade (continued)

No.	aPTT	PT	TIL	BT	TT	INR	DD	PC	FL	CT	References	Country
10	-	-	√	-	-	-	-	-	-	-	Nailufar, *et al.*, 2016 [4]	Indonesia
11	√	√	-	√	-	-	-	-	-	-	Kwon *et al.*, 2016 [32]	South Korea
12.	√	√	-	√	-	-	-	-	-	√	Chen *et al.*, 2015 [33]	China
13.	-	-	√	-	-	-	√	-	√	-	Ma *et al.*, 2015 [34]	China
14.	-	-	√	-	-	-	√	-	-	-	Xu *et al.*, 2014 [8]	China
15.	√	√	-	√	-	-	-	-	-	√	Choi, 2014 [35]	South Korea
16.	√	√	√	-	-	√	√	-	√	-	Liu *et al.*, 2013 [36]	China
17.	-	-	√	-	-	-	-	-	-	-	Yuan, et al., 2012 [37]	China
18.	-	-	√	√	-	-	-	-	-	-	Simkhada, *et al.*, 2012 [9]	South Korea
19.	-	-	-	√	-	-	-	-	-	√	Angelina *et al.*, 2011 [38]	Indonesia
20.	-	-	√	-	-	-	-	-	-	-	Arslan *et al.*, 2011 [39]	Turkey
21.	-	-	√	-	-	-	-	-	-	-	Kamiya, *et al.*, 2010 [40]	Japan

Note: aPTT (Partial Thromboplastin Time), PT (Prothrombin Time), TIL (Thrombus Infark Length), BT (Bleeding Time), TT (Thrombin Time), INR (International Normalized Ratio), DD (D-Dimer), PC (Platelet Count), FL (Fibrinogen Level), CT Clotting/ Coagulatin Time

Bleeding time (BT) can be defined as the time taken from the puncture of the blood vessel to the cessation of bleeding while Clotting Time is the time interval from the puncture of the blood vessel to the formation of fibrin threads. [49-50]. The length of the thrombus infarct is the length of the appearance of the caudal thrombus formation which is burgundy in color due to the induction of thrombolytic agents [51]. Platelet Count is a basic hematological examination to assess the number of platelets in the body using various methods such as peripheral blood smear with staining, platelet counting using a counting chamber with phase contrast microscopy, and automatic cell counting [52-53]. Fibrinogen is a protein that comes from the liver and is converted into fibrin during the blood clotting process. Evaluation of abnormally elevated fibrinogen (Fibrinogen Levels) helps in the diagnosis of suspected clotting (Bleeding time, BT) or bleeding (Clotting Time, CT) disorders [54]. Fibrinogen levels reflect the accompanying inflammatory process and levels can

Table 2. Studies reporting in vivo assays of the potential of microbial enzymes as antithrombosis agent using rats/ mice as animal model.

No	Thrombolytic Enzyme	Administration	Induction Agent	Reference	Country
1.	Unspecified protease (*Bacillus* sp.)	Intravena	Carrageenan	[24]	Portugal
2.	Unspecified protease (*Stenotrophomonas* sp.)	Intravena, oral	k-carrageenan	[4]	Indonesia
3.	Brevithrombolase (*Brevibacillus* sp.)	Intravena	k-carrageenan	[7]	India
4.	Natokinase (*Bacillus natto*)	Intravena	Carrageenan	[8]	China
5.	Douchi Fibrinolytic Enzyme (DFE)	Oral	Carrageenan	[37]	China
6	*Streptomyces* enzyme	Intravena	Carrageenan	[9]	South Korea
7.	Natokinase (*Bacillus natto*)	Intravena	k-Carrageenan	[40]	Japan

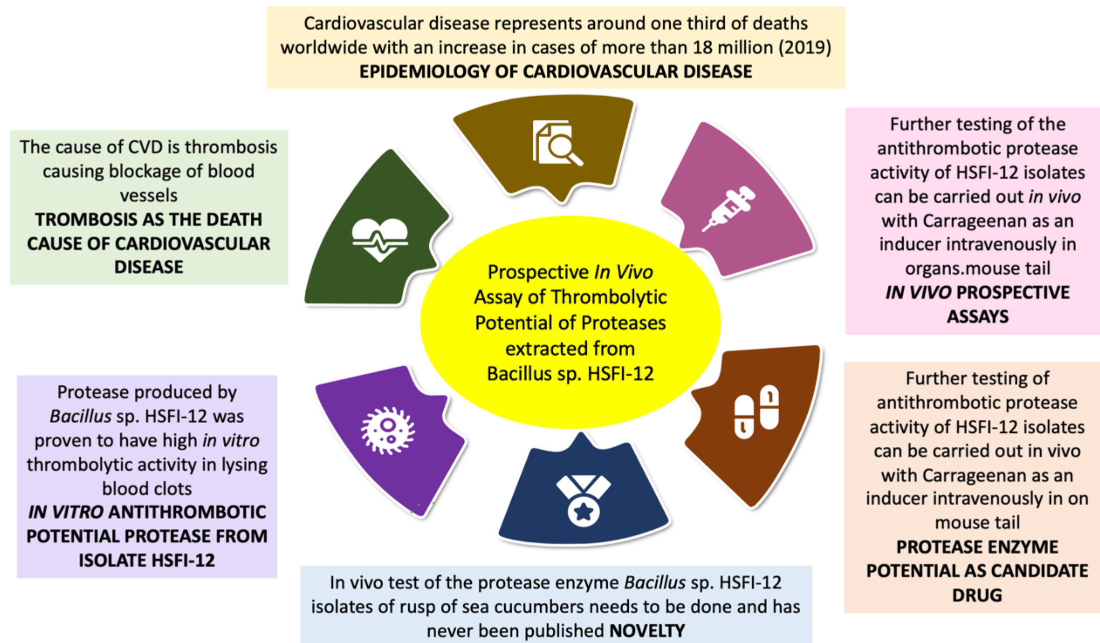

Figure 1 Factors contributing the necessity to conduct prospective *in vivo* assays on the antithrombotic potential of protease extracted from *Bacillus* sp. HSFI-12

exceed 7 mg/mL during acute inflammation, increasing the risk of CVD [55].

Fibrin degradation products (FDP) and **D-dimers** are widely involved in vascular disease. FDP is a product of the degradation of fibrin and fibrinogen. D-dimer is a specific crosslinked fibrin derivative that is an endogenous product of fibrinolytic degradation of fibrin. The FDP and D-dimer are easily measured and are generally considered useful laboratory markers for coagulation activation. Both have also been found to be correlated with the rate of thrombotic events [56-57].

It could be inferred from the data in **Table 1** a study aiming to examine the antithrombotic activity of the protease extract of *Bacillus* sp. HSFI-12 as a thrombolytic agent *in vivo* can be carried out with available methods. Parameters that could be reported include: Partial Thromboplastin Time (aPTT), Prothrombin Time (PT), Thrombin time (TT), Bleeding Time, Clotting Time, Measuring the length of thrombus infarct, Platelet Count, Fibrinogen Levels, D-Dimers and Fibrin Degradation Products (FDP) by intravenous carrageenan induction method in rat tails. Based on the definitions, each of these parameters measures different type of hematology experimental data. Thus, these parameters complete each other.

Table 2 shows the variety of materials used as induction agent in the *in vivo* assays of antithrombotic enzymes using rats or mice which are reported in the last decade. As shown in Table 2 carrageenan induction is widely used in experimental animals when conducting the *in vivo* assays with intravenous dosing (on tail) of antithrombotic agents. Two of 7 studies reported oral administration of antithrombotic agents. The induction of carrageenan was often chosen for the evaluation of *in vivo* assays of compounds having antithrombotic effects in the discovery of thrombolytic agents for several reasons [33,41-43]. One of which is because carrageenan can affect the inactivation of Hageman factor, which is followed by endogenous coagulation [9, 38, 44].

Based on **Table 2**, carrageenan induction coud be chosen for the evaluation of *in vivo* assays of compounds having antithrombotic effects in the discovery and development of thrombolytic drugs [33]. As seen in Table 2, carrageenan induction is widely applied in thrombus formation in experimental animals. Carrageenan can affect the inactivation of Hageman factor, which is followed by endogenous coagulation [9, 38, 43].

Cardiovascular disease (CVD) is the most common cause of death worldwide. To date, the development of thrombolysis treatment is still ongoing to control CVD-related mortality [42]. The fibrinolytic agents, streptokinase, urokinase and plasminogen activator are still frequently used for the treatment of thrombosis. However, these fibrinolytic agents have limitations in terms of their cost, short half-life, allergic reactions, as well as other side effects.

In the search for new antithrombotic agents that are capable of preventing thrombus formation without impairing hemostasis, molecules from marine organisms emerged as promising solutions [43].

Figure 1. shows the scheme explaining prospective *in vivo* assays on the antithrombotic potential of protease extracted from *Bacillus* sp. HSFI-12 and the reasons why it is important to be done. From this literature study it could be concluded that:

1. Due to the high morbidity and mortality of CVD, *in vivo* study is prospective to develop antithrombosis agent from protease of *Bacillus* sp. HSFI-12 following its competitive thrombolytic activity previously reported in the *in vitro* study.
2. The recommended assays to be conducted in the prospective *in vivo* study include the measurement of Thrombus Infarct Length (TIL), Bleeding Time (BT), Partial Thromboplastin Time (aPTT), and Prothrombin Time (PT).
3. The recommended material for thrombosis induction is carrageenan, while the recommended administration of antithrombotic enzyme is by intravenous intervention on tail rather than oral treatment.

Based on the findings of this review study, it is critical to perform in vivo tests on the protease extracted from Bacillus sp. HSFI-12. This is to see if the bacterial thrombolytic protease can be used as a candidate antithrombotic agent/drug, which is critical in combating thrombosis as a death factor and risk factor in CVD. Furthermore, because it has not previously been reported, such a study provides originality and novelty.

AUTHORS' CONTRIBUTIONS

OYD and SNE designed the overall study, AS and MYR screened and summarized all obtained literatures. SD evaluated the generation of tables and figures, and also analysed the bias of the study. The main text was written by OYD and SNE. The manuscript was first written by NI, and then revised and proof-read by SNE.

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