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Antiplatelet Activity of *Piper cubeba* Fruit Ethanol Extract

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ABSTRACT

The search for a new generation of antiplatelet agents from natural resources with favorable effects is promising. Preliminary research on the screening of antiplatelet agent from Indonesia medicinal plants have identified 10 out of 139 plant extracts. One of these active plant extracts is *Piper cubeba* fruit ethanol extract. The antiplatelet activity of *P. cubeba* fruit ethanol extract in different pathways of platelet aggregation was investigated to elucidate the mechanism of action of the extract as antiplatelet agent. Dry powder of *P. cubeba* fruit (500 g) was extracted with ethanol and the dried extract was used for the experiment. Cubebin is the major compound found in the fruit of *P. cubeba*. For standardization, we determined the levels of cubebin in the *P. cubeba* fruit ethanol extract by using densitometry method. Human platelet-rich plasma (487.5µL) was added to the extract solution (2.5 µL in Dimethyl Sulfoxide (DMSO) and incubated at 37°C for 3 minutes. The platelet aggregation was induced with 10 µL platelet receptor agonists (ADP, arachidonic acid, epinephrine, or thrombin) and the aggregation was measured for 8 minutes in an aggregometer. In this study, 2.97% cubebin was detected in the ethanol extract of P. cubeba fruit. The extract inhibited platelet aggregation induced by ADP, arachidonic acid, epinephrine, and thrombin with the IC_{50} of 813.30; 895.90; 883.40; and 1.090 µg/ ml respectively. This results suggested that the extract has moderate antiplatelet activity in several pathways of platelet aggregation.

Keywords: Cubeb, Cubebin, Platelet, Cardiovascular diseases, Thrombosis, Herbal medicine.

Introduction

Antiplatelet agents play crucial role in the therapy of cardiovascular and cerebrovascular diseases.[1,2] Antiplatelet drugs are important for antithrombotic treatment in patients at high risk of arterial thrombosis.[3,4] Antiplatelet agents prevent the formation of second messengers, by interacting with intracellular signaling pathways, blocking membrane receptors, or inhibiting platelet aggregation.[2] The four main classes of antiplatelet drugs that are currently available in the market are Cyclooxygenase inhibitors (Aspirin), Thienophyridine derivatives (Clopidogrel and Ticlopidine), Phosphodiesterase inhibitors (Cilostazol, Dipyridamole), and Glycoprotein IIb/IIIa receptor blockers (Abciximab).[5]

Aspirin is the most widely used antiplatelet agent because aspirin is effective in reducing the incidence of cardiovascular diseases such as ischemic stroke. Despite the considerable benefit, about 15% - 25% of patients have reported resistance to aspirin.[6,7] This aspirin resistance can increase the risk of recurrent ischemic stroke and even death in patients. In addition, aspirin which is classified as a Non-Steroidal Anti-Inflammatory Drug (NSAID) has known for its side effect (stomach irritation and bleeding).[8] The search for a new generation of antiplatelet bioactive compound from natural resources with high efficacy and favorable side effects is a promising approach for combating cardiovascular diseases.[9]

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Preliminary research have screening 139 plant extracts resulted in 10 active extracts having antiplatelet activity. One of the active extracts is the ethanol extract of cubeb fruit (*Piper cubeba* Lf).[10] This study aimed to investigate the antiplatelet activity of *P. cubeba* fruit ethanol extract in different pathway of platelet aggregation. Platelets are equipped with a variety of receptors that synergize to trigger a range of functional responses.[11] Platelet aggregation is caused by various agonists that bind to and activate specific receptors expressed on platelets.[12,13] Platelets under physiological conditions are resting in the blood circulation, but become active due to soluble platelet agonists such as thrombin, ADP, thromboxane A2 (TXA2), and others.[14] Specific receptors on the platelet surface can initiate platelet adhesion. Under high shear conditions, platelet adhesion is mediated by the interaction of glycoprotein (GP) Ib/V/IX receptors on the platelet surface with vWF and the interaction of GP VI and GPIa with collagen on the injured vascular surface.[15,16] Platelet agonists such as ADP (ligand of P2Y1 and P2Y12 receptors), thromboxane A2 (ligand of TXA2 receptor), and thrombin (ligand of PAR1 receptor) activate their respective receptors to induce platelet activation and shape change. The ultimate step after the binding of platelet agonist to their respective receptors is platelet aggregation.[12,17] This aggregation provokes more platelet cross-linking and ultimately leading to thrombus formation.[18] Platelets can be activated by collagen and platelet-activating factor (PAF) which results in the enzymatic metabolism of arachidonic acid (AA) that contributes to further platelet aggregation and thrombus formation.[19,20] *P. cubeba* fruit contains several lignan compounds such as cubebin, hinokinin, clusin, dihydrocubebin, dihydroclusin, cubebinin, yatein, cubbinulide (cordigerine), dihydroyatein, isoyatein, cubebinone.[21] Plants of the genus *Piper* have many species that share similar secondary metabolites content. Due to this similarity, they might have similar pharmacological activity. There are four acid amides namely piperine, pipernonaline, piperoctadecalidine, and piperlongumine isolated from *Piper longum* L. fruit shown to inhibit

platelet aggregation in rabbits induced by collagen, AA, and PAF.[22] Another study showed that (-)-3',4'-O,O-demethylenehinokinin and 3,4-methylenedioxy cinnamaldehyde isolated from *Piper philippinum* have anti-platelet activity in the *in vitro* model.[23] Further, piplartine (piperlongumine; 5,6-dihydro-1-[1-oxo- 3- (3,4,5-trimethoxyphenyl]-2 (1H) pyridinone) from *Piper tuberculatum* inhibited collagen, arachidonic acid, collagen, or ADP-induced platelet aggregation, but not in platelet aggregation induced by thrombin.[24] Piperine obtained from *Piper nigrum* and *Piper longum* was shown to have antiplatelet activity by inhibiting AA release through decreasing cPLA2 activity on collagen-induced platelets.[20] Additionally, *Piper betle* extract inhibited AA- and collagen-induced platelet aggregation, although it showed little effect on thrombin-induced platelet aggregation.[25] Medicinal plants offer abundant source of antiplatelet agents and are considered safe to be further developed as drugs.[26] This study aims to obtain antiplatelet agents derived from medicinal plants, to find complementary and alternative compounds with relatively few side effects. Studies on natural product for therapeutic properties of medicinal plants have become popular in recent times.[27,28] Therefore, natural products isolated from medicinal plants could be developed as an alternative antiplatelet agent to overcome drug resistance and/or to minimize side effects, especially bleeding risk. This study contributes to the discovery and development of antiplatelet drugs originally derived from natural resources.

Materials and Methods

Plant material and chemicals

Fruits of *P. Cubeba* were collected on from Tegal Sari, Giri Purwo, Kulonprogo District, Yogyakarta Province, Indonesia, in March 2017. The plant was authenticated by a botanist (Dr. Djoko Santosa), of the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia (voucher specimen number 16.20.9/UNI/FFA/BF/PT/2021). Aspirin (Sigma), human thrombin (Sigma, catalog no. T6884-250UN), epinephrine (PT. Phapros Tbk.), Adenosine 5' diphosphate monosodium salt (Calbiochem), arachidonic acid (Sigma, catalog no. A9673), ethanol (Merck), sodium citrate (Merck), Whatman paper no.1, DMSO (Merck), ethyl acetate (Merck), dichloromethane (Merck), NaCl (Merck), Bovine Serum Albumin (BSA) (Merck), Phosphate Buffer Saline (PBS), plate TLC silica F_{254} (Merck), 50% sulfuric acid spray reagent.

Plant extraction

Powdered *P. cubeba* fruit (500 grams) was macerated with ethanol 2.5 L for 5 days with stirring every 24 hours. The filtrate was evaporated using a vacuum rotatory evaporator at 50°C and left at the room temperature until dryness.

Reagents preparation

Aspirin (20 mg) was added with 100 mL DMSO, and then vortexed to obtain a stock solution with a concentration of 200 µg/mL aspirin. The extract (20 mg), added to 100 mL DMSO, and then vortexed to obtain a stock solution with a concentration of 200 µg/mL extract. Epinephrine (1mg/mL) was added with 0.9% NaCl solution to the final volume of 600 mL. The solution was homogenized to obtain a stock solution with a concentration of 10 mM. ADP 22.50 mg dissolved in 100 mL of DMSO, and further homogenized to obtain a stock solution of 10µM. Human thrombin (100 IU/mL) was diluted in PBS buffer pH 6.5 containing BSA 0.1% v/v. Arachidonic acid (100 mg) was dissolved in DMSO to obtain a stock solution of 100 mg/mL.

Subject

Blood from donors with aged 18 - 45 years, not smoking, and not taking anticoagulants and non-steroidal anti-inflammatory drugs (NSAIDs) in the past two weeks. The procedure for taking blood met the ethical clearance of the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Universitas Gadjah Mada (number Ref: KE/FK/622/EC/2015).

Platelet preparation

The human blood was added with 3.8% sodium citrate in a ratio of 1:9. Whole blood obtained was centrifuged at 1000 rpm for 15 minutes to obtain platelet-rich plasma (PRP, the supernatant). The lower phase was centrifuged again at 3500 rpm for 15 min to obtain platelet-poor plasma (PPP, the second supernatant). Only blood with the number of platelet more than $2x10^5$ platelets/µL was used for the experiment.

Antiplatelet assay

The antiplatelet experiments were performed according to previous method.[16] Shortly, PRP (487.5 µL) in a glass tube was added with 2.5 µL test samples (in DMSO) at different concentrations and then incubated at 37°C for 3 minutes. Platelet agonist solution (10 µL of ADP, epinephrine, thrombin, or arachidonic acid) was added to the mixture and the platelet aggregation was measured for 8 minutes in an aggregometer (Chronolog 490). Aspirin was used as the standard antiplatelet drug to compare the activity of the extracts. The platelet aggregation baseline value was set according to aggregation of PPP, and the percentage of platelet aggregation was relative to the solvent (DMSO). The IC_{50} was calculated based on the dose-response curve obtained from nonlinear regression equation (percent of inhibition vs log of concentration. The data was processed and analyzed using Graphpad Prism 8.0.1 software.

The determination of the levels of cubebin by densitometry method

Cubebin concentration series was prepared with the range of 0.1-1.0 mg/mL. *P. cubeba* fruit ethanol extract (30.0 mg) was dissolved in ethanol (10.0 mL), and 10 µL of the solution was dripped into TLC plate (silica gel F254) using the mobile phase of dichlormetane:etyl acetate (8:2). The spot of cubebin was quantified using TLC-densitometer (Camac) equipped with WinCAT Software. The cubebin content in the extract was calculated based on the linear regression equation obtained from the standard curve. The analysis was done at a quadruplet.

Statistical analysis

The data were analyzed in SPSS version 23 software by using One-Way Anova followed by Tukey post hoc test. The data were presented as mean ± SD from three independent experiments.

Results and Discussion

We found that *P. cubeba* fruit ethanol extract (PCE) inhibited platelet aggregation induced by ADP, arachidonic acid, epinephrine, and thrombin with the IC_{50} of 813.30; 895.90; 883.40; and 1090.00 µg/mL, respectively (Fig. 1 A, B, C, D). Fig. 1 showed that the strongest antiplatelet activity of PCE was in the platelet aggregation induced by ADP (agonist of P2Y1 platelet receptor). Thus, further study focusing on the mechanism action on this pathway of platelet aggregation is a promising research. However, the activity of the extract is still lower compared to the reference antiplatelet drug, aspirin. Aspirin provides inhibition of platelet aggregation induced by ADP, arachidonic acid, and epinephrine with the IC_{50} of 2.00; 3.34; and 1.17 µg/mL, respectively (Fig. 2). The highest antiplatelet activity of Aspirin was found in platelet aggregation induced by epinephrine. Interestingly, Aspirin was unable to inhibit platelet aggregation in platelets induced by thrombin. This suggested that Aspirin might interfere platelet aggregation through PAR1 and PAR4 receptors. Previous study showed that platelet aggregation induced by thrombin involving PAR1 and PAR4 receptors, and aspirin failed to inhibit the platelet aggregation.[29] Aspirin irreversibly inhibits the COX-1 enzyme by acetylating a serine residue at position 530 and induces long-term functional platelet damage, thereby inhibiting platelet aggregation by interfering the biosynthesis of TXA2, prostacyclin, and other prostaglandins.[30,31] In this study, we found that *P. cubeba* fruit ethanol extract inhibited platelet aggregation through several pathways (Figure 2 A, B, C, D). The extract was able to inhibit ADP-induced platelet aggregation presumably because the extract inhibited P2Y1 and P2Y12 receptors. These receptors are targets of antiplatelet drugs, such as Clopidogrel, Ticlopidine, and Ticagrelor.[5]

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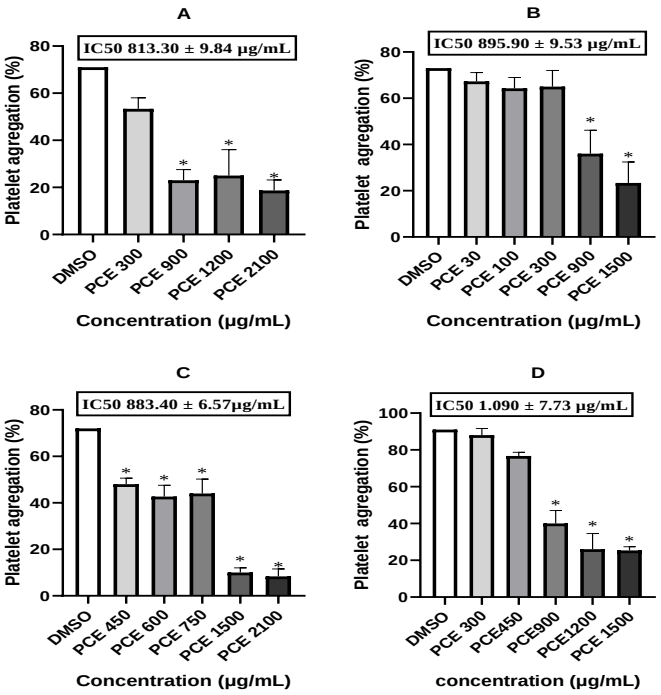

Figure 1: Antiplatelet activities of *Piper cubeba* extract (PCE) in platelet induced by ADP (A), arachidonic acid (B) epinephrine (C), and thrombin (D). The data are mean ± SD (One Way Anova followed by Tukey post hoc test; $p < 0.05$; n=3; * indicates a significant difference compared to the DMSO group (solvent).

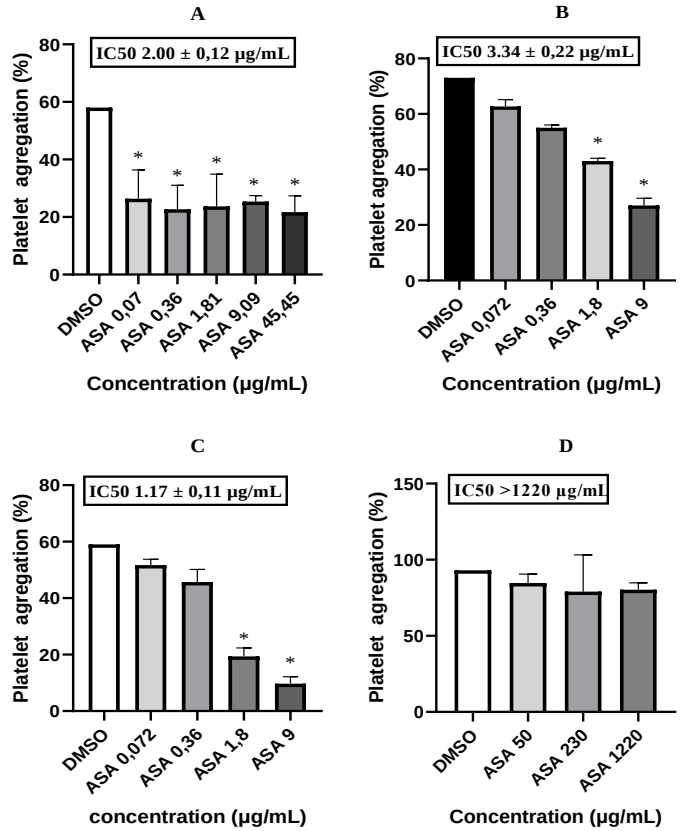

Figure 2: Antiplatelet activities of Aspirin (ASA), the reference antiplatelet drug in platelet induced by induced by ADP (A), arachidonic acid (B) epinephrine (C), and thrombin (D). The data are mean ± SD (One Way Anova followed by Tukey post hoc test; $p < 0.05$; n=3; * indicates a significant difference compared to the DMSO group (solvent).

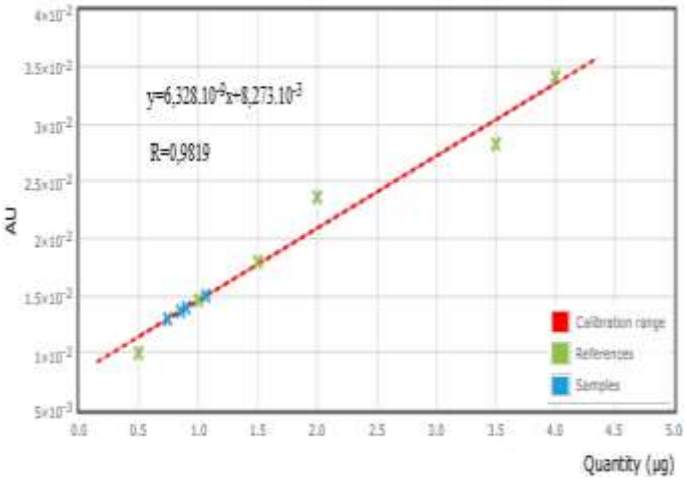

Figure 3: The linear regression curve of Cubebin used for the determination of Cubebin content in the *Piper cubeba* fruit extract by TLC densitometry method at wavelength of 288 nm.

The ability of the extract to inhibit arachidonic acid-induced platelet aggregation may be due to the inhibition of COX-1 enzymatic activity, thereby preventing the synthesis of arachidonic acid into TXA2.
Another possibility is that the extract was able to inhibit the interaction of TXA2 with its receptor. Epinephrine is endogenous ligand that induces platelet aggregation through interaction with adrenergic receptors. Epinephrine interaction with A2A adenosine receptor (A2AR) induces a decrease in adenylate cyclase activity.[32] Thus, the *P. cubeba* fruit ethanol extract might inhibit platelet aggregation by targeting A2AR. As the extract also inhibited platelet aggregation induced by thrombin, it might interact with PAR1 and PAR4 receptors, thus inhibits platelet aggregation induced by thrombin. Cubebin is the major compound found in the fruit of *P. cubeba*. There is no evidence that Cubebin is antiplatelet agent. Further experiment will be focused in this regard. However, several lignan compounds from the plant, such as lignans from Saururus chinensis,[33] Justicia spesies,[34,35] *Schisandra chinensis*[36] demonstrated antiplatelet activity. For standardization and quality control purpososes, we determined the levels of cubebin in the *P. cubeba* fruit ethanol extract by using densitometry method. We found that the extract contained 2.97% cubebin (Figure 3). Our finding was in line with previous study showing that cubeb fruit extract contains 3% cubebin.[37]

Conclusion

P. cubeba fruit ethanol extract inhibited platelet aggregation induced by ADP, epinephrine, arachidonic acid, and thrombin with the IC_{50} of 813.30; 883.40; 895.90; and 1.090 µg/mL respectively. This finding indicated that *P. cubeba* fruit ethanol extract showed moderate antiplatelet activity in several pathways of platelet aggregation. Further research is needed to determine the active compound in the extract. Additionally, a thorough investigation on its mechanism of action as antiplatelet agent is also a promising study.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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