Original Article

Thai Journal of Pharmaceutical Sciences

The evaluation on anti-platelet and antithrombosis activities of *Cinnamomum sintoc* bark extract

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Received: Jun 11, 2019
Accepted: Nov 02, 2019
Published: Dec 10, 2019

ABSTRACT

Introduction: One of the factors contributing to cardiovascular diseases is platelet aggregation. Although several antiplatelet agents have been introduced into clinical practices, discovery of antiplatelet agents is still challenging. Plants from the genus of *Cinnamomum* were previously reported to have antiplatelet activity. This study aimed to determine the activity of *Cinnamomum sintoc* (CS) bark extract inhibiting platelet aggregation and thrombosis. **Materials and Methods:** Antiplatelet activity of CS bark methanolic extract (CS methanolic extract [CSME]) (5–300 μg/mL) was determined by measuring the ability of the extract to inhibit platelet aggregation induced by epinephrine. Meanwhile, the antithrombosis activity was determined by calculating the survival mice and by measuring the thrombus formation in the mice lung capillaries after challenging with thrombus-inducing agent, combination of collagen, and epinephrine. **Results:** The results showed that CSME inhibited platelet aggregation induced by epinephrine with the IC_{50} of 117.9 ± 8.8 μg/mL. However, CSME at low, medium, and high doses (100, 400, and 600 mg/kg BW, respectively) failed to protect the mice from death and paralysis after thrombotic challenge. **Conclusions:** CSME demonstrated an *in vitro* antiplatelet activity, but it failed to protect thrombosis in the thrombotic mice.

Keywords: Cardiovascular protective, *Cinnamomum sintoc,* platelet, thrombus

INTRODUCTION

To date, cardiovascular disease (CVD) ranks the first cause of death globally. Mostly, CVD is mediated by the presence of obstruction or blockage in the blood capillary vessels involving thrombotic reactions. The emergence of infarction or blockage of blood vessels is initiated by atherosclerosis that in later step disrupts the endothelium wall and leads to rupture. Consequently, endothelial tissue debris enters the bloodstream and triggers platelet activation and thus leads to thrombus formation.[1] Platelets play a significant role in the development of thrombosis. Several endogenous agents have been identified as platelet receptor activators that cause platelet aggregation. These substances include adenosine diphosphate (ADP), collagen, thromboxane A2 (T × A2), thrombin, serotonin, and epinephrine. They act as an agonist for their respective receptors and work synergistically to induce platelet aggregation.[2,3] Epinephrine is a soluble ligand that induces platelet aggregation by the binding with α2-adrenergic receptor. In platelets, activation of this receptor by epinephrine results in the reduction of cyclic adenosine monophosphate and increase of intracellular calcium ions. These events trigger shape changes and contraction of platelet as well as activation of glycoprotein (GP) IIb/IIIa receptor. Activation of GP IIb/IIIa provokes the binding of platelets to fibrinogen that represents the common final pathway of platelet aggregation and thrombus formation.[4,5] In addition, previous *in vivo* study indicated that α2-adrenergic-knockout mice were protected from thromboembolism on induction with the combination of pro-thrombotic agents, epinephrine, and collagen.[6]

Antiplatelet, an agent with the ability to inhibit platelet aggregation, remains the drug choice in combating platelet-related disorders such as stroke, ischemia, and myocardial infarction. At present, many drugs have been developed as antiplatelet agents. This included clopidogrel, abciximab, and vorapaxar, for $P2Y_{12}$, GP IIb/IIIa, and protease-activated receptor-1 antagonist, respectively. However, aspirin, the old drug inhibitor of cyclooxygenase-1 remains the first drug of choice in the clinics either as a single or combination with other drugs.[7,8] Current antithrombosis agents are classified into seven major groups: (a) Vitamin K antagonists, (b) heparins, (c) inhibitors of platelet aggregation other than heparins, (d) enzymes, (e) thrombin direct inhibitors, (f) direct factor Xa inhibitors, and (g) other antithrombosis agents.[9] Although these drugs retain their efficacy, some limitations such as undesirable side effects such as hyporesponsiveness,[10] slow-onset,[11] and drug resistance[12] were reported. Thus, development and discovery of alternative antithrombosis agents, especially from natural resources is still promising.

Several studies have reported that plants from the genus of *Cinnamomum*, such as *Cinnamomum cassia*,[13] *Cinnamomum tenuifolium,*[14] *Cinnamomum philippinense,*[15] *Cinnamomum altissimum,* and *Cinnamomum pubescens,*[16] demonstrated antiplatelet activities. Another plant species within this genus that has not been explored yet is CS. Until now, no data are available regarding the antiplatelet and antithrombosis activities of CS. However, our preliminary study identified CS as a plant with antiplatelet activity.[17] Here, we aimed to investigate the antiplatelet activity of CS bark methanolic extract (CS methanolic extract [CSME]) in platelet induced by epinephrine. In addition, the antithrombosis potency of CSME was also determined in mice induced by collagen-epinephrine.

MATERIALS AND METHODS

Materials

Plant material

The bark of CS was obtained from the local market of herbal medicine in Beringharjo, Yogyakarta, Indonesia. The plant material was authenticated by a botanist Dr. Djoko Santosa, M.Si.; with the certificate of determination number: UGM/FA/2046/M/03/02) of the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The bark was dried in oven (50°C for 48 h) and crushed into smaller size before powdered. The dried powder of CS bark was used for the extraction process.

Chemicals and instruments

Dichloromethane, methanol, ferric chloride, ethyl acetate, and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany), porcine gelatine, ticagrelor, catechin, ADP, calfskin collagen, and thin-layer chromatography (TLC) plate silica C-18 were purchased from Sigma (Missouri, USA), epinephrine (Hospira; Illinois, USA), and yohimbine HCl was purchased from Liftmode (Chicago, USA). Aggregometer (Chrono-Log®; Pennsylvania, USA) was used to measure platelet aggregation.

Animal

Balb/c strain male mice (2 months of age) were obtained from the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The mice were maintained in animal cages in the animal facility with a controlled environment and normal light cycle exposure. The mice have free access to the normal diet, and the water was given *ad libitum*. Acclimatization of the mice in the environment was done at least a week before the experiments. The animal experiment protocols were approved by Ethical Committee of Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (Ethical clearance number: KE/FK/1228/EC/2017).

Plant Extract Preparation

CS stem bark powder (400 g) was macerated twice (each with 2 L of dichloromethane for 24 h) to remove essential oil components and other non-polar compounds that might interfere with the antiplatelet activity measurement. After filtering, the filtrate was collected and the residue was macerated again twice (each with 2 L of methanol for 24 h). The filtrates obtained from the first and second extraction were combined and evaporated until dryness by using rotary evaporator instrument to obtain 23.6 g dried CSME. The dried extract was stored at 4°C until use.

TLC Analysis

Chemical profile determination of the extracts was carried out using TLC. The mixture of methanol-water (2:1) and TLC Silica gel 60 RP-18 F254 (Merck, Darmstadt, Germany) was used as the mobile phase and stationary phase, respectively. The extracts were dissolved in their respective solvents and applied to the stationary phase and put into a TLC chamber containing saturated mobile phase. After the mobile phase reached 8 cm of distance, the plate was taken out and dried. Visualization of the chemical components was done under ultraviolet (254 nm and 366 nm) and visible light after derivatization with Cerium (IV) sulphate reagent.

Platelet Preparation

Human plasma was obtained from the blood of 12 volunteers who met the inclusion criteria including: (a) Healthy, (b) not infected with bacteria and viruses (HIV, Hepatitis), (c) not having a disease or disorder in the blood (e.g., Hemophilia), (d) not taking nonsteroidal anti-inflammatory drug or blood affecting drugs in the past 2 weeks, and (e) not being pregnant. Blood collection was carried out by experienced technical personnel through arm veins as much as 12 mL per blood draw. Sodium citrate was used as an anticoagulant agent.[18] The blood was pipetted 1 mL for platelet counts, and the rest was centrifuged for 15 min at 1000 rpm. The supernatant containing high concentration of platelet was separated and labeled as platelet-rich plasma (PRP). The residue was centrifuged for 15 min at 3500 rpm, and the supernatant was separated and labeled as platelet-poor plasma (PPP).

Antiplatelet Assay

The platelet aggregation was determined in an aggregometer instrument[17] based on the principle of light transmission aggregometry using Chrono-Log® Aggregometer. Shortly, the addition of platelet aggregators to the hazy PRP causes

the platelet to aggregate so that the plasma becomes clearer indicated by the rise of the spectrophotometric transmittance value. The platelet aggregation was induced by epinephrine (at the concentration of 3.9 μM). Yohimbine HCl (an antagonist of alpha-2 adrenergic receptor) was used as a positive control whereas DMSO was used as a solvent vehicle.

As much as 487.5 μl, PRP was put into the cuvette using a micropipette containing a magnetic stirrer bar. Then, 2.5 μl of extract (in DMSO) was added and this mixture was incubated for 3 min at 37°C. Epinephrine (10 μl) was added and the transmittance value was measured after 10 min (endpoint). The platelet aggregation was given by the transmittance value at 10 min. About 100% of platelet aggregation was assumed to be proportional to the transmittance of PPP, and this value was set as a measurement baseline. The experiments were performed 3 times independently. The inhibition of platelet aggregation was calculated using the formula: (1–(platelet aggregation upon extract treatment/platelet aggregation of DMSO)) × 100%. The protocol was approved by the Institutional Ethical Committee of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (Ethical clearance number: KE/FK/1228/EC/2017).

Antithrombosis Assay

In vivo, antithrombosis activity test was performed using male Balb/c mice as animal experimental model.[19] Twenty-five mice were divided into five groups containing five mice per group. The mice were pretreated daily with CSME at the dose of 100, 400, and 600 mg/kg body weight (BW) orally for 5 days. Ticagrelor 11 mg/kg BW and sodium carboxymethyl cellulose (CMC-Na) were used as positive control and solvent vehicle, respectively. One hour after the administration on the last day, the mice were injected with epinephrine (0.072 mg/kg BW) and collagen (6 mg/kg BW) through the vein of the tail. Shortly after injection, the mice were observed for 10 min. The observations included paralysis (limb dysfunction and respiratory failure) and death. The percentage of thrombosis protection was calculated as: (1–[Number of death or paralysis mice]/total) × 100%. At the end of the study, the surviving mice were sacrificed, and the lungs, hearts, and brains were collected for histopathology examination.

Detection of Tannins Content

Gelatin precipitation

Tannin detection in the CSME was conducted according to the Bele *et al*.[20] based on gelatin precipitation. Shortly, 10 mL CSME in methanol (1 mg/mL) was mixed with 2 mL of gelatin 1% and observed until precipitation was formed. The formation of white sediment at the bottom of the flat-bottom flask indicated the presence of tannin in the extract.

Condensed tannins cross-linking test by formaldehyde

The method was performed according to a previous study.[21] Briefly, 10 mL of CSME (1 mg/mL) in a flat-bottom flask was added with 2 mL of 10% formaldehyde and three drops of HCl, and this solution was heated on a water bath. The formation of insoluble red deposits at the bottom of the flask indicated the presence of condensed tannins in the extract.[21]

Color test by ferric chloride

Detection of hydrolyzable tannins in the CSME was done using ferric chloride ($FeCl_3$) reagent (10%).[22] In short, 10 mL of the extract solution (1 mg/mL) was added with three drops of $FeCl_3$. Hydrolyzable and condensed tannins would give blue and green color, respectively.

Gas Chromatography-Mass Spectrometer Analysis

The determination of non-tannin constituents in the CSME was done using gas chromatography coupled with mass spectroscopy (gas chromatography-mass spectroscopy [GC-MS]) method. The column for gas chromatography was Rxi®-1 ms (30 m, 0.25 mmID, and 0.25 μm) produced by Restek (Cat. Number #13323; PA, USA). The column conditions are as follows: Column oven temperature: 75°C; injector temperature: 250°C; pressure: 100 kPa; and carrier: Helium, with the flow rate of 1.50 mL/min. The column temperature program: Initial temperature of 75°C for 5 min and then increased 20°C/min until the final temperature of 300°C (maintained for 10 min). The instrument was equipped with a mass spectrometer instrument utilizing an electron impact ionizing method. The obtained mass spectra were compared with spectrum library database.

Statistical Analysis

The data obtained from the antiplatelet and antithrombosis evaluations were presented as a mean ± standard error of mean. We performed one-way ANOVA (analysis of variance) followed by Dunnett's *post hoc* test to determine the level of significance.

RESULTS

TLC Profile of CSME

The first extraction of CS bark using dichloromethane resulted in CS bark dichloromethane extract (CS dichloromethane extract [CSDE]). The first extraction aimed to remove the non-polar constituents of CS bark. The second extraction was done using methanol resulted in CSME. To confirm the effectivity of the removal of the non-polar compounds constituents, a reverse phase TLC analysis was performed. Eugenol was included in the analysis as a reference compound. Figure 1 indicated that the first extraction successfully removed the non-polar constituents, indicated by the presence of non-polar compounds (lower Rf values) in the CSDE. Consequently, second extraction using methanol produced CSME in which the non-polar components have been deprived. This extract was used for the evaluation of antiplatelet and antithrombosis activities.

Antiplatelet Activity of CSME

We measured the antiplatelet activity of CSME at the doses of 5, 10, 50, 100, 200, and 300 μg/mL. Epinephrine (3.9 μM) was used as a platelet agonist to induce platelet aggregation, whereas yohimbine HCl (1 μM), the well-known antiplatelet agent was used as a positive control to inhibit platelet aggregation. Figure 2 indicated that CSME dose-dependently inhibited platelet aggregation induced by epinephrine with the IC_{50} of 117.9 ± 8.8 μg/mL.

Antithrombosis Activity of CSME in *Mice*

The *in vivo* antithrombosis activity was done to confirm the effectiveness of CSME in the intact model by employing thrombotic mice. The mice were pretreated orally with the extract at the doses of 100, 400, or 600 mg/kg BW for 5 days, and the thrombosis was induced by the combination of epinephrine and collagen. We found that CSME at those concentrations failed to protect the mice from being paralysis or death compared to the negative control ($P > 0.05$). As expected, ticagrelor, the reference drug showed a significant protective effect from thrombosis ($P < 0.05$) [Table 1]. The histopathological examination indicated that the induction of collagen-epinephrine led to the exclusive thrombi formation in the lung arteries, but not in the arteries of brain and hearth. This indicated that the induction of collagen-epinephrine led to death or paralysis which was caused by thrombi formation in the lung arteries. Thrombus is characterized by the presence of fibrin and platelet that can be observed as pink to red distinct mass when stained with hematoxylin-eosin. We found that the size of the thrombi varied among the groups. Massive multifocal thrombi were present in the group treated with the vehicle (CMC-Na). Interestingly, a lower level of multifocal thrombi was observed in the group treated with CSME. Ticagrelor, however, showed a stronger protection effect indicated by a minimal thrombi formation [Figure 3].

To measure the level of thrombotic severity, the number of thrombus for each group was calculated and semi-quantitatively analyzed. We found that the number of thrombus in the mice pretreated with CSME was not significantly different compared to those pretreated with CMC-Na ($P > 0.05$). The average number of thrombus in each group is presented in Table 2.

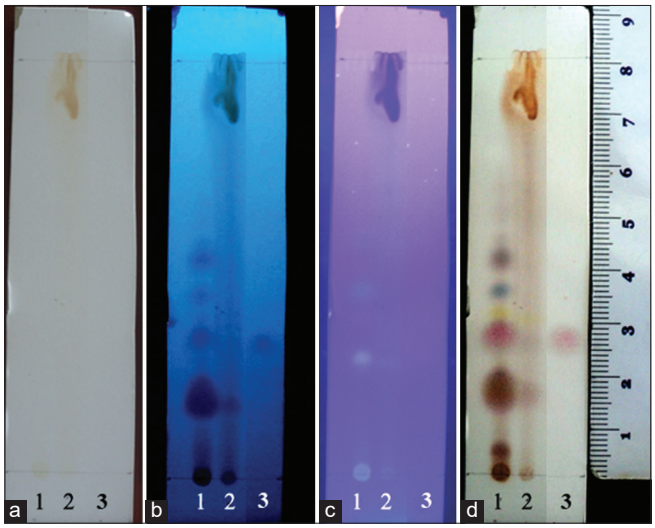

Figure 1: TLC profiles of *C. sintoc* bark extracts. (1) *C. sintoc* bark dichloromethane extract (CSDE); (2) *C. sintoc* bark methanolic extract (CSME); (3) Eugenol. Dried powder of *C. sintoc* bark was pre-macerated with dichloromethane to yield CSDE. The residue was re-macerated with methanol to produce CSME which is used in this study. Clearly, first maceration of the plant material with dichloromethane effectively removed essential oils and other nonpolar components. TLC was done in a reversed phase system using stationary phase of silica gel 60 RP-18 F_{254}s and the mobile phase of methanol-water (2:1). The spots were visualized with: (a) visible light; (b) UV_{254}; (c) UV_{366}; (d) visible light after derivatization with Cerium(IV) sulphate spray reagent. The scale in the right side is in centimetre (cm) unit

Tannins Detection in CSME

As the plant genus of *Cinnamomum* is known for its tannins content,[13] the presence of tannin in the CSME was analysis. We performed common methods (section 2.7) for tannins analysis and we confirmed that CSME contained tannins [Figure 4]. White precipitates [Figure 4a] were formed after CSME solution was added with gelatin (1%). This suggested that the extract contained tannins. The formation of insoluble red precipitates after addition of formaldehyde indicated that the CSME contained condensed tannins [Figure 4b]. These precipitates were the end product of cross-linking reaction between procyanidins and acidic formaldehyde.[23] In addition, generation of green color after addition of $FeCl_3$ to the CSME solution [Figure 4c] is another evidence supporting the presence of condensed tannins.[24]

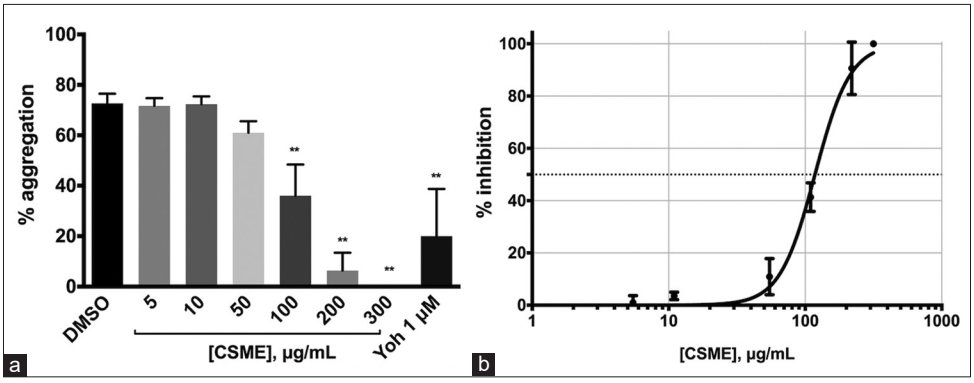

Figure 2: The *in vitro* antiplatelet activity of CSME. (a) Inhibition of platelet aggregation by CSME (μg/ml) in epinephrine-induced human platelet. (b) Semi log-dose regression curves representing the antiplatelet activities of CSME in platelet induced by epinephrine. Human PRP (in cuvette with magnetic stirrer) containing CSME, DMSO or yohimbine HCl, was pre-incubated for 3 min in an aggregometer. Epinephrine (3.9 μM) was added and the platelet aggregation was determined after 10 min. The percent inhibition of platelet aggregation of CSME was relative to the solvent vehicle (DMSO). The data are mean ± standard errors from three independent experiments. **p < 0.001 (ANOVA/Dunnett), compared to the solvent (DMSO)-treated group

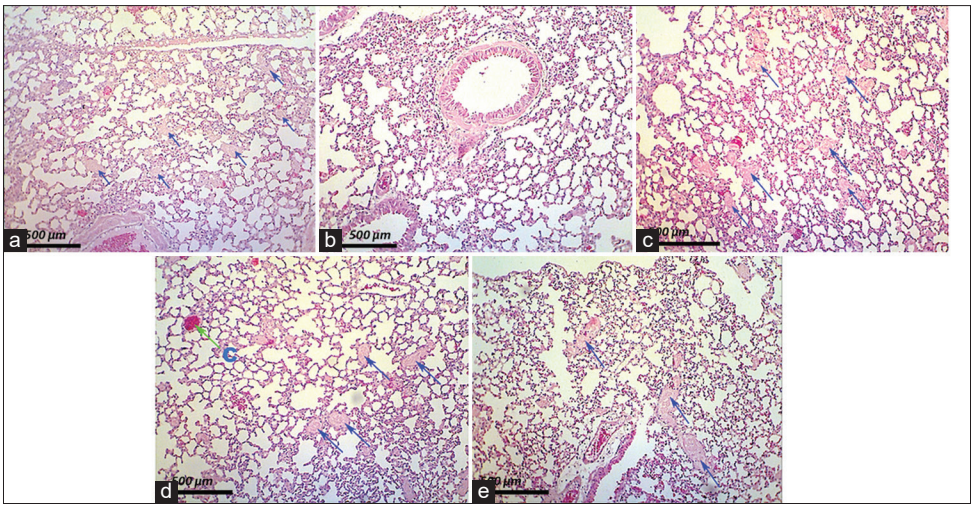

Figure 3: Representative photos showing the histology of the lung tissues of the mice. The mice were pretreated with CMC-Na (a), Ticagrelor 11 mg/kg BW (b), CSME 100 (c), CSME 400 (d), or CSME 600 mg/kg BW (e) before the induction of thrombosis using collagen-epinephrine. Multifocal thrombi were found in the lung of all group, except for Ticagrelor (b). The lung tissues were stained with hematoxylin-eosin and the photos were taken at 10x magnification (blue arrow: thrombus, C: congestion)

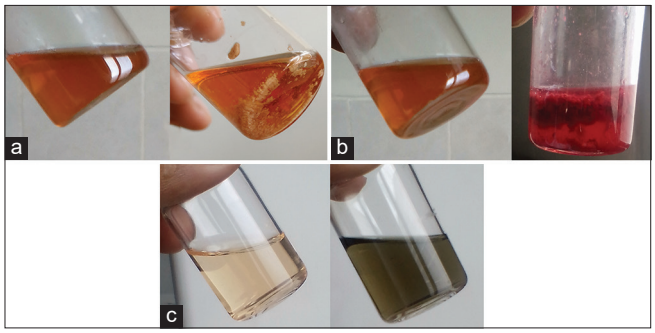

Figure 4: Qualitative test for tannins. (a) CSME was dissolved in ethanol before gelatin addition (left); white precipitates were formed after the addition of gelatin (right). (b) Red insoluble deposits were formed after the addition of acidic formaldehyde followed by heating (right). (c) Dark greenish color was formed after the addition of FeCl3 (right)

GC-MS Analysis of CSME

The CSME was analyzed using GC-MS to reveal the non-tannins components of the extract. Clearly, 10 compounds have been successfully identified [Figure 5] are 3-allyl-6-methoxyphenol, methyleugenol, cis-methylisoeugenol, p-acetamidophenol, methyl myristate, methyl palmitate, methyl linoleate, methyl octadec-9-enoate, methyl stearate, and methyl 11-eicosenoate. Methyl palmitate and methyl octadec-9-enoate represent the two major non-tannin compounds in the CSME.

DISCUSSION

To date, CVDs remain the leading diseases cause of death globally. Platelet aggregation plays a crucial role in the progression of CVD. The effort of finding antiplatelet agents is a promising approach to combat CVD. In our preliminary study,[17] we found that CS is one of the promising plants with potent antiplatelet activity. To further investigate the effect of CS bark on cardiovascular systems, we prepared CSME and evaluated its efficacy as antiplatelet and antithrombosis.[19] As

Table 1: Antithrombosis evaluation of CSME in the thrombotic mice induced by collagen-epinephrine

Groups	Number of subject	Percent of protection (%)	Onset of paralysis (s)	Onset of death (s)
CMC-Na	5	0	96±33	260±35
Ticagrelor	5	100*	n.d.	n.d.
CSME 100	5	0n.s	58±4n.s	204±33n.s
CSME 400	5	0n.s	72±45n.s	120±60n.s
CSME 600	5	20n.s	90±60n.s	340±151n.s

The mice were treated with CSME in different doses and they were challenged with a thrombotic inductor (epinephrine-collagen). The number of survival mice, onset of paralysis, and onset of death were calculated. Data are mean±standard deviation (n=5). n.d.: not detected or no dead animal in ticagrelor-treated group. Thus, it was excluded from the statistical analysis. n.s: not significant, * P<0.05; ANOVA followed by Dunnett's *post hoc* t test, compared to the CMC-Na-treated group. The data are mean±standard deviation. CSME: *Cinnamomum sintoc* methanolic extract, CMC-Na: Sodium carboxymethyl cellulose

Table 2: The number of thrombus between groups

Groups	Number of subject (n)	Thrombus number (Mean±standard deviation)
CMC-Na	5	6.0±2.4
Ticagrelor	5	0.0±0.0*
CSME 100	5	9.5±4.2n.s
CSME 400	5	9.9±5.5n.s
CSME 600	5	3.2±3.5n.s

The mice were sacrificed at the end of the experiments, and the number of thrombus per view in the lung was calculated under microscope (×10). The calculation was done in a triplicate for each lung. n.s. Not significant; *P<0.05; ANOVA followed by Dunnett's *post hoc* test, compared to the CMC-Na-treated group. CSME: *Cinnamomum sintoc* methanolic extract, CMC-Na: Sodium carboxymethyl cellulose

the *in vitro* antiplatelet assay was performed in hydrophilic environment involving human PRP, removal of the essential oils and the other nonpolar components before extraction (in

methanol) was performed to exclude possible interferences in the bioassays due to solubility problem [Figure 1]. To assess the antiplatelet activity of CSME, we first tested the ability of CSME to inhibit platelet aggregation in ADP-induced human PRP. The method represents the versatile and the most common *in vitro* method for evaluation platelet aggregation.[25] Yohimbine HCL (Yoh) was used as a positive control in the experiment, and it was tested at a single working concentration. In this experiment context, the idea of utilizing Yohimbine HCL was to confirm the responsiveness of the bioassay against the respective drug rather than to compare the potency against CSME. For the 1st time, we demonstrate that CSME exerted antiplatelet activity with the IC_{50} of 117.9 ± 8.8 μg/ml. Our finding is in line with the previous study demonstrating that the methanolic extract of *Cinnamomum cassia,* another plant with the same genus (*Cinnamomum*) exhibited antiplatelet activity.[26] Interestingly, the antiplatelet activity of CSME was relatively stronger compared to the methanolic extract of *C. cassia*. In addition, CSME also exhibited a superior antiplatelet activity compared to *Allium* sp. (IC_{50} 494 μg/ml),[27] a well-known antiplatelet plant, and other previously reported plants such as *Wendtia calycina* (IC_{50} 820 μg/ml)[28] and kiwi fruit (IC_{50} 1600 μg/ml).[29]

Platelet aggregation contributes to the progression of atherothrombosis leading to CVD.[30] Therefore, the potency of CSME as antithrombosis agent was evaluated. By employing a thrombotic mice model, we found that although CSME showed potent antiplatelet activity in the *in vitro* experiment, it failed to retain the activity in the *in vivo* thrombotic mice model. CSME at the dose up to 600 mg/kg BW was not able to protect the thrombotic mice from death and paralysis, as well as thromboembolism. The histopathology examination revealed that all the mice suffered multifocal thrombi in the lung arteries, suggested that the mice died due to thrombosis. Many factors might contribute to the discrepancy between the *in vitro* and *in vivo* experiments. The first factor is likely due to poor absorption of the bioactive compounds in CSME that limits the bioavailability. *Cinnamomum* is a genus of plants that is well-known for its tannins content.[13,31] Our investigation showed that CSME contains a high tannin content (especially condensed-tannins) indicated by its ability to strongly aggregate gelatin solution [Figure 4]. Although tannin was reported previously as an antiplatelet agent[32] and there was a positive correlation between tannin content and antiplatelet activity of CS,[31] these studies were done in the *in vitro* experimental model, which omitted the absorption factor. Many studies have shown that most tannins have poor bioavailability.[33,34] When given orally, tannins concentration in the blood does not reach therapeutic doses required for the activity. The second factor determining the fate and bioactivity of a compound is metabolism. Phases I and II metabolisms of tannins generate complex structure of tannins and form conjugation (methyl, glucuronide, and sulfate) derivatives, respectively.[35] In addition, the untransformed tannins that reach colon or small intestine are further metabolized by microbiota into small-weight phenolic molecules that alter their biological activities.[36] These could be the reason for the lack of efficacy of CSME in the *in vivo* antithrombosis assay in this study. Nevertheless, further study is needed to identify the active compound and to investigate the impact of absorption, distribution, metabolism, and excretion of the CSME and its active compound on the *in vivo* efficacy.

Considering the presence of tannin in the CSME, separation of the tannin from the CSME is needed to confirm whether the tannin separation affects the activity. The previous study showed that tannins removal impaired *in vitro* antithrombosis activity of several plant extracts.[37] An effective tannins removal method could be done by dissolving the CSME in methanol-water mixture (3:1) and subjected to a liquid-liquid partition using dichloromethane to yield tannin-free extract.[38] Re-evaluation of the *in vitro* antiplatelet activity of the tannin-free extract of CSME will reveal whether tannin is the compound responsible for the antiplatelet activity. Although tannin is a major component in the CSME, the contribution of non-tannin compounds in the antiplatelet activity of CSME could not be ruled out. Thus, we analyzed the non-tannins constituent of CSME to get insight into the non-tannin compounds that might be responsible for the antiplatelet activity. Our phytochemical investigation using gas-chromatography revealed that CSME contains 10 non-tannin compounds [Figure 5]. However,

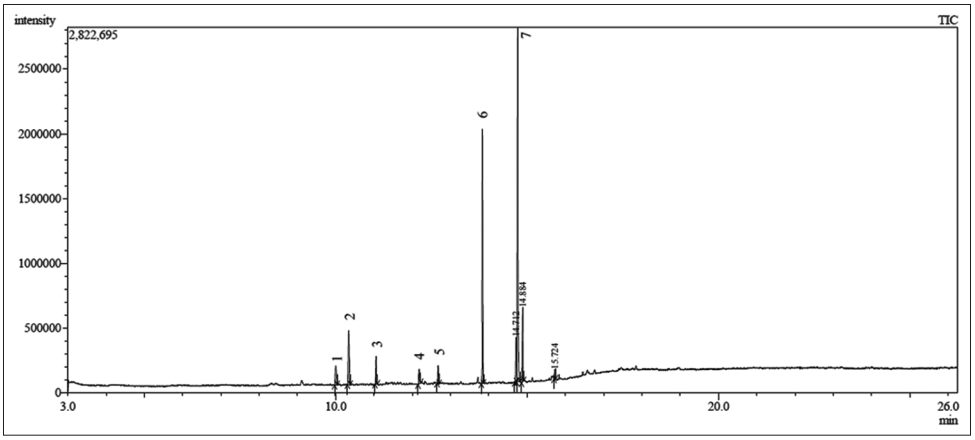

Figure 5: Non tannin components of CSME. The phytochemicals analysis was performed using gas chromatography. Ten compounds were detected in CSME: (1) 3-allyl-6-methoxyphenol; (2) Methyl eugenol; (3) Cis-methyl isoeugenol; (4) P-acetamidophenol; (5) Methyl myristate; (6) Methyl palmitate; (7) Methyl linoleate; (8) Methyl octadec-9-enoate; (9) Methyl stearate; (10) Methyl 11-eicosenoate. Methyl palmitate (6) and Methyl octadec-9-enoate (8) are the major compound.

none of these 10 identified compounds were reported to have antiplatelet or antithrombosis activities.

CONCLUSIONS

We found that CSME demonstrated *in vitro* antiplatelet activity (IC_{50}: 117.9 ± 8.8 μg/mL) in platelet induced by epinephrine. However, CSME failed to exert antithrombosis activity in the *in vivo* experiment indicated by its inability to protect thrombotic mice from paralysis and death due to thromboembolism.

ACKNOWLEDGEMENT

The authors thank the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia for the financial support (PUPT grant number 2401/UN1.P.III/DIT-LIT/LT/2017, and to the Research Unit for Natural Product Technology, Indonesian Institute of Sciences for the opportunity of taking master program (T.W.). The data published in this article was used by T.W and A.R.P to obtain their academic degrees in the Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia.

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