

The Antiplatelet Aggregation Effect of Extract and Ethyl Acetate Fraction of Velvet Bean Seed (*Mucuna pruriens* L.) in Dyslipidemic Rat

Wahyu Widowati¹, Hana Ratnawati¹, Udju Djunaedi Rusdi²,
Wahyu Winarno³, Felix Kasim¹

¹Medical Research Center, Faculty of Medicine, Maranatha Christian University, Jl. Prof. Drg. Suria Sumantri 65 Bandung, 40164; ² Department of Microbiology, Faculty of Medicine, University of Jenderal Achmad Yani, Jl. Terusan Jend Sudirman, PO Box 148, Cimahi 40533; ³Department of Agricultural Production, State Polytechnic of Jember, Jl Mastrip PO Box 164, Jember 68101
Email: wahyu_w60@yahoo.com

ABSTRACT

Cardiovascular disease (CVD) is the first cause of death in the world, CVD has complex and multifactorial process including atherogenic lipoprotein, oxidized low density lipoprotein (LDL), endothelial dysfunction, plaque stability, vascular inflammation, thrombotic and fibrinolytic disorder, exercises and genetic factor. Inhibiting the platelet aggregation is one of the CVD prevention. Velvet bean seed (*Mucuna pruriens* L.) can be found abundantly in Indonesia, but has not been used as herbal medicine. Ethanol extract and ethyl acetate of velvet bean seed contain high flavonoids and antioxidants properties which is expected could inhibit platelet aggregation. The objectives of the research were to determine the activity of ethanol extract and ethyl acetate fraction of velvet bean seed towards clotting and bleeding time in dyslipidemic rats. This research used completely randomized design in dyslipidemic rats which were given by ethanol extract of velvet bean seed at the concentrations of 50, 100 and 200 mg/kg BW/day and ethyl acetate fraction of velvet bean seed at the concentrations of 15, 30 and 60 mg/kg BW/day and 42.2 mg/kg BW/day aspirin for ten days. Clotting and bleeding time were measured at days 0, 10, and 20. Data were analyzed using One way analysis of variance and continued with Duncan's post Hoc test with 95 % level of significancy. The results showed that administration of 60 mg/kg BW/day ethyl acetate fraction of velvet bean seed and at the concentrations of 100 and 200 mg/kg BW/day ethanol extract of velvet bean seed, prolong the clotting time at day 10, ethyl acetate fraction at the concentration of 60 mg/kg BW/day, 200 mg/kg BW/day ethanol extract of velvet bean prolong bleeding time at day 10.

Keywords : antiplatelet, flavonoid, platelet aggregation, *Mucuna pruriens* L., cardiovascular disease, dyslipidemic

INTRODUCTION

Cardiovascular disease (CVD) is the first cause of death in the world; around 19.8 % from total death in 1993 and become 24.4 % in 1998. An estimated 17.1 million people died from CVD in 2004, representing 29% of all global deaths. Of these deaths, an estimated 7.2 million were due to CVD and 5.7 million were due to stroke (AHRQ, 2003; WHO, 2009). Incidence of CVD is increasing in developed countries, the incidences are estimated to be three to four times higher in developing countries. Globally, CVD is estimated 31 % of the worldwide mortality. Each year 17.2 million people die of CVD, 80 % of them in the developing world and emerging economies. By 2030, almost 23.6 million people will die from CVD, mainly from heart disease and stroke. These are

projected to remain the single leading causes of death. The largest percentage increase will occur in the Eastern Mediterranean Region. The highest increase in number of death will occur in the South-East Asia Region (WHO, 2009). Based on the data of Medical Department and Yayasan Penyakit Jantung (Cardiac Disease Foundation) showed that CVD is the leading cause of death in Indonesia. Based on World Health Organization (WHO) data, around 12 million people died of heart attack in the world. According to the family health survey (survei kesehatan rumah tangga) in 1992, CVD causes around 16 % of death and become 26.4 % in 2001 (Irawan, 2007).

Atherosclerosis is the main cause of CVD with complex and multifactorial process involving genetic and environment factors. The risk factors of CVD are atherogenic lipoprotein,

oxidized low-density lipoprotein (LDL), endothelial dysfunction, plaque instability, vascular inflammation, thrombotic and fibrinolytic disorder, exercises and genetic factor (Wijaya, 1998; Halliwell and Gutteridge, 1999). Atherosclerosis is an inflammatory disease and high plasma cholesterol concentrations, in particular those of LDL cholesterol, is one of the principal risk factor of atherosclerosis. If LDL particles become trapped in an artery, they can undergo progressive oxidation and be internalized by macrophages by means of the scavenger receptors on the surfaces of these cells (Ross, 1999). The internalization leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters. Oxidized LDL may also be involved in atherogenesis by inducing smooth muscle cell proliferation and smooth muscle foam cell generation (Holvoet *et al.*, 1998). Subendothelial accumulation of foam cells plays a key role in the initiation of atherosclerosis. These foam cells may be generated by the uptake of oxidized LDL and/or malondialdehyde (MDA)-modified LDL (Holvoet *et al.*, 1998; Ross, 1999). Numerous *in vitro* studies using a variety of oxidation methods and measurements have shown that polyphenolics from red wine, green tea and chocolate can inhibit LDL oxidation (Anderson *et al.*, 2001).

Platelet aggregation is an important factor in thrombosis with uncontrollable clotting blood (Wu *et al.*, 2007). Formation of platelet aggregates in an atherosclerotic vessel can cause total blockage of blood flow, leading to myocardial infarction and thromboembolic diseases. Atherosclerosis promotes increased adhesiveness in the endothelial wall for platelets, and a loss of anticoagulant properties, which contribute to atherosclerotic plaque formation. Platelets adhesion is initiated when platelets contact with exposed connective tissue of injured tissues. Adhesion stimulates platelets to secrete a variety of factors, including fibrinogen and von Willebrand factor, which facilitates aggregation and platelet plaque on the vessel wall. Activated platelets also secrete thromboxane A₂ that further stimulates platelet aggregation, and the activated immune cells also secrete platelet activating factor (PAF) which stimulate platelet aggregation (Boik, 1996; Geraldo, 2010). The activated platelets change shape, put out pseudopodia, discharge their granules, and stick to other platelets thus initiating the process of platelets aggregation (Resh and Ernst, 1995; Klepser and Klepser, 1999; El-Sabban, 2009). The rupture of plaques is the most important mechanism for the progression of vascular disease because this rupture exposes thrombogenic subendothelial matrix of protein and collagen, triggering a cascade of platelet activity (Geraldo, 2010).

Flavonoids are component of a wide variety of edible plants, fruits, and vegetables and of beverage appears to have antioxidative properties toward LDL, lipid peroxidation (Fuhrman *et al.*, 1995; Sesso *et al.*, 2003). Flavonoid from *Garcinia cambogia* inhibit atherosclerosis, endothelial

damaged, leucocyte activation, platelet adhesion and platelet aggregation (Koshy *et al.*, 2001). According to the previous research by Widowati and Retnaningsih (2007) antioxidant activity in ethanol extract and ethyl acetate fraction of velvet bean seed (*Mucuna pruriens* L.) compared with α -tocopherol, ascorbic acid and *butylated hydroxytoluen* (BHT), ethanol extract and ethyl acetate fraction of velvet bean seed contain higher flavonoid compared to the others fraction (hexane fraction, butanol and water fraction) (Widowati *et al.*, 2007; Widowati *et al.*, 2010), thus this compound can be possibly used as antiaggregation platelet agent.

Many drugs as antiplatelet agents for treating CVD, however some have several collateral effects and resistance in long term therapy, such as the known clinical aspirin resistance (Geraldo, 2010). Thus, the purpose of this research is to develop new antiplatelets agents with low collateral effects, from ethanol extract and ethyl acetate fraction of velvet bean seed.

MATERIALS AND METHODS

Extraction and Fractionation

The velvet bean seed (*Mucuna pruriens* L.) was collected from Sukoharjo District, Central Java, Indonesia, during April, 2007. Five kilograms of velvet bean seeds were milled and soaked in distilled ethanol (EtOH) and extracted by maceration technique; evaporated filtrate and yielded 516.845 g (10.335 %) ethanol extract of velvet bean seed. The ethanol extract (133.889 g) was partitioned with solvent mixture of 75% *n*-hexane and 25 % water, produced 30.466 g (22.71 %) hexane fraction and the water filtrate residue was partitioned with solvent mixture of 95 % ethyl acetate and 5 % water, produced 0.498 g (0.37 %) ethyl acetate fraction and the water filtrate residue was partitioned with solvent mixture of 90 % *n*-butanol and 10 % water, produced 3.578 g (2.67 %) ethyl acetate fraction. Finally, the water filtrate residue was evaporated and produced 4.358 g (3.26 %) water fraction. In this study, we used ethanol extract and ethyl acetate fraction of velvet bean seed, since the previous *in vitro* study showed that ethanol extract and ethyl acetate fraction of velvet bean seed had the highest antioxidant activity.

Animals and Diets

Thirty adult male Wistar rats obtained from School of Life Science, Bandung Institute of Technology, were housed in standard cages provided with food and water *ad libitum*. Rats were adapted for 7 days until the body weight were 175-200 g. The rats were feed by high fat diet (3000 g standard diet mixed with 250 g duck egg yolk, 500 g palm oil, 1250 g wheat flour, 500 g lamb fat and hot water). The rats which feed with high fat diet showed the average percentage

of crude fat was 20.78 % compared to the standard diet rats which was only 7.37 % .

In spite of high fat diet, each rat was also given with 1 mL/day fructose liquid 60 % (120 g/200 mL aquadest). The high fat diet and fructose liquid were given for 2 weeks until the body weight achieved 200 – 250 g, but the high fat diet was still given continually for 20 days. The ethanol extract and ethyl acetate fraction of velvet bean seed were given only for 10 days.

Rats were divided into 9 groups (3 rats) with different treatments. The first group, as negative control, was given standard diet. The second group, as positive control, was given high fat diet. The third, fourth, and fifth groups were treated with high fat diet plus ethyl acetate fraction 15 mg/kg BW, 30 mg/kg BW and 60 mg/kg BW daily. The sixth, seventh and eighth groups were treated with high fat diet plus ethanol extract 50 mg/kg BW, 100 mg/kg BW and 200 mg/kg BW daily. Group ninth was treated with high fat diet and aspirin 42,2 mg/kg BW daily.

Sample Preparation for The Lipid Test and MDA

After the treatments, 1.5 mL blood from orbital vein was collected in the tube contained heparin. It was centrifuged at 3,000 rpm for 10 minutes and the plasma was used for measuring the total cholesterol, LDL-cholesterol, high-density lipoprotein (HDL-cholesterol), triglyceride, and MDA level.

Total plasma cholesterol and triglyceride were measured according to the instruction manuals by the diagnostic kits from Abbott Clinical Chemistry (Abbott Clinical Chemistry, 2006), HDL-cholesterol, LDL-cholesterol were measured according to the instruction manuals of the diagnostic kits from Daiichi Pure Chemicals Co., Ltd (Daiichi Pure Chemical, 2008).

After feeding high fat diet and fructose for 2 weeks, the lipid profile of negative control group (standard diet) was as follow : total cholesterol 58.33 mg/dL, LDL 8.00 mg/dL,

HDL 33.00 mg/dL, triglyceride 63.67 mg/dL and the dyslipidemic rats had total cholesterol 70.67 mg/dL, LDL 14.33 mg/dL, HDL 24.33 mg/dL, and triglyceride 112.33 mg/dL. Thus, feeding with high fat nutrient induced dylipidemic rats.

Clotting Time Assay

The rat's tail was injured, blood collected with capillary tube for 30 seconds. Capillary tube was broken every 15 seconds until the fibrin filament appeared at the broken capillary tube as clotting time. Clotting time was measured at days 0, 10 and 20.

Bleeding Time Assay

The rat's tail was injured, blood absorbed with filter paper for 15 seconds. The interval time between the first dropping blood until the flowing blood stopped was calculated as bleeding time. Bleeding time was measured at days 0, 10 and 20.

Statistical Analysis

To verify the statistical significance of all parameters, the data were calculated the values of means and standard deviation (M ± SD) and 95 % confidence interval (CI) of means. This research used completely randomized design. To compare several groups, analysis of variance (ANOVA) was used. P-values of less than 0.05 were considered as statistically significant. Furthermore to know the best treatment, Duncan's post-Hoc test at 95 % confidence interval was used. Statistical analysis was done using SPSS 16.0 version.

RESULTS AND DISCUSSION

Based on the statistical analysis, at day 10 showed that HDL, LDL and MDA significantly different among treatment, high fat diet significantly increased the LDL-cholesterol, decreased the HDL-cholesterol and decreased MDA compared to standard diet (Table 1). Extract and ethyl acetate fraction of

Table 1. HDL, LDL and MDA level in dyslipidemic rats treated with ethanol extract and ethyl acetate fraction of velvet bean seed for 10 days

| Treatment | LDL level (mg/dL) | HDL level (mg/dL) | MDA level (mmol/L) |
|----------------------------------------|-------------------|-------------------|--------------------|
| Negative control | 8.00±1.00 ab | 33.00±2.67 c | 5.52±0.67 ab |
| Positive control | 14.33±1.53 cd | 24.33±1.53 a | 11.77±1.13 c |
| Ethyl acetate fraction 15 mg/kg BW/day | 10.00±2.00 ab | 25.33±3.22 ab | 5.62±0.18 ab |
| Ethyl acetate fraction 30 mg/kg BW/day | 8.33±2.52 ab | 26.67±2.3 abc | 4.06±0.55 ab |
| Ethyl acetate fraction 60 mg/kg BW/day | 7.33±1.53 a | 29.67±1.15 abc | 3.39±0.22 a |
| Ethanol extract 50 mg/kg BW/day | 11.33±2.52 bc | 26.33±3.51 abc | 6.03±2.35 b |
| Ethanol extract 100 mg/kg BW/day | 10.33±1.53 ab | 28.67±5.51 abc | 5.54±1.35 ab |
| Ethanol extract 200 mg/kg BW/day | 11.67±3.22 bc | 31.67±3.51 bc | 5.14±1.86 ab |
| Aspirin 42,2 mg/kg BW/day | 15.33±1.53 d | 26.33±3.51 abc | 9.60±1.61 c |

Results are expressed means ± SD, Duncan post Hoc test are shown by little letter, different letter at the same column are significantly different among treatment groups at confidence interval 95 %

velvet bean seed decreased the LDL level and ethanol extract of velvet bean seed 200 mg/kg BW/day increased HDL level in dyslipidemic rats. Extract and ethyl acetate fraction of velvet bean decreased the MDA level in dyslipidemic rats.

After treatment with various doses of ethanol extract, ethyl acetate fraction of velvet bean seed, and aspirin 42.2 mg/kg BW/day for 10 days, the effect on clotting time was shown in Table 2. Based on the statistical analysis, at day 0, clotting time showed no significant difference among groups because at day 0 rats had not been given with ethanol extract, ethyl acetate and aspirin yet. At day 10, clotting time showed significant difference among groups because rats had been given ethanol extract and ethyl acetate fraction. At day 20, clotting time showed no significant difference among groups because the ethanol extract, ethyl acetate and aspirin had been stopped at day 10, indicated that ethanol extract and ethyl acetate fraction had no effect on clotting time after the treatment had been stopped. To determine the difference of the clotting time (days 0, 10 and 20) among treatment groups, we analyzed using Duncan's post Hoc test (Table 2), which showed the effect of treatments (ethanol extract and ethyl acetate fraction of velvet bean seed) towards clotting time. At day 10, the clotting time of the dyslipidemic rats treated with ethyl acetate fraction 60 mg/kg BW/day and ethanol extract 100 and 200 mg/kg BW/day were significant difference compared to positive control and had similar effect with aspirin as anticoagulant.

Based on statistical analysis, at day 0, the bleeding time showed no significant difference among treatment groups. While, at day 10, bleeding time showed significant difference among treatment groups, since rats had been given ethanol extract and ethyl acetate fraction. At day 20, bleeding time showed significant difference among treatment groups although the ethanol extract, ethyl acetate and aspirin had been

stopped at day 10, indicated that ethanol extract and ethyl acetate still influenced the bleeding time. To determine the difference of the bleeding time (0, 10 and 20 days) among treatment groups, we analysed using Duncan's post Hoc test (Table 3), which showed that on day 0, no significant difference in bleeding time among treatment groups. At day 10, the bleeding time showed that ethyl acetate group (60 mg/kg BW/day) was significant difference compared to positive control, even showed a better result compared to group treated with aspirin as anticoagulant. At day 20, the shortest bleeding time was only in positive control (dyslipidemic rats), negative control, rats given ethyl acetate fraction 60 mg/kg BW/day and rats given with aspirin were significant difference compared to positive control. Although aspirin and ethyl acetate fraction had been stopped at day 10, it means that aspirin and ethyl acetate fraction could prolong the bleeding time until day 20.

Based on the clotting time data (Table 2) at day 10 indicated that dyslipidemic rats were given with extract and ethyl acetate prolonged clotting time compared to positive control. The bleeding time (Table 3) at days 10 and 20 in dyslipidemic rats were shorter than negative control. At day 10 showed that the dyslipidemic rats which were given ethanol extract and ethyl acetate fraction prolonged the bleeding time. This research was verified by previous study that hyperlipidemic in rats exhibit significant increase in ADP or collagen-induced platelet aggregation and cholesterol/phospholipid molar ratio in platelets. The increase in cholesterol/phospholipid ratio was responsible for hyperaggregation of platelet in animals (Nobukata, 1999). This data was verified with previous research that high cholesterol consumption can induce dyslipidemic and endothelial damaged, also effect thrombocyte adhesion in collagen furthermore activate thrombocyte (Aprami, 1993;

Table 2. The clotting time of dyslipidemic rats treated with ethanol extract and ethyl acetate of velvet bean seed

| Treatment | Clotting time 0 day | Clotting time 10 day | Clotting time 20 day |
|----------------------------------------|------------------------|-------------------------|-------------------------|
| Negative control | 105 ± 15.00 | 80 ± 17.32 ab | 85 ± 8.66 |
| Positive control | 90 ± 0.00 | 65 ± 22.91 a | 70 ± 8.66 |
| Ethyl acetate fraction 15 mg/kg BW/day | 80 ± 8.66 | 90 ± 0.00 abc | 95 ± 22.91 |
| Ethyl acetate fraction 30 mg/kg BW/day | 90 ± 30.00 | 105 ± 15.00 bc | 95 ± 22.91 |
| Ethyl acetate fraction 60 mg/kg BW/day | 90 ± 0.00 | 115 ± 8.66 c | 110 ± 17.32 |
| Ethanol extract 50 mg/kg BW/day | 95 ± 8.66 | 100 ± 17.32 bc | 80 ± 22.91 |
| Ethanol extract 100 mg/kg BW/day | 95 ± 8.66 | 110 ± 17.32 c | 80 ± 17.32 |
| Ethanol extract 200 mg/kg BW/day | 100 ± 17.32 | 110 ± 17.32 c | 100 ± 17.32 |
| Aspirin 42,2 mg/kg BW/day | 95 ± 8.66 | 115 ± 8.66 c | 115 ± 8.66 |

Results are expressed means ± SD, Duncan post Hoc test are shown by little letter, different letter at the same column are significantly different among treatment groups at confidence interval 95 %

Tabel 3. The bleeding time of dyslipidemia rats given ethanol extract and ethyl acetate of velvet bean seed

| Treatment | Bleeding time 0 day | Bleeding time 10 day | Bleeding time 20 day |
|----------------------------------------|------------------------|-------------------------|-------------------------|
| Negative control | 95 ± 8.66 | 100 ± 17.32 bc | 100 ± 8.66 bc |
| Positive control | 70 ± 8.66 | 65 ± 8.66 a | 65 ± 8.66 a |
| Ethyl acetate fraction 15 mg/kg BW/day | 65 ± 8.66 | 85 ± 17.32 ab | 85 ± 8.66 abc |
| Ethyl acetate fraction 30 mg/kg BW/day | 70 ± 17.32 | 95 ± 8.66 abc | 85 ± 8.66 abc |
| Ethyl acetate fraction 60 mg/kg BW/day | 70 ± 8.66 | 120 ± 30.00 c | 95 ± 8.66 bc |
| Ethanol extract 50 mg/kg BW/day | 80 ± 17.32 | 90 ± 25.98 abc | 80 ± 17.32 ab |
| Ethanol extract 100 mg/kg BW/day | 75 ± 15.00 | 95 ± 8.66 abc | 80 ± 17.32 ab |
| Ethanol extract 200 mg/kg BW/day | 75 ± 15.00 | 110 ± 8.66 bc | 95 ± 17.32 ab |
| Aspirin 42,2 mg/kg BW/day | 70 ± 17.32 | 115 ± 0.00 bc | 105 ± 0.00 c |

Results are expressed means ± SD, Duncan post Hoc test are shown by little letter, different letter at the same coloumn are significantly different among treatment groups at confidence interval 95 %

Wijaya, 1998). Active thrombocyte release ADP and thromboxane A2 and initiate aggregation of thrombocyte and vasoconstriction. Thrombocyte aggregation is important factor in thrombose formation in uncontrol blood clotting (Wu *et al.*, 2007). The high LDL and low HDL level in dyslipidemic rats (Table 3) were shorter clotting and bleeding time compared to negative control, this research was verified that elevating levels of LDL and low levels of HDL are widely used for predicting risk to atherosclerosis (Sevanian *et al.*, 1998).

Velvet bean seed ethanol extract and ethyl acetate fraction contain high antioxidant and flavonoids (Widowati and Retnaningsih, 2007; Widowati *et al.*, 2007, Widowati *et al.*, 2010), were capable to prolong the clotting and bleeding time at day 10 in dyslipidemic rats (Table 2,3). This research was verified with previous study that flavonoids could reduce LDL oxidation, an important step in atherogenesis (Setorki *et al.*, 2009) through free radicals scavenger mechanism. Based on the data (Table 1) displayed that velvet bean seed extract and ethyl acetate fraction had high antioxidant activity (Widowati and Retnaningsih, 2007), and were capable to reduce lipid peroxidation and decreased MDA, LDL level compared to dyslipidemic rats which were not given extract and ethyl acetate fraction as antioxidant and flavonoid sources. Antioxidant activity of polyphenols were shown to possess many biological properties including the inhibition of platelet aggregation, vasorelaxing activity, modulation of lipid metabolism, and inhibition of LDL oxidation (Agli *et al.*, 2004). In Table 2, the LDL in dyslipidemic rats 14.33 mg/dL was higher than negative control (8.00 mg/dL) and will highly influenced the oxidative damage (MDA 11.77 mmol/L), due to the high LDL level accelerating atherogenicity (Parthasarathy *et al.*, 1999). The oxidation of LDL was led by free radical-mediated chain reaction, yielding phosphatidylcholine

hydroperoxide (PCOOH) cholesteryl ester hydroperoxide (CEOOH) as the primary oxidations product (Noguchi *et al.*, 1998). It means that high LDL level increases oxidized LDL (ox-LDL) and MDA level (Table 2) and play role in atherosclerosis. LDL level increases amounts of thiobarbituric acid reactive materials (often attributed to increase amounts of MDA, 4-hydroxynonenal, and related carbonyl compounds), lipid peroxides, cholesterol oxides, decrease amounts of specific phospholipids, showed increase lysophospholipids and diminish antioxidants level (Sevanian *et al.*, 1998). The evidence indicate that oxidative modification of LDL may play a causative role in atherosclerosis (Sevanian *et al.*, 1998). Oxidized LDL is cytotoxic to endothelial cells, and nLDL is not toxic (Sevanian *et al.*, 1998; Halliwell and Gutteridge, 1999). The previous research indicated that atherogenicity was due to oxidative damage to LDL cholesterol. Modified or oxidized LDL (ox-LDL) has been shown to accelerate several steps in atherosclerosis including endothelial damage, monocyte/macrophage recruitment, increased uptake of LDL by foam cells (Hannekens, 1999). The oxidation of LDL is important in atherosclerosis, it is likely to occur in the subendothelial intima rather than the plasma (Parthasarathy, 1999). In Table 1, showed that dyslipidemic rats, high LDL concentration increased ox-LDL. The MDA level in dyslipidemic rat was higher 11.77 mmol/L compared to normal rat was 5.52 mmol/L, furthermore the bleeding time at 10 day of dyslipidemic rats were shorter than those in normal rats. This research result supports previous research, that oxLDL could accelerate atherosclerosis stages including endothelial damage, monocyte/macrophage recruitment, increased uptake of LDL by foam cells, alteration in vascular tone (Hannekens, 1999; Parthasarathy *et al.*, 1999). The oxidative modified LDL is taken up by macrophage, which results in its unregu-

lated uptake and eventual formation of foam cells as the early stages in cardiovascular disease (Noguchi *et al.*, 1998). Atherosclerosis is a systemic inflammatory disease characterized by the accumulation of monocytes/macrophages and lymphocytes in the intima of large arteries. Rupture or erosion of the advanced lesion initiates platelet activation and aggregation on the surface of the disrupted atherosclerotic plaque (Massberg *et al.*, 2002). The term of endothelial dysfunction has been used to refer several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling (Cai and Harrison, 2000), damaging the platelet activity, leukocyte adhesion, and thrombosis and is intimately involved in the development of atherosclerosis (Heitzer *et al.*, 2001). Increased production of oxygen-derived free radicals such as the superoxide anion has been linked to impaired endothelial vasomotor function (Heitzer *et al.*, 2001). Ox-LDL is the key event in endothelial injury and dysfunction. LDL in the subendothelial space, undergoes progressive oxidation and activates the expression of macrophage chemotactic protein 1 (MCP-1) and macrophage colony stimulating factor (M-CSF) in the endothelium. MCP-1 and M-CSF promote the entry and maturation of monocytes to macrophages, which further oxidize LDL. Ox-LDL is specifically recognized by the scavenger receptor of macrophages and once internalized, formation of foam cells. Ox-LDL and modified LDL induce endothelial dysfunction, associated with changes of the adhesiveness to leukocytes or platelet and to wall permeability (Virgili and Scaccini, 2001).

Extract and ethyl acetate fraction of velvet bean could prolong the clotting and bleeding time, and was verified with the previous research that using apple juice as flavonoids source (total flavonoids 1.36 ± 0.03 g/100 mL equivalent catechin and total anthocyanin 3.05 ± 0.85 mg/100 g) in cholesterolemic rabbit, significantly decreased fibrinogen and factor VII levels compared to high-cholesterol group (Setorki *et al.*, 2009). Based on the data (Table 2,3) displayed velvet bean seed extract and ethyl acetate fraction could prolong the clotting and bleeding time due to contained high flavonoid (Widowati *et al.*, 2007). The previous research showed that ethyl acetate fraction of velvet bean seed 500 µg/mL exhibited antiaggregation platelet against adenosine diphosphate (ADP) inducer by *in vitro* test (Widowati and Ratnawati, 2009). Flavonoids are subgroup of polyphenol, capable of: 1). inhibiting atherosclerosis, endothelial disruption, leukocyte activity, adhesion, aggregation platelet, decrease LDL level in normal and hypercholesterolemic rats, also inhibit *in vitro* LDL oxidation; 2). decreasing atherogenesis, inhibit hyperlipidemia, inhibit triglyceride accumulation in blood and liver, decrease phospholipid and fatty acids in rat's tissue (Koshy *et al.*, 2001; Hidgon, 2005). Giving 10 mL and 5

mL apple juice in cholesterolemic rabbits, the atherosclerotic thickness of right and left coronary arteries was 0.8 – 1.4 and 0.92 – 1.92, respectively and the plaque degree for both was grade 1. High cholesterol groups, right and left coronary arteries, the atherosclerotic thickness of right and left coronary arteries was 3.47 ± 0.37 and 3.28 ± 0.26 , respectively and the plaque degree in both was grade 3. Giving apple juices as flavonoid sources decreased the plaque degrees (Setorki *et al.*, 2009). Atherosclerotic changes were absent in normal diet group, whereas in the intimal surface of the coronary arteries from high-cholesterol diet group were seen many fat-laden macrophages. The cytoplasm of the macrophages filled with lipid droplets (foam cell) as the result of lipid digestion by the macrophage. Apple juice as flavonoids source may be useful in preventing hypercholesterolemic atherosclerosis and lowering the related risk of coronary artery disease (Setorki *et al.*, 2009).

Flavonoids play role as an antiinflammation, antioxidant, antiallergic, hepatoprotective, antitrombotic, neuroprotective and anticarcinogenic (Ganapaty *et al.*, 2007). Antiaggregation platelet activity of flavonoid is through inhibition of thromboxane A_2 mechanism. The inhibited thromboxane A_2 stimulates *adenyl cyclase* further increase cAMP and the increasing cAMP will reduce ion Ca (calcium) concentration in thrombocyte, furthermore inhibit aggregation and adhesion (Hoffbrand and Pettit, 1987), prolong clotting and bleeding time.

Flavonoids protect against atherosclerosis, through reducing the susceptibility of LDL to oxidation (Benito *et al.*, 2002), and vasodilator properties observed *in vitro* (Benito *et al.*, 2002)

The research results showed that giving velvet bean seed extract 200 mg/kg BW/day increased HDL level (31.67 mg/dL) compared to positive control (24.33 mg/dL) (Table 2). This results were verified with previous study that HDL concentration inhibited atherosclerosis progression and advance cardiovascular disease (Sevanian *et al.*, 1998; Santoso and Setiawan, 2005).

Based on Table 2 showed that aspirin prolonged clotting time in dyslipidemic rats and prolonged bleeding time in dyslipidemic rats (Table 3), aspirin is antiplatelet agent, medication that blocks the formation of blood clots by preventing the clumping of platelets. Aspirin as anti-thrombotic compounds is through the inhibition of platelet cyclooxygenase-1 (COX-1) by irreversible acetylation of a specific serine moiety, thereby blocking the formation of thromboxane A_2 (TXA₂) for the life time of the platelets (McKee *et al.*, 2002; Ohmori *et al.*, 2006). Table 1 showed that aspirin has no antioxidant effect, can not decrease the MDA level, has no anticholesterol activity, and can not decrease the LDL level in dyslipidemic rats.

CONCLUSIONS

Ethanol extract and ethyl acetate fraction of velvet bean seed exhibited antiplatelet aggregation in dyslipidemic rats, treated with ethanol extract 100 mg/kg BW/day, 200 mg/kg BW/day and ethyl acetate fraction 60 mg/kg BW/day prolonged the clotting time at day 10, ethanol extract 200 mg/kg BW/day and ethyl acetate fraction 60 mg/kg BW/day prolonged the bleeding time at day 10.

ACKNOWLEDGEMENT

We are grateful to Directorate General for Higher Education, Ministry of National Education of Republic Indonesia, for Research Grant of Hibah Bersaing (2007, 2008) for financial support.

REFERENCES

- Abbott Clinical Chemistry. (2006). Triglyceride. Abbott Park, IL 60064 USA.
- Abbott Clinical Chemistry. (2006). Cholesterol. Abbott Park, IL 60064 USA.
- Agli, M.D., Busciala, A. and Bosisio, A. (2004). Vascular effects of wine polyphenols. *Cardiovascular Research* 63: 593 – 602.
- AHRQ (2003). Effect of supplemental antioxidants vitamin C, vitamin E, and coenzyme Q10 for the prevention and treatment of cardiovascular disease. PubMed Navigation. The Agency for Healthcare Research and Quality. <http://www.ahrq.gov/clinic/epcsums/antioxsum.htm>. [15th April 2009].
- Anderson, K.J., Teuber, S. S., Gobeille, A., Cremin, P., Waterhouse, A. L. and Steinberg, F. (2001). Walnut polyphenolics inhibit *in vitro* human plasma and LDL oxidation. *J. Nutr.* 131: 2837–2842.
- Aprami, T.M. (1993). Peranan antioksidan dalam pencegahan aterosklerosis. Bagian/UPF Ilmu Penyakit Dalam Fak. Kedokteran UNPAD. Bandung.
- Benito, S., Lopez, D., Sáiz, M.P., Buxaderas, S., Sánchez, J., Puig-Parellada, P. and Mitjavila, M. T. (2002). A flavonoid-rich diet increases nitric oxide production in rat aorta. *Br J Pharmacol* 135: 910–916.
- Boik J. (1996). Cancer and natural medicine. Oregon Medical Press. Princeton, Minnesota.
- Cai, H. and Harrison, D.G. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 87:840–844.
- Daiichi Pure Chemicals Co. Ltd. (2008). High density lipoprotein cholesterol test. Ibaraki.
- Daiichi Pure Chemicals Co. Ltd. (2008). Low density lipoprotein cholesterol test. Ibaraki.
- El-Sabban, F. (2009). Garlic as an antithrombotic and antiplatelet aggregation agent. *J. Chin. Clin. Med.* 4:288-294.
- Fuhrman, B., Lavv, A. and Aviram, M. (1995). Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am.J. Clin. Nutr.* 61:549-54.
- Ganapaty S., Chandrashekhar V.M., Chitme H.R. and Narsu M.L. (2007). Free radical scavenging activity of Gossypin and Nevadensin: An in-vitro evaluation. *Indian J. Pharmacol.* 6: 281-283.
- Geraldo, R.B., Belo, M.L., Dias, L.R.S., Vera, M.A.F., Nagashima, T., Abreu, P.A., Santos, M.B., Albuquerque, M.G., Cabral, L.M., Freitas, A.C.C., Kalil, M.V., Rodrigues, C.R., Castro, H.C. (2010). Antiplatelet activity and structure-activity relationship study of Pyrazolopyridine Derivatives as potential series for treating thrombotic diseases. *J Atheroscler Tromb* 17: 2-11.
- Halliwell, B., and J.M.C. Gutteridge. (1999). *Free Radicals in Biology and Medicine*. Oxford University Press, New York.
- Hannekens, C.H. (1999). Antioxidant vitamins and cardiovascular disease. In : Papas, A.M. (ed.). *Antioxidant Status, Diet, Nutrition and Health*, p 463-478. CRC Press, Washington, D.C.
- Heitzer, T., Schlinzig, T., Krohn, K., Meinertz, T. and Münzel, T. (2001). Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Am. Heart Assoc.* 104:2673-2678.
- Hoofbrand, A. V. and Pettit, J.E. (1987). Kapita selekta haematologi (*Essential Haematology*). Edisi 2. Aru W. S, S. Bambang, A. Idrus. (eds.). Pusat Penerbitan Departemen Ilmu Penyakit Dalam FK UI, Jakarta.
- Holvoet, P., Vanhaecke, J., Janssens, S., de Werf, F.V. and Collen, D. (1998). Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *J. Am. Heart Assoc.* 98:1487-1494.
- Irawan. B. 2007. *Penyakit Jantung Koroner*. <http://www.nursyifa.net/index.html>. [5th November 2008].
- Klepser, T.B. and Klepser, M.E. (1999). Unsafe and potentially safe herbal therapies. *Am J Health Syst Pharm* 56:125-138.
- Koshy, A.S., Anila, L., and Vijayalakshmi, N.R. (2001). Flavonoids from *Garcinia combagia* lower lipid levels in hypercholesterlemic rats. *Food Chemistry* 72:289-294.
- Massberg, S., Brand, K., Grüner, S., Page, S., Müller, E., Müller, I., Bergmeier, W., Richter, T., Lorenz, M., Konrad, I., Nieswandt, B. and Gawaz, M. (2002). A Critical role

- of platelet adhesion in the initiation of atherosclerotic lesion formation. *J. Exp. Med.* **196**:887-96.
- McKee, S.A., Sane, D.C. and Deliargyris, E.N. (2002). Aspirin resistance in cardiovascular disease: a review of prevalence, mechanisms, and clinical significance. *Thromb Haemost* **88**: 711-715.
- Nobukata, H., Ishikawa, T., Obata, M. and Shibutani, Y. (1999). Age-related changes in coagulation, fibrinolysis, and platelet aggregation in male WBN/Kob rats. *Thromb. Res.* **98**:507-516
- Noguchi, N., Okimoto, Y., Cynshi, O., Kodama, T. and Niki, E. (1998). Inhibition of oxidative modification of Low Density Lipoprotein by novel antioxidant BO-653 prepared by theoretical design. In : Packer, L and Ong, A.S.H. (eds.). *Biological Oxidants and Antioxidants, Molecular Mechanism and Health Effects*, p:139-152. OACS Press, Illionis.
- Parthasarathy, S., Santanam, N. and Auye, N. (1999). Antioxidants and Low Density Lipoprotein Oxidation. In : Pappas, A.M. (ed.). *Antioxidant Status, Diet, Nutrition and Health*, p:347-370. CRC Press. Washington, D.C.
- Ohmori, T, Yatomi, Y., Nonaka, T., Kobayashi, Y., Madoiwa, S., Mimuro, J., Ozakis, Y. and Sakata, Y. (2006). Aspirin resistance detected with aggregometry cannot be explained by cyclooxygenase activity: involvement of other signaling pathway(s) in cardiovascular events of aspirin-treated patients. *J. Thromb. Haemost.* **4**: 1271-1278.
- Resh, K.I. and Ernst, E. (1995). Garlic (*Allium sativum*) a potent medicinal plant. *Fortschr Med* **113**:311-315.
- Ross, R. (1999). Atherosclerosis an inflammatory disease. *The New England J. Med.* **340**:115-126.
- Santoso, M., dan Setiawan, T. (2005). Penyakit Jantung Koroner. *Artikel Cermin Dunia Kedokteran* No.147.
- Sesso, H.D., Gaziano, J.M., Liu, S. and Buring, J.E. (2003). Flavonoid intake and the risk of cardiovascular disease in women. *Am.J. Clin. Nut.* **7**:1400-1408.
- Setorki, M., Asgary, S., Eidi, A., Rohani, A.H. and Esmaei, N. (2009). Effects of apple juice on risk factors of lipid profile, inflammation and coagulation, endothelial markers and atherosclerotic lesions in high cholesterolemic rabbits. *Lipids in Health and Dis.* **8**:1-9.
- Sevanian, A., Duncan, R., Hwang, J. and Hodis, H.N. (1998). Human LDL oxidation, atherosclerosis and cardiovascular disease. In : Packer, L and Ong, A.S.H. (eds.). *Biological Oxidants and Antioxidants, Molecular Mechanism and Health Effects*, p:127-138. OACS Press, Illionis.
- Virgili, E. and Scaccini, C. (2001). Cardiovascular disease and nutritional phenolics. In: Pokorny, J., Yanishlieva, N. and Gordon, M. (eds.). *Antioxidants in Food*, p:87-96. CRC Press, Washington D.C.
- World Health Organization (2009). Cardiovascular diseases (CVDs). Fact sheet No 317. WHO Media centre.
- Widowati, W. and Retnaningsih, Ch. (2007). Antioxidant activity of ethanol extract and fraction of Velvet bean (*Mucuna pruriens* L.). Poster presentation at the Sixth Princess Chulabhorn International Science Congress (PC-VI). The Interface of Chemistry and Biology in the "OMICS" Era: Environment & Health and Drug Discovery" November 25-29, 2007, Bangkok Thailand.
- Widowati, W., Lili, M.P. dan Ratnasari, A.I. (2007). Potensi Fraksi Aktif Antioksidan, Antikolesterol Kacang Koro (*Mucuna pruriens* L.) Dalam Pencegahan Aterosklerosis. Laporan Hasil Penelitian Hibah Bersaing Tahun Anggaran 2007/2008. Direktorat Jenderal Pendidikan Tinggi, Departemen Pendidikan Nasional.
- Widowati, W., Ratnawati, H., Rusdi, U.D., Winarno, W, Immanuel, V. (2010). Phytochemical Assay and Antiplatelet Activity of Fractions of Velvet Bean Seeds (*Mucuna pruriens* L.). *Hayati Journal of Biosciences*.**17**:85-90
- Wijaya, A. (1998). Faktor Resiko Penyakit Kardiovaskuler Perspektif Baru. Forum Diagnosticum. Laboratorium Klinik Prodia. Bandung.
- Wu, C.M., Wu, S.C., Chung, W.J., Lin, H.C., Chen, K.T., Chen, Y.C., Hsu, M.F., Yang, J.P., Wang, J.M. and Lin, C.N. (2007). Antiplatelet effect and selective binding to cyclooxygenase (COX) by molecular docking analysis of flavonoids and lignans. *Int. J. Mol. Sci* **8**:830-841.