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Phytochemicalassay and Antiplatelet Activity of Fractions of Velvet Bean Seeds (*Mucuna pruriens* L.)

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Platelet aggregation is an important factor contributing to the formation of thrombus due to an uncontrolled blood clotting. An antiplatelet agent is a compound which decreases platelet aggregation and inhibits thrombus formation. The objectives of this study were to determine the class of compound employing phytochemical assay and to determine the *in vitro* antiplatelet activity of four fraction, namely hexane, ethyl acetate, butanol, and water fractions of velvet bean seeds (*Mucuna pruriens* L.) using epinephrine (EPN) as agonist of platelet aggregation. The antiplatelet activities were tested in human platelet rich plasma with hyperaggregation. To determine the activities, EPN was arranged at 4 level of concentrations (300, 150, 75, and 30 ì M), and antiplatelet agents were at 500 µg/ml. The results indicated that ethyl acetate, butanol and water fraction contained high flavonoids and moderate phenols. The water, butanol and ethyl acetate fractions of velvet bean seeds exhibited potential inhibition of EPN-induced platelet aggregation at all concentrations. The strongest antiplatelet agent was water fraction and had the same antiplatelet activity as aspirin at level 150, 75, and 30 ì M of EPN. Butanol fraction had the same antiplatelet activity as aspirin at the lowest EPN (30 ì M).

Key words: platelet, *Mucuna pruriens* L., epinephrine, flavonoid, aspirin

INTRODUCTION

Hyperaggregatebility is one of the risk factors in pathogenesis of thrombosis and atherosclerosis, the most common cause of premature death in developed world as many as 30% of all deaths (Olas & Wachowicz 2005). Platelets play a crucial role not only in homeostasis but also in the development of cardiovascular disease (CVD). The increasing acute clinical manifestations of coronary atherosclerotic disease are caused by plaque disruption and subsequent platelet-thrombus formation (O'Kennedy *et al*. 2006). Inhibiting the platelet aggregation is one of the efforts for preventing cardiovascular disease. Atherosclerosis is the pathological consequence from the modification of oxidized LDL in arterial wall which can be stimulated by free radicals. Preventing the oxidation of LDL has a meaning to prevent coronary heart disease. Giving antioxidant supplement both vitamin E and flavonoid (vegetable, fruit, tea) to CVD patients increase the defense towards the attack of free radicals (Olas & Wachowicz 2005).

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It has been found that platelet inhibitory drugs, such as aspirin, physiologically reduce the incidence of myocardial infarction, stroke, and death from cardiovascular diseases in prevention trials. A number of prospective randomized clinical trials have shown that 50-500 mg of aspirin per day reduces the risk of primary or secondary cardiovascular events (Olas & Wachowicz 2005). The usage of aspirin has negative side effects: low dose aspirin increases the risk of major bleeding and intestinal ulceration, and for a prolong therapy, it usually causes many side effect (Schror 1997; Ohmori *et al*. 2006). The aspirin resistance is a clinical inability of aspirin to protect individuals from arterial thrombotic events or when laboratory methods indicate the failure of aspirin to inhibit platelet activity (Cattaneo 2004; Ohmori *et al*. 2006). It is estimated that between 8-45% of patients suffering an ischemic stroke or cardiovascular disease are aspirin resistant (McKee *et al*. 2002; Ohmori *et al*. 2006). It is an urgent need to search natural compounds having antiaggregation properties with minimal side effects and safer. One of the natural compounds with antiplatelet activity is flavonoid. It can prevent atherosclerosis, endotelial damage, leukocyte activation, adhesive

aggregation, and platelet secretion (Koshy *et al*. 2001; Bucki *et al*. 2003). Natural antioxidants having minimal side effects can be obtained from plants containing phenolic and polyphenolic compounds, such as flavonoids (Bucki *et al*. 2003; Koleckar *et al*. 2008). Flavonoids bioavailability and its relationship with antioxidant and antiplatelet activity, resulting in cardiovascular protection (Violi 2002).

The natural compounds including flavonoids can be obtained ubiquitously in Indonesia, such as in velvet bean *Mucuna pruriens* L., and it has not been used optimally. Previous research demonstrated the total polyphenolic content of the methanol extract of *M. pruriens* was 33.04 mg/g gallic acid, a noticeable amount of total phenols (Rajeshwar *et al*. 2005). *M. pruriens* contains isoflavones such as daidzin 0.041; glycitein 0.011; genistein 0.050; aglucones total 0.131 mg/l (Handajani 2001). Methanol extract of *M. pruriens* (MEMP) has high antioxidant activity (Rajeshwar *et al*. 2005).

MATERIALS AND METHODS

The four fractions (hexane, ethyl acetate, n-butanol, and water fraction) were obtained from ethanol extract of velvet bean seeds (*M. pruriens* L.).

The four fractions of velvet bean seeds were tested by phytochemical assay including flavonoid assay, phenolic, saponin, triterpenoid, steroid, terpenoid, tanin, and alkaloid assay. The four fractions and aspirin as positive control were diluted with dimethyl sulfoxide (DMSO 1%) achieving at level 500 ìg/ml. Epinephrine (EPN) was diluted with distilled water at various concentrations of 300, 150, 75, 30 ì M. Platelet rich plasma (PRP) obtained from hyperaggregation individual (Helena Laboratories 2008).

9 ml blood was collected from hyperaggregation individual and added with 1 ml 3.8% sodium citrate as anticoagulant. The blood was centrifuged at 100 x g for 10 minutes. The platelet rich plasma (PRP) was removed from the cells with a plastic pippete and placed in a plastic tube. The PRP was maintained at room temperature for 30 minutes. Platelet poor plasma (PPP) was prepared by recentrifuging the remaining blood samples at 1600 x g for 10 minutes. PPP was then removed, placed in a plastic tube, and the tube was maintained at room temperature (Chun-Han *et al*. 1993; Helena Laboratories 2008).

Aggregation activity was measured by Platelet Aggregation Chromogenic Kinetic System (PACKS-4). 450 ìl of PPP was pippeted into a cuvette. 450 ìl PRP and 40 ìl the antiplatelet agents (aspirin, hexan fraction, ethyl acetate fraction, butanol fraction, water fraction) were also pippeted into cuvettes with stir bar and incubated at 37 ^{o}C for 3-5 minutes. The PPP and PRP cuvettes were inserted into appropriate channels and the instrument was set up. 50 ìl of the aggregating reagent dilutions (Epinephrine) at level 300, 150, 75, 30 ì M was added to the PRP cuvette and the percent aggregation was recorded (Helena Laboratories 2008).

All treatments consisted of 20 levels arranged in a factorial design. The first factor was the inducing concentration (four level concentrations) and the second factor was the antiplatelet agent (five level antiplatelet agents). The treatments were replicated three times. To verify the statistical significance of the parameters, the data was calculated for the values of means and standard deviation (M $\pm$ SD) and 95% confidence interval (CI) of means. To compare several treatments, we used analysis of variance (ANOVA) with two-factorial completely randomized design. P-values of less than 0.05 were considered as statiscally significant. Furthermore, to know the difference of means among treatments and to know the best treatmet, we used Duncan's Post-Hoc test 95% confidence interval. Statistical analysis used SPSS 16.0 program.

RESULTS

The phytochemical assay showed that hexane fraction of velvet bean seeds contained lowest flavonoids and phenolics compound compared with the other fractions (Table 1). Ethyl acetate, butanol and water fractions contained high flavonoid and moderate phenolic compounds, but they did not contain terpenoid and triterpenoid compounds. Butanol fraction contained the highest tanin compared with the other fractions. Water fraction contained moderate alkaloids and butanol and ethyl acetate fractions contained less alkaloids. Hexane, ethyl acetate and butanol fractions conatained less steroids. Butanol and water fractions contained less saponins, whereas hexane and ethyl acetate fractions did not contained saponins (Table 1).

Observing the antiplatelet activity, epinephrine (EPN) agonist induced platelet aggregation at all level concentrations, the lower concentration of EPN as agonist caused the reduction of platelet aggregation (Table 2). Aspirin as positive control decreased platelet aggregation at all concentrations of EPN. After aspirin, the most effective fraction showing the antiplatelet activity was

Table 1. The result of phytochemical assay of velvet bean seed fractions (hexane fraction, ethyl acetate, buthanol, and water fraction)

Sample (fractions)	Compound content							
	Tanins	Flavonoids	Phenols	Saponins	Terpenoids	Triterpenoids	Steroids	Alkaloids
Hexane	-	+	+	-	+	-	+	-
Ethylacetate	++	+++	++	-	-	-	+	+
Buthanol	+++	+++	++	+	-	-	+	+
Water	++	+++	++	+	-	-	-	++

+++: high content, ++: moderate content, +: less content, -: undetected.

Table 2. The antiplatelet acitivity of velvet bean seed fractions

Antiplatelet agents	EPN 300 ì M	EPN 150 ì M	EPN 75 ì M	EPN 30 ì M
Control (Hyperaggregation)	82.633 ± 8.228b D	74.067 ± 3.761ab C	68.267 ± 9.215a C	66.067 ± 2.732a C
Aspirin	31.333 ± 8.912a A	28.600 ± 4.029a A	27.433 ± 1.756a A	27.000 ± 1.825a A
Hexane fraction	68.233 ± 9.127b CD	60.767 ± 3.272ab B	55.033 ± 4.168a B	53.933 ± 4.509a B
Ethyl acetate fraction	69.733 ± 5.064b D	69.000 ± 4.784b C	69.800 ± 5.237b C	58.867 ± 2.950a B
Butanol fraction	54.433 ± 8.315b B	54.367 ± 2.318b B	51.033 ± 10.934b B	31.133 ± 3.134a A
Water fraction	47.600 ± 8.0206b B	34.067 ± 4.350a A	31.267 ± 3.027a A	27.500 ± 4.939a A

The data showed means ± standard deviation. The different small letters at the same row (among EPN concentrations) and capital letters at the same column (among antiplatelet agents) show significant at the 5% (Duncan's Post Hoc test).

the water fraction followed by butanol fraction at all concentrations of EPN, except at 30 mM of EPN. The antiplatelet aggregation at 30 ì M concentration of EPN showed similar activity among aspirin, water fraction and butanol fraction of velvet bean (Table 2).

DISCUSSION

Previous research showed that velvet bean seeds are rich in novel alkaloids, saponins, and sterols. The seeds of all *Mucuna* species contain a high concentration of L-dopa; velvet bean seeds contain 7-10% L-dopa (Del Carmen *et al*. 1999). Crude content of velvet bean showed the presence of alkaloid, flavonoid, tannin, saponin, quinone, and steroid/triterpenoid (Yulizia 2008). The velvet bean seeds contain the bioactive alkaloids mucunine, mucunadine, mucuadinine, pruriendine and nicotine, ß-sitosterol, glutathione, lecithin, oils, venolic and gallic acids. The seeds with seed coat showed the presence of a number of bioactive subtances including tryptamine, alkylamines, steroids, flavonoids, coumarins, cardenolides. The major phenolic constituent of *Mucuna* beans was L-dopa (Rajeshwar *et al*. 2005). The phenolic and flavonoid contents from our research verify the previous research, but alkaloids, saponins, and sterols concentration of *Mucuna* seeds can not be validated. The different content of alkaloids and saponins may be caused by different farming of strain of *Mucuna*.

Antiplatelet agents (aspirin and four fractions of velvet bean) decreased the EPN-induced platelet aggregation compared with control (hyperaggretation individual) at all level concentration of epinephrine (Table 2). The concentration level of EPN as an induceraffects the platelet aggregation, the higher concentration of EPN the higher the platelet aggregation (Hoffbrand & Pettit 1996). Blood platelets can be activated by different compounds including coagulation factors (thrombin), hormones (epinephrine, vasopresin), low molecular weight substances [serotonin, adenosine diphosphate (ADP), lipid derivatives (platelet aggregating factor (PAF), thromboxane A2 (TXA2)], and other protein substances (collagen or immune complexes). The responses of platelet include mainly adhesion (to foreign surfaces such as collagen or glass), shape change, aggregation and secretion of active compounds from three different storage granules (dense granules, a-granules and lysosomes), shedding of microvesicles, formation of platelet procoagulant activity and retraction of fibrin clots (Willoughby *et al*. 2002; Olas & Wachowicz 2005; Fogari & Zoppi 2005). Epinephrine stimulates human blood platelets by an $á_2$-adrenergic receptor mechanism (Kjeldsen *et al*. 1995). $á_2$-adrenergic receptors may more directly activate phospholipase A_2 via release of G protein ßã-subunits. Phospholipase A_2, which releases arachidonic acid from platelet lipids to form thromboxane A2 (TXA2), plays an important role in epinephrine-mediated platelet activation (Kjeldsen *et al*. 1995). Epinephrine agonists require aggregation for secretion to occur. After platelet activation induced by an agonist, intracellular signalling is necessary for cytoskeletal reorganisation, fibrinogen receptor exposure (integrin aIIbb3), and granule secretion to occur. Two differential pathways central to platelet activation are the phosphoinositide hydrolysis pathway and the eicosanoid synthesis pathway (Olas & Wachowicz 2005). The previous research showed that infused epinephrine in doses of 0.1 and 0.2 µg/kg per minute on 40 healthy men aged 20 to 40 years increased in the capacity for TXB_2 production by platelets (Kjeldsen *et al*. 1995). Infused epinephrine up to 0.04 µg/kg per minute in healthy men, increased platelet count and size (Lande *et al*. 1988). Epinephrine in the presence of fibrinogen and Ca^{2+} (*in vitro*) induces both primary and secondary aggregation, potentiates aggregation (Kjeldsen *et al*. 1995).

Aspirin is the best antiplatelet agent at all level of inducer concentrations and as an anti-thrombotic compounds through the inhibition of platelet cyclooxygenase-1 (COX-1) by irreversible acetylation of a specific serine moiety, thereby blocking the formation of thromboxane A2 (TXA2) for the lifetime of the platelets (McKee *et al*. 2002; Ohmori *et al*. 2006).

Water fraction of velvet bean seeds is comparable to aspirin because of the moderate content of phenols and the high content of flavonoids (Table 1). Phenols and flavonoids show commonly high antioxidant and antiplatelet activities. The previous research showed that

methanol extract of velvet bean seeds contained a noticeable amount of total phenols (Rajeshwar *et al*. 2005). Phenolic compounds exhibit a wide range of biological effects, including antiplatelet, anti-inflammatory, anticancer, antimutagenic, and antifungal properties. It is also a potent antioxidant, reactive oxygen species scavenger and metal chelators (Olas & Wachowicz 2005). The extract contains many compounds including flavonoids, tanins, phenols, saponins, terpenoids, triterpenoids, steroids, alkaloids. Each fraction of velvet bean contains different compounds (Table 1). This result confirm the previous studiy that *M. pruriens* contained isoflavones (the subclass of flavonoid) (Handajani 2001). Eriodictyol and patuletin are flavonoids from *Leuzea carthamoides* inhibiting platelet aggregation with agonist collagen (COL) and arachidonic acid (AA) (Koleckar *et al*. 2008).

Rutin is a flavonoid inhibiting platelet aggregation in human platelets stimulated by COL agonist depend on its concentration (250 and 290 µM) (Sheu *et al*. 2004). The antiplatelet activity of rutin (flavonoid) may involve the following pathways: rutin inhibits the activation of phospholipase C, followed by inhibition of protein kinase C activity and TXA2 formation, thereby leading to inhibition of the phosphorylation of platelet protein of M(r) 47000 (P47) a marker of protein kinase C activation and intracellular Ca^{2+} mobilization, and finally resulting in inhibition of platelet aggregation (Sheu *et al*. 2004). The *in vitro* combination of 2 flavonoids, namely quercetin and catechin, demonstrated that they are synergistic in reducing platelet formation of H_2O_2 and inhibiting platelet function by interfering the activation of phospholipase C pathway (Violi 2002). Flavonoid bioavailability and its relationship with antioxidant activity and platelet function (Violi 2002). Flavonoids have antioxidant activity, inhibits platelet aggregation and production of superoxide anion, resulting in cardiovascular protection (Violi 2002).

Catechin and eugenol (flavonoids) also inhibit cyclooxygenase (COX) activities and platelet aggregation (Huss *et al*. 2002). The isolation of flavonoids from *Cephalotaxus wilsoniana* (Cephalotaxaceae) had antiplatelet and anti-inflammatory effect (Wu *et al*. 2007). The ginkgetin flavonoid was isolated from *Cephalotaxus wilsoniana* and *Justicia species* had inhibitory effect on cyclooxygenase-1 (COX-1). The flavonoids including ginkgetin, taiwanhomoflavone C, justicidin B, and justicidin D were isolated from *Cephalotaxus wilsoniana* and *Justicia* showed inhibition of secondary aggregation induced by adrenaline (Wu *et al*. 2007).

The butanol and water fractions of velvet bean seeds show promising antiplatelet activities. These results suggest that the antiplatelet activities of the fractions may be mediated by TXA_2 receptor blockade with TXA_2 synthase inhibiting and suppressing cytosolic $[Ca^{2+}]$ mobilization (Jin *et al*. 2005), and inhibiting the activation of phospholipase C, followed by inhibition of protein kinase C activity and TXA_2 formation, thereby leading to inhibition of the phosphorylation of P47 and intracellular Ca^{2+} mobilization, finally resulting in inhibition of platelet aggregation (Mc Kenzie 2004; Sheu *et al*. 2004). TXA_2 formation cause the bond between platelets is weak and platelet aggregation is reversible (primer aggregation), secondary aggregation need longer time and produces irreversible aggregation platelet (Mc Kenzie 2004). Supplementation studies with polyphenol-rich foods or extracts indicate that the compounds may exert effects *in vivo* as well. For instance, the ingestion of dealcoholized red wine, grape juice, or polyphenol extracts reduced blood pressure and inhibited platelet aggregation in laboratory animals (Erlund *et al*. 2008).

The flavonoid-rich beverages such as red wine and purple grape juice are vasodilators and improve endothelial function, probably because of a nitric oxide-dependent mechanism (Lin *et al*. 2007).

Quercetin and catechin are flavonoids antioxidant, synergistically inhibit platelet function (*in vitro* assay) by blunting the release of hydrogen peroxide (H_2O_2) from platelets, subsequently reducing phospholipase C activation, calcium mobilization, and inositol phosphate synthesis. Flavonoids inhibit platelet aggregation because of their antioxidant activity, either by inhibiting the formation of endogenous mediators derived from phospholipid peroxidation, by blocking enzymatic free radical production, or by reducing platelet sensitivity to agonists by preventing lipid peroxidation (Murphy *et al*. 2003). Based on the experimental model of diabetes tested animal, the combination of acetylsalicylic acid and á-tocopherol as antioxidant led to beneficial changes that can help protect tissues from thrombotic incidents (Gonzales-Correa *et al*. 2006). Various agonists may stimulate platelet reactive oxygen pecies (ROS) production and aggregation, by regulating AA metabolism or via COX inhibition (Iuliano *et al*. 1997). In the presence of haemoglobin, ROS-induced platelet aggregation is enhanced (Iuliano *et al*. 1992). The resting platelets also generated a low amount of ROS. AA stimulates platelet ROS production, which is inhibited by hydroxychavicol flavonoid (HC), supporting HC flavonoid as a ROS scavenger (Chang *et al*. 2002). Our data (Table 2) showed that water fraction, butanol and ethyl acetate fraction of velvet bean seeds decreased platelet aggregation (as an antiplatelet activity) probably due to antioxidant activities. Previous research showed that *M. pruriens* had an ability to scavenge DPPH radicals, ABTS radicals and ROS. Velvet bean seeds significantly inhibit the oxidation of lipids and deoxyribose sugar. *M. pruriens* exhibited bivalent iron chelating activity (Dhanasekaran *et al*. 2008). Methanol extract of *M. pruriens* shows strong antioxidant activity by inhibiting DPPH and hydroxyl radicals, nitric oxide and scavenging superoxide anion, scavenging hydrogen peroxide, and reducing power activities when compared with different standards such as BHT, L-Ascorbic acid, curcumin, quercetin, and á-tocopherol (Rajeshwar *et al*. 2005). AA-induced thromboxane production or induced by the other agonists is not solely mediated by ROS production. The other possible reason

is that platelet ROS production can be mediated by COX as well as other enzymes such as platelet isoforms of NADPH oxidase, xanthine oxidase, mitochondrial respiration (Krotz *et al*. 2002). HC flavonoid inhibits the enzymes responsible for platelet ROS formation (Finazzi-Agro *et al*. 1982; Chang *et al*. 2007).

Aspirin showed higher antiplatelet activity compared with butanol and water fraction of velvet bean seeds, particularly at high concentration of epinephrine. Because aspirin is pure compound, it will produce high antiplatlet activity. The butanol and water fractions of velvet bean seeds contain complex compounds. To obtain the pure compound with high antiplatelet activity is required on further study.

The ethyl acetate, butanol, and water fraction contained high flavonoid and moderate phenol. The water, butanol, and ethyl acetate fraction of velvet bean seeds exhibited potential inhibition of EPN induced platelet aggregation at all concentrations. The strongest antiplatelet was water fraction and had similar antiplatelet activities with aspirin at level 150, 75, and 30 ì M of EPN inducer. Butanol fraction had similar antiplatelet activity with aspirin at lowest EPN inducer (30 ì M).

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