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Original Research Article

Identification of Cubebin from Cubeb (*Piper cubeba*) as An Antiplatelet Agent

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ABSTRACT

Platelets play a key role in arterial thrombosis. Platelet aggregation is part of a sequential mechanism leading to the formation of a thrombus. Antiplatelet agent is needed for the inhibition of platelet aggregation. *Piper cubeba* or Cubeb is an Indonesian traditional herbal medicine. Cubeb fruit is rich in lignans, such as cubebin, hinokinin, clusin, dihydrocubebin, dihydroclusin, cubebinin, yatein, cubbinulide (cordigerine), dihydroyatein, isoyatein, and cubebinone. Previous studies have indicated that several lignans exert antiplatelet activity. Additionally, the extract of *Piper cubeba* fruit exhibits antiplatelet activity. This study aimed to evaluate the antiplatelet activities of *P. cubeba* fruit fractions and identify active compound. The fruit was macerated in ethanol for 5 days. The extract was fractionated by vacuum liquid chromatography (using dichloromethane with ethylacetate added in a polar gradient. The main compound was isolated and identified based on its infrared, H-nuclear magnetic resonance, (H-NMR), C-NMR, and mass spectra. The antiplatelet activity was determined by an *in vitro* antiplatelet aggregation assay employing human platelets induced with several platelet receptor agonists Adenosine Diphosphate (ADP), epinephrine, thrombin, and arachidonic acid). The ethanol extract of *P. cubeba* fruit and its fractions demonstrated antiplatelet activity induced by epinephrine, ADP, thrombin, arachidonic acid and ristocetin. The major compound in the active fraction was identified as cubebin. Cubebin demonstrated antiplatelet activity in platelet aggregation induced by thrombin with the IC_{50} of 14.62±1.16 µM. Cubebin is a novel antiplatelet agent isolated from *P. cubeba*.

Keywords: *Piper cubeba*, medicinal plant, cardiovascular diseases, platelet aggregation .

Introduction

Platelets play a key role in arterial thrombosis.[1] Platelet aggregation is part of a sequential mechanism leading to the formation of a thrombus (blood clot), which can be induced by various agonists known to activate aggregation via different biochemical pathways.[2,3] Platelets play an important role in cardiovascular disease, both in the pathogenesis of atherosclerosis and in the development of acute thrombotic events.[4,5] Most platelet agonists, such as thrombin, adenosine diphosphate (ADP), thromboxane A2 (TXA2), and epinephrine, stimulate G-protein coupled receptor-type surface receptors, while others, such as collagen and von Willebrand factor, stimulate immunoglobulin-like receptors.[1,6,7] Thrombin does not activate cyclooxygenase-1 (COX-1) to induce platelet aggregation but rather activates Protease Activated Receptors 1 (PAR1) and Protease Activated Receptors 4 *(*PAR4) receptors, so aspirin is unable to inhibit aggregation initiated by thrombin.[8] Arachidonic acid is converted to tromboxane A2 (TXA2), which binds to the thromboxane receptor on platelets, causing aggregation. Inhibiting COX-1 prevents the formation of TXA2 from arachidonic acid.[9] Ristocetin induces the binding of von Willebrand Factor (VWF) to the Glycoprotein Ib (GpIb) complex by altering the electrostatic forces between GpIb and VWF in the microcirculation.[10]

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Antiplatelet therapy is a method that helps reduce the ability of platelets to clot during primary hemostasis. Various antiplatelet drugs reversibly or irreversibly inhibit platelet activation, which reduces the tendency of platelets to stick together and damage the vascular endothelium.[11,12] The main classes of antiplatelet drugs are cyclooxygenase inhibitors (aspirin), *Purinergic receptor type Y subtype 12 (*P2Y12) receptor antagonist thienopyridine derivatives (thienopyridine derivatives), phosphodiesterase inhibitors (cilostazol and dipyridamole), glycoprotein IIb/IIIa receptor blockers (abciximab), and protease-activated receptor 1 inhibitors (vorapaxar).[13] Many efforts have been devoted to discovering novel therapeutic strategies, but more novel antiplatelet agents are needed.[14,15,16]

Natural products are an excellent great source of new medicines to discover highly potent and safe antiplatelet drugs.[17,18] The use of plants as traditional medicine has been practiced by people worldwide for centuries.[19] The World Health Organization stated that traditional and alternative medicines have a very important role in supporting human health.[20] The WHO conducted a comprehensive study on the use of plants as traditional medicine around the world and has declared the "Traditional Medicine Strategy 2014-2023" policy to ensure that traditional medicine products meet the quality, safety, efficacy, and availability aspects according to scientific principles and guidelines. Indonesia has huge plant biodiversity, including medicinal plants, which increases the chance of discovering new drugs or bioactive compounds from natural sources, particularly medicinal plants.

In the previous study, we showed that that *Piper cubeba* (cubeb) fruit ethanol extract demonstrated antiplatelet activities.[21] The cubeb fruit contains several lignan compounds, such as cubebin, hinokinin, clusin, dihydrocubebin, dihydroclusin, cubebinin, yatein, cubbinulide (cordigerine), dihydroyatein, isoyatein, and cubebinone.[22,23,24] No study is available regarding the antiplatelet activity of the compounds found in cubeb. The genus *Piper* is comprised of multiple species each of which contains similar secondary metabolites. This similarity in chemical constituents leads to similarities in the pharmacological

activity among species. Several studies have demonstrated the antiplatelet activity of secondary metabolites commonly found in *Piper spp*. Piperine, pipernonaline, piperoctadecalidine, and piperlongumine from *Piper longum* L. inhibit platelet aggregation.[25] *Piper philippinum* contains (-)-3',4'-O,O-demethylenehinokinin and 3,4-methylenedioxy cinnamaldehyde, which have antiplatelet activity.[26] *Piper tuberculatum* contains piplartine, which inhibits platelet aggregation induced by collagen, arachidonic acid, and ADP.[27] One study showed that piperine isolated from *P nigrum* and *P. longum* has antiplatelet activity by a different mode of action. The mode involved inhibiting the release of arachidonic acid and inhibiting TXA2 synthase activity without affecting COX-1. These inhibitory effects interfere with the platelet aggregation pathway.[28] These studies indicate that plants in the genus *Piper* are a potential source of antiplatelet agents. This study aimed to evaluate the antiplatelet activity of *P. cubeba* fractions and identify the active compound responsible for the antiplatelet activity.

Materials and Methods

Plant material and chemicals

P. cubeba fruits were collected from Tegal Sari, Giri Purwo, Kulonprogo District, Yogyakarta Province, Indonesia (GPS location -7.724351792488525, 110.13547666534014). The plant was authenticated on September 2021 with voucher specimen number 16.20.9/UNI/FFA/BF/PT/2021. Aspirin (Sigma, St. Louis, MO, USA), human thrombin (Sigma, cat. no. T6884-250UN), epinephrine (PT. Phapros Tbk.), adenosine 5' diphosphate monosodium salt (Calbiochem, San Diego, CA, USA), arachidonic acid (porcine liver, sodium salt) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), FR-171113 (Sigma SML0028), ethanol (Merck, Kenilworth, NJ, USA), sodium citrate (Merck), Whatman paper no. 1, DMSO (Merck), ethyl acetate (Merck), dichloromethane (Merck), NaCl (Merck), bovine serum albumin (Merck), phosphate-buffered saline (PBS), F_{254} TLC silica plates (Merck), and 50% sulfuric acid spray reagent.

Extraction

The *P. cubeba* fruit powde (1 kg) was macerated with ethanol (5 L) for 5 days. The solvent was evaporated using a vacuum rotatory evaporator (Heidolph) and allowed at room temperature in until dryness (128.2 g).

Fractionation by vacuum liquid chromatography (VLC)

Five grams of ethanol extract was fractionated using gradient elution with dichloromethane solvent (100%), dichloromethane: ethyl acetate (9:1 v/v), dichloromethane: ethyl acetate (8/2 v/v), dichloromethane: ethylacetate (6:4 v/v), dichloromethane: ethyl acetate (4:6 v/v), dichloromethane: ethyl acetate (2:8 v/v), and ethyl acetate (100%). The fractions were analyzed by thin-layer chromatography (TLC), and each fraction was tested for antiplatelet activity using an aggregometer.

Antiplatelet activity test

Solution preparation

Several stock solutions were used, including 200 µg/mL of the cubeb fruit fraction (PCF 1–8) in DMSO, 50 g/mL epinephrine in 0.9% NaCl, 500 M ADP in dimethyl sulfoxide (DMSO), 60 mg/mL arachidonic acid in distilled water, 50 µg/µL ristocetin in 0.9% NaCl, 49.91 IU/mL thrombin in PBS buffer, pH 6.5, and 1.6 M FR171113 in DMSO.

Blood sample

To obtain platelets for the experiment, blood was taken from non-smoking human donors (age 18–45 years) who were not pregnant, and had not taken anticoagulants or non-steroidal anti-inflammatory drugs within the last 2 weeks. The procedure for collecting the blood was reviewed by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Universitas Gadjah Mada (registration number Ref: KE/FK/0804/EC/2018). The blood was supplemented with 3.8% sodium citrate at a 1:9 ratio and then centrifuged at 1,000 rpm (Gemmy PLC-03 plc 03) for 15 min to obtain the platelet-rich plasma (PRP) in the supernatant. The residue (lower phase) was centrifuged again at 3,500 rpm for 15 min to obtain the platelet-poor plasma (PPP). Only PRP with > 2×10^5 platelets/µL was used in the experiment.

Measurement of platelet aggregation

The measurement of platelet aggregation was done according to previous method.[21] Briefly, 487.5 µL aliquot of the PRP was added to 2.5 µL of the test sample and incubated in an aggregometer (Chronolog 490 model Aggregometer, Chrono-Log Corp) at 37°C for 3 min. Then, 10 µL of platelet receptor agonist solution was added to the mixture to obtain the final concentration of the platelet receptor antagonist of 5.45 µM epinephrine, 10 µM ADP, 2.42 µg/µL arachidonic acid, 1 µg/µL ristocetin, 0.10 IU/mL thrombin, and FR 171113 at concentration of 0.5; 1.0; 2.0; 4.0; and 8.0 µM. Aggregation was measured for 10 min using an aggregometer. The PPP was used as the baseline, so the percentage of aggregation in each treatment was compared with the solvent (2.5 L DMSO).

Column chromatography

The active fraction obtained from VLC was further separated by column chromatography. The stationary phase was F254 silica gel, and the mobile phase was dichloromethane: ethyl acetate using a polar gradient. TLC was performed to monitor the chemical profile of each fraction. Fractions with the same TLC profile were combined, and the solvent was removed with a vacuum evaporator and separated. Then, the compounds were purified by preparative TLC.

Preparative TLC

Preparative TLC was used to isolate the main compound from the most active fraction obtained from column chromatography. Dichloromethane: ethyl acetate 8:2 (v/v) and F254 silica gel (0.5 mm thick on a glass plate) were used as the mobile and stationary phases, respectively. The purity of the isolated compound was analyzed by TLC and high-performance liquid chromatography (HPLC).

Identification of the compounds

The chemical structure of the main compound from the most active fraction was identified by analyzing the infrared (Cary 630 FTIR Agilent Technologies), H-nuclear magnetic resonance (H-NMR) (JNM-ECZ500R, 500 MHz Super Conductive Magnets), C-NMR (JNM-ECZ500R, 500 MHz Super Conductive Magnets), and mass spectra (Thermo Scientific™ Q Exative™ Hybrid Quadrapole-Orbitrap Mass Spectrometer).

Statistical analysis

The antiplatelet activity was determined by comparing the aggregation value (reflected as amplitude in the aggregometer) of the tested samples with the solvent group. The data were analyzed in SPSS version 23 software (One-way Anova followed by Tukey post hoc test). The data were presented as mean ± SD from three independent experiments. GraphPad Prism version 8 software was used to determine the IC_{50}.

Results and Discussion

The *P. cubeba* fruit was extracted using a 1:5 ethanol ratio to obtain a 12.82% extraction yield (ratio of extract to dried plant material). Furthermore, 5 grams of the extract was separated by VLC. Silica gel was used as the stationary phase and dichloromethane and ethyl acetate in a gradient (dichloromethane: ethyl acetate (9:1 v/v), dichloromethane: ethyl acetate (8/2 v/v), dichloromethane: ethyl acetate (6:4 v/v), dichloromethane: ethyl acetate (4:6 v/v), dichloromethane: ethyl acetate (2:8 v/v), and ethyl acetate) were used as the mobile phase. This fractionation resulted in 8 fractions (Figure. 1).

The chemical profile of the fractions obtained from VLC was analyzed by TLC, and the fractions were tested for antiplatelet activity using 4 different inducers (platelet agonists), such as ADP, epinephrine, thrombin, and arachidonic acid. The antiplatelet activities of PCF 1–8 are shown in Figure 2. This assay was performed to determine the antiplatelet mechanism of action of the fractions. Figure 2 shows that fraction 3 (PCF 3) demonstrated the strongest antiplatelet activity of platelets induced by thrombin and epinephrine with inhibitory rates of 29.61% and 74.35%, respectively (tested at 200 µg/mL). PCF 3, which was platelet aggregation induced by ADP and arachidonic acid, exhibited weak antiplatelet activity (13.33% and 10.42%, respectively)

at 200 µg/mL. The other fractions failed to inhibit platelet aggregation with the four different inducers.

Fraction 3 had the greatest inhibitory effect on platelet aggregation induced by epinephrine and thrombin. Fraction 8, induced with ADP, revealed the strongest inhibitory effect, whereas fractions 1 and 7 showed the strongest inhibitory effect on platelet aggregation induced by arachidonic acid and ristocetin, respectively. As fraction 3 had the greatest percent inhibition of platelet agonists, fraction 3 was selected for further separation using column chromatography with gradient elution of dichloromethane: ethyl acetate as the mobile phase and silica gel as the stationary phase. The main compound from the most active fraction was isolated by preparative TLC using dichloromethane: ethyl acetate (8:2 v/v) as the mobile phase on a 0.5 mm thick silica gel plate. The purity of the isolated compound was analyzed by TLC (Figure 3) and HPLC (Figure 4). The isolated main compound was identified as cubebin based on the IR, H-NMR, C-NMR, and mass spectra. The ^{1}H and ^{13}C NMR spectroscopic data of the isolated compound was summarized in Table 1 and compared with previous publication.[29]

Cubebin ESI-MS 357 m/z (calc. for $C_{20}H_{20}O_6$); IR vmax in cm^{-1}: 3336 (OH), 2992 (C=C). The complete spectra data were presented in Supplementary Figure 1-4.

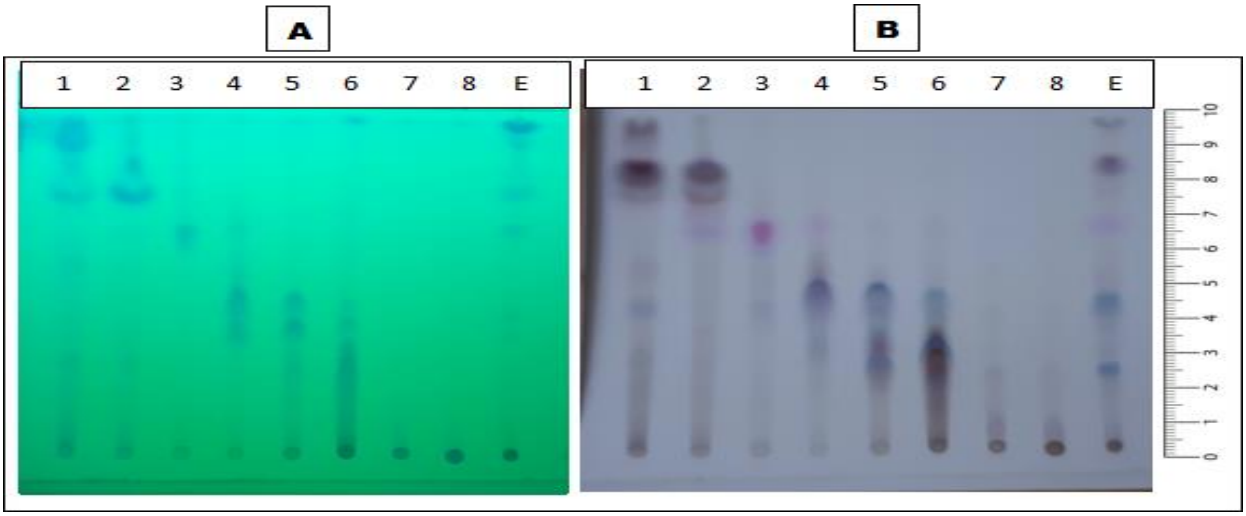

Figure 1: TLC profile of Piper cubeba fraction (PCF 1-8) at UV 254 (A) and visualization with H_2SO_4 50% (B), dichloromethane-ethyl acetate (8:2) as mobile phase. The numbers in the upper lane indicated the fractions (1-7) obtained from the column chromatography, and ethanolic extract of P. cubeba (E).

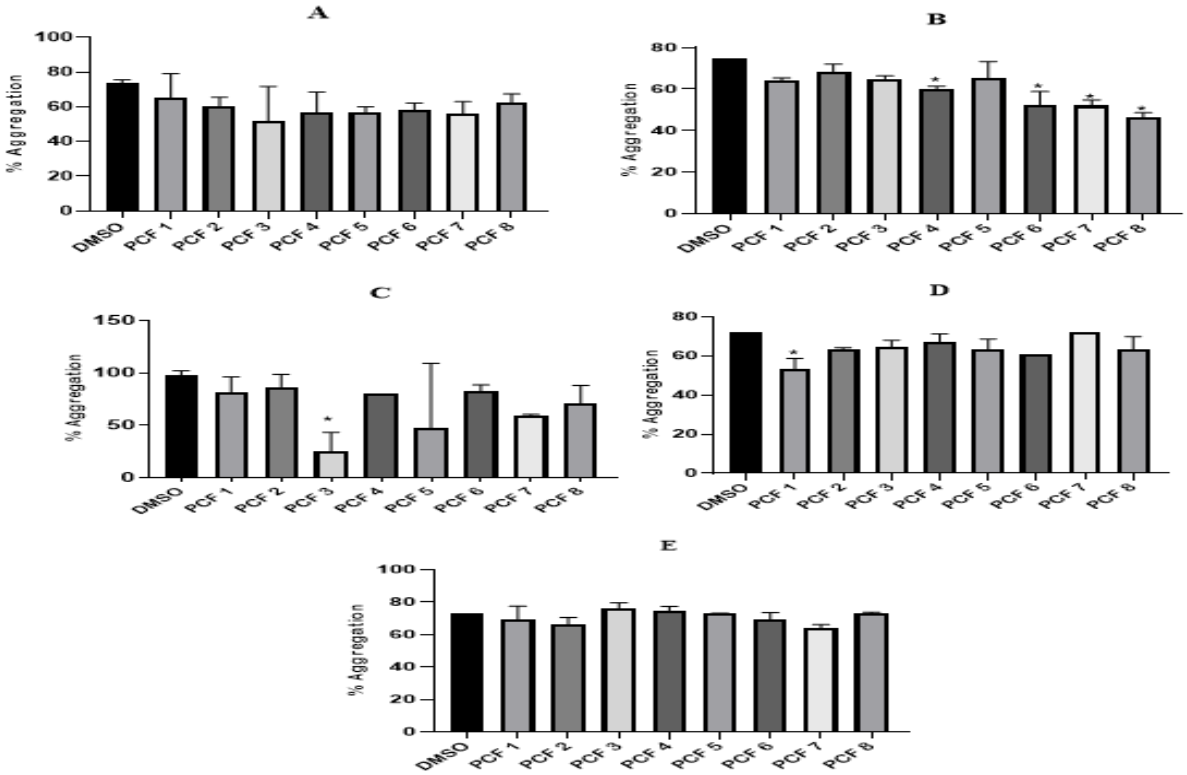

Figure 2: Antiplatelet activity of the fractions. The Piper cubeba fractions (PCF 1–8) were tested at 200 µg/mL. Platelet aggregation was induced with epinephrine (A), ADP (B), thrombin (C), and arachidonic acid (D), and ristocetin (E). ADP (A), arachidonic acid (B) epinephrine (C), and thrombin (D). 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).

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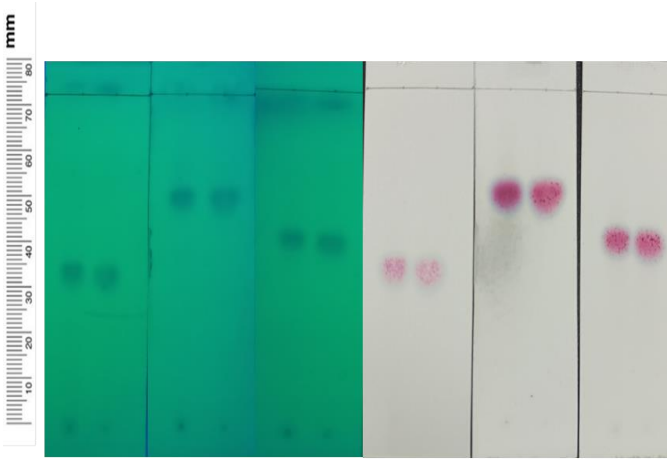

Figure 3: TLC profile of the isolate from P. cubeba at UV 254 (A) and after derivatization using 50% H_2SO_4 reagent (B). The stationary phase was F254 silica gel and the mobile phases were (1) dichloromethane: ethyl acetate (6:1); (2) chloroform: ethyl acetate (10:3); (3) dichloromethane: ethyl acetate (9:2)

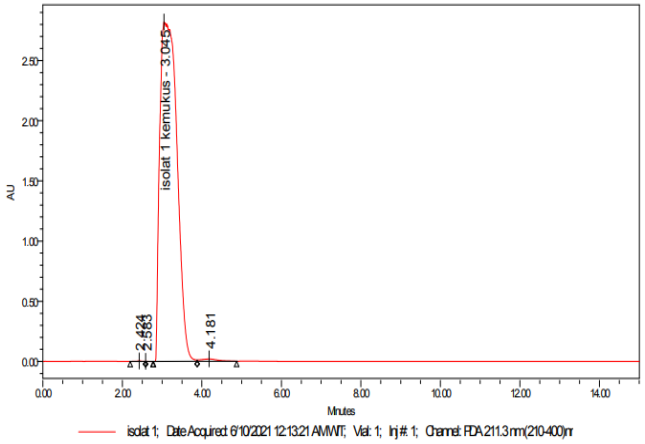

Figure 4: Purity of the isolated compound analyzed by HPLC (PDA detector)

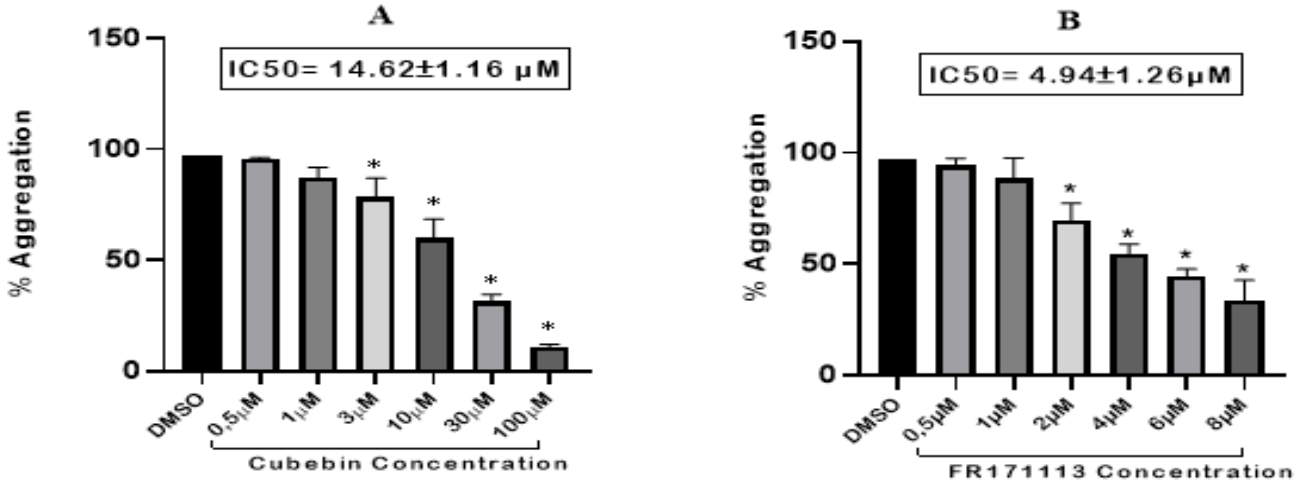

Figure 5: Antiplatelet activity of cubebin from P. cubeba compare to FR171113 as reference. Platelet aggregation was induced with thrombin 1IU/mL. The data are mean ± SD (One Way Anova followed by Tukey post hoc test); $p < 0.05$; n=3; * indicates a significant difference compared to the DMSO group (solvent).

1H-NMR (500 MHz, MeOD): 6.53 (1H, d, J = 1.32 Hz, H-2), 6.68 (1H, d, J = 7.9 Hz, H-5), 6.50 (1H, d, J = 1.85 Hz, H-6),2.51 (2H, dd, J = 4.9 Hz, J = 7.65 Hz, H-7), 1.94 (1H, m, H-8), 5.07 (1H, d, J = 2.45 Hz, H-9), 6.53 (1H, d, J = 1.8, H-2'), 6.66 (1H, d, J = 8.0 Hz, H-5'), 6.50 (1H, d, J = 1.85 Hz, H-6'), 2.38 (2H, m, H-7'), 1.94 (1H, m, H-8'), 3.68 (1H, t, J = 8.0 Hz, H-9'), 3.49 (1H, t, J = 7.6 Hz, H-9), 5.86 (2H, m, OCH2O), 5.86 (2H, m, OCH_2O).

13C-NMR (500 MHz, MeOD):134.02 (C1), 109.00 (C-2), 147.08 (C-3), 145.09 (C-4),108.06 (C-5), 121.06 (C-6), 38.00 (C-7), 53.02 (C-8), 103.02 (C-9), 134.08 (C-1'), 108.09 (C-2'), 147.07 (C-3'), 145.08 (C-4') '), 108.04 (C-5'), 121.05 (C-6'), 38.07 (C-7'), 45.08 (C-8'), 71.04 (C-9'), 100.08 (OCH2O), 100.08 (OCH_2O). The NMR spectra were compared with those from a previous study[29] (Table 1).

The mass spectroscopy data showed that the molecular weight of the isolated compound was 356 (detected at 357 as M+H). With a molecular weight of 356, the molecular formula was predicted to be $C_{20}H_{20}O_6$. The compound had an index of hydrogen deficiency (IHD) value of 11, indicating that the structure of the compound consisted of six sp2 bonds and five rings. The spectra of the isolated compound were similar to the spectra of cubebin, a major compound in *P. cubeba* fruit. These data indicate that the isolated antiplatelet compound was cubebin. In line with previous studies, cubebin represented the major compound in the *P. cubeba* fruit extract.[30,31] In this study, we found that cubebin demonstrated antiplatelet activity by inhibiting platelet aggregation induced by thrombin with IC_{50} of 14.62±1.16 µM (Figure 5). However, the activity of cubebin is still lower than FR171113, a potent synthetic agent antagonist of PAR 1 (IC_{50}: 4.94±1.26 µM). This suggested that cubebin might interact with Protease Activated Reseptor 1 (PAR 1) responsible for platelet aggregation induced by thrombin. Further study employing receptor binding assay is required to confirm this interaction. Previous studies found that cubebin demonstrated antibacterial,[30] antifungal,[32] anti-inflammatory,[33] and anticancer activities.[34] In this study, for the first time, we identified cubebin, a lignan isolated from *P. cubeba* fruit as antiplatelet agent. As *P. cubeba* fruit contain several lignans,[22] further investigation on the antiplatelet potential of these lignans are challenging. This study indicated that *P. cubeba* is a promising plant for the development of antiplatelet agents from natural products.

Conclusion

In this study, we found that the fractions of the ethanol extract of *P. cubeba* fruit demonstrated antiplatelet activities in different pathways of platelet aggregation induced by epinephrine, ADP, thrombin, arachidonic acid or ristocetin. The active major compound identified in the fraction was cubebin. Cubebin demonstrated antiplatelet activity with the IC_{50} of 14.62±1.16 µM. This finding contribute to the

development of antiplatelet agents derived from natural products for combating cardiovascular diseases.

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.

Acknowledgments

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Table 1: ^{1}H and ^{13}C NMR spectroscopic data of the isolated compound in MeOD

No. of C	C-NMR δ_C (ppm)		H-NMR δ_H (*mult.*, *J* in Hz, ΣH)	
	Isolat P.Cubeba	**Cubebin***	**Isolat P. Cubeba**	Cubebin*[29]
1	134.02.00	134.03.00	-	-
2	109.00.00	109.05.00	6.53 (*d*, *J* = 1.32 Hz, 1H)	6.55 (*d*, *J* = 1.6 Hz, 1H)
3	147.08.00	147.05.00	-	-
4	145.09.00	145.07.00	-	-
5	108.06.00	108.04.00	6.68 (*d*, *J* = 7.9 Hz, 1H)	6.66 (*d*, *J* = 7.6 Hz, 1H)
6	121.06.00	122.01.00	6.50 (*d*, *J* = 1.85 Hz, 1H)	6.49 (*dd*, *J* = 7.9 Hz, *J* = 1.7 Hz, 1H)
7	38.00.00	38.00.00	2.51 (*dd*, *J* = 4.9 Hz, *J* = 7.65 Hz, 2H)	2.46 (*dd*, *J* = 13.8 Hz, *J* = 7.9 Hz, 1H) 2.30 (*dd*, *J* = 13.6 Hz, *J* = 7.5 Hz, 1H)
8	53.02.00	53.04.00	1.94 (*m*, 1H)	1.83 (*m*, 1H)
9	103.02.00	102.09.00	5.07 (*d*, *J* = 2.45 Hz, 1H)	4.90 (*d*, *J* = 2.9 Hz, 1H)
1′	134.08.00	134.08.00	-	-
2′	108.09.00	109.02.00	6.53 (*d*, *J* = 1.8, 1H)	6.51 (*d*, *J* = 1.7 Hz, 1H)
3′	147.07.00	147.06.00	-	-
4′	145.08.00	145.07.00	-	-
5′	108.04.00	108.03.00	6.66 (*d*, *J* = 8.0 Hz, 1H)	6.65 (*d*, *J* = 7.8 Hz, 1H)
6′	121.05.00	121.07.00	6.50 (*d*, *J* = 1.85 Hz, 1H)	6.44 (*dd*, *J* = 7.9 Hz, *J* = 1.6 Hz, 1H)
7′	38.07.00	38.07.00	2.38 (*m*, 2H)	2.39 (*m*, 2H)
8′	45.08.00	45.09.00	1.94 (*m*, 1H)	1.94 (*m*, 1H)
9′	71.04.00	71.00.00	3.68 (*t*, *J* = 8.0 Hz, 1H) 3.49 (*t*, *J* = 7.6 Hz, 1H)	3.76 (*dd*, *J* = 8.3 Hz, J = 7.6 Hz, 1H) 3.47 (*t*, *J* = 8.2 Hz, 1H)
OCH_2O	100.08.00	101.00.00	5.86 (*m*, 2H)	5.83 (*m*, 2H)
OCH_2O	100.08.00	101.00.00	5.86 (*m*, 2H)	5.83 (*m*, 2H)

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