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The Effect of Ethanol Extract of *Punica granatum* Linn. Leaves on Lipid Profiles of Dyslipidemic Rat

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ABSTRACT

It has been reported that peel of *Punica granatum* has antidyslipidemic activity. The aim of this research was to investigate the antidyslipidemic activity of *Punica granatum* leaf. The dried *Punica granatum* leaves were extracted with 96% ethanol. Rats were divided into several groups, which were normal, positive control, simvastatin at a dose of 10 mg/kg bw as reference group, and *Punica granatum* extract at a dose of 100 mg/kg. Before treatment, male Wistar rats were fed with high cholesterol chow for 3 months, and then plant extract was given for 4 weeks. Blood samples were collected at week 0, 12, 14, and 16 to measure the levels of total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, and triglyceride. Furthermore, at the last day of extract treatment, the aorta was isolated and haematoxylin-eosin stained. Administration of ethanol leaf extract of *Punica granatum* at a dose of 100 mg/kg for 2 weeks significantly reduced the total cholesterol and LDL-cholesterol levels up to 27.6% and 34.79%, respectively, compared to positive control group. After 4 weeks of extract treatment, the reduction of total cholesterol and LDL-cholesterol level were up to 59.9% and 75.05%, respectively. There was no effect of extract on HDL-cholesterol and triglyceride level. Furthermore, histological study showed that ethanol extract of *P. granatum* reduced aortic wall thickness significantly compared to untreated group. *Punica granatum* leaf is potential to be developed as an antidyslipidemic drug.

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INTRODUCTION

Dyslipidemia is a condition of metabolic syndrome disorder which is characterized by increased levels of total cholesterol, LDL, triglycerides, apolipoprotein (apo) B, and decreased levels of HDL. It has been reported that low HDL levels, high triglycerides, and cholesterol levels have a significant and independent relationship to myocardial infarction or stroke (Kolovou et al., 2005). Therefore, dyslipidemia has become a risk factor for various cardiovascular diseases such as atherosclerosis, coronary artery disease, myocardial infarction, and hypertension.

The prevalence of dyslipidemia is very high in Indonesia. Based on Indonesian Heart Association (PERKI) (National Institute of Health Research and Development, Ministry of Health, Republic of Indonesia, 2013), Nangroe Aceh, West Sumatra, Bangka Belitung, and Riau Islands in Indonesia have a prevalence of more than 50%. Furthermore, Indonesia Basic Health Research (Riskesdas) 2013 data shows that the prevalence of abnormal cholesterol, HDL, LDL, and triglyceride levels at age more than 15 years subject in Indonesia were 35.9%, 22.9%, 60.3%, and 13%, respectively.

Currently, synthetic drugs are used to treat dyslipidemia if life style modification is not successful. However, high side effects are still the major problem for synthetic. This condition has prompted the need for a natural product that can be used as a dietary supplement to treat or even prevent the development of dyslipidemia.

Pomegranate (*Punica granatum* Linn.) has been known and considered sacred in some countries. Empirically, the whole part of the pomegranate plant is often used by communities to treat a variety of diseases. Many researchers discovered that pomegranate flower extracts can overcome the condition of hyperglycemia in type II diabetes and obesity (Li et al., 2005; Huang et al., 2005; Jafri et al., 2000; Bhaskar &Kumar, 2012). Concentrated pomegranate fruit juice can improve lipid profiles in diabetic patients with hyperlipidemia (Esmaillzadeh et al., 2004). In addition, pomegranate fruit juice may slow the oxidation of cholesterol by up to half, and lowered the LDL level (Abaas et al., 2014). Furthermore, pomegranate leaves have been reported to have antihyperglycemic and antihyperlipidemic activity in streptozotocin-induced diabetic rats (Patel et al., 2014). However, the antihyperlipidemic activity of pomegranate leaves has not been fully explored

using dyslipidemic rat model. Therefore, in this study, antidyslipidemia of pomegranate leaves will be studied using dyslipidemic rat model.

METHODS

Materials

The following materials were used in this study: high-cholesterol chow 10 kg (20 pieces of chicken liver, 1 kg of cow fat, 1 kg of goat fat, 75 quail eggs, 20 duck eggs, 3 kg of wheat flour and 2 kg of corn flour), normal chow, cholic acid, pure cholesterol, propylthiouracil (PTU), vegetable oil, aquadest, NaCl 0.9% solution and CMC-sodium. The chemicals used were ethanol, xylol, formaldehyde, paraffin wax, haematoxylin, and eosin (Merck, Germany). Total cholesterol kit, HDL kit, and triglyceride kit was supplied by PT. Rajawali Nusindo, Jakarta, Indonesia.

Test Animals

The test animals were 20 male Wistar rats aged 10–12 weeks and weighing 200–350 g. The animals were obtained from the Animal Laboratory of School of Pharmacy, Bandung Institute of Technology. All animal experimental protocols have been approved by the Institutional Animal Ethics Committee (IAEC) in accordance with the Committee's guidelines for the Objective of Supervision and Control of Animal Experiments (No. 05/KEPHP-ITB/2016).

The test animals were placed in cages under suitable environments based on animal testing standards. There were four groups consisting of five rats each. Group one was the normal group that was fed with normal chow, without any extract treatment. While group two (positive control group), three and four were fed with high cholesterol chow for 12 weeks to develop dyslipidemic rats. Moreover, for group three consisted of dyslipidemic rats that were treated with simvastatin as a reference drug. Furthermore, group four were treated with *P. granatum* leaf extract at dose of 100 mg/kg bw.

Antidyslipidemic Rat Model

Dyslipidemia was induced in the animals through the oral administration of a high-cholesterol diet and water *ad libitum*, as well as PTU, pure cholesterol, and cholic acid. PTU 12.5 mg/kg bw/day was suspended in 0.3% CMC-Na with aquadest as the solvent. Cholic acid 200 mg/kg bw/day and pure cholesterol 100 mg/kg bw/day were dissolved in vegetable oil. PTU, cholic acid and pure cholesterol were administered orally to the rats for 5 consecutive days each week. The high-cholesterol feed composition was prepared from 20 pieces of chicken livers, 1 kg of cow fat, 1 kg of goat fat, 75 quail eggs, 20 duck eggs, 3 kg of wheat flour, and 2 kg of corn flour. Cholesterol-rich feed was administered daily for 12 weeks. Afterward, simvastatin and *P. granatum* leaf extract were given daily for 4 weeks (Nurfatwa, 2016).

Collection of Rat Blood

Rat blood was collected for the evaluation of lipid profile. Rats were put inside restriner and then blood was collected through lateral venous blood vessels in the rat tails. A total of 0.3 mL of blood was collected into Eppendorf tubes and centrifuged for 5 min at 10,000 rpm. After centrifugation, the serum was collected and stored at −20 °C. Lipid profile analysis included the measurement of total cholesterol, triglycerides, High-density lipoprotein (HDL)-cholesterol, and Low-density lipoprotein (LDL)-cholesterol (Safitri, 2016). Total cholesterol of serum collected at before induction, 12 weeks after induction, and every 2 weeks during treatment were measured. While for the other lipid profiles, measurements were done for serum collected at week 2 and 4 of simvastatin or extract treatment.

Quantification of Lipid Markers

Commercially available kits were used to measure the levels of total cholesterol, triglycerides, and HDL according to the manufacturer's instructions. All kits were purchased from PT. Rajawali Nusindo, Jakarta, Indonesia. While for LDL cholesterol, the level was determined using the Friedewald equation as follows (Sukandar, 2016):

$$\text{LDL} = \text{Total Cholesterol} - \text{HDL} - (\text{Triglyceride}/5)$$

Aorta Histology

The test animals were sacrificed using CO_2, then heart and aorta of rats were dissected and isolated for histology. The aorta was soaked in buffered formalin for 1 week. The aorta then was immersed in a container which contains 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol, and absolute ethanol respectively for 4 hours. Furthermore, the aorta was immersed in xylol in 3 different containers each for 1 hour to remove residual alcohol from the aorta. Paraffin in solid form was further heated at temperature of 60-65ºC until liquid and the aorta then was blocked with paraffin. Next, paraffinized aorta was sliced using a microtome with a thickness of 3 µm and placed on the object glass.

Haematoxylin-Eosin Staining

The object glass that has been filled with pieces of the aorta was put into containers containing xylol, absolute ethanol, 95% ethanol, 90% ethanol, 80% ethanol, and 70% ethanol for 2 minutes to remove paraffin. The object glass was then washed with clean water to remove alcohol and put in a container filled with hematoxylin dye for 15 minutes. Furthermore, it washed again to remove any residual haematoxylin and put into the container which contains eosin dye for 2 minutes. Then,

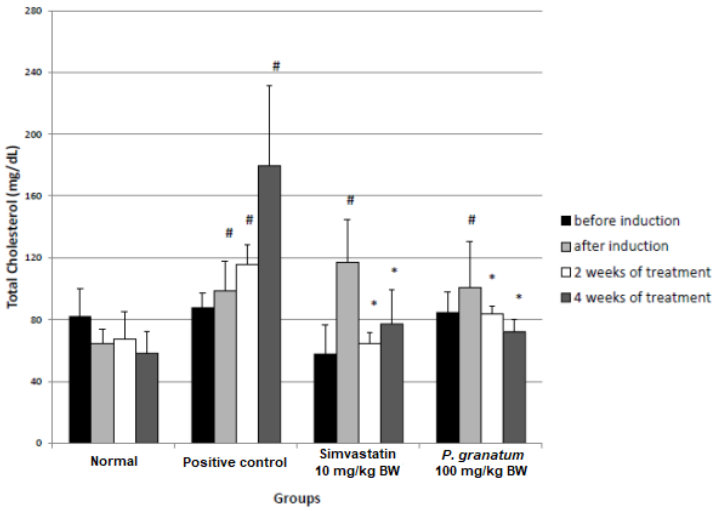

Figure 1. Effect of extract on cholesterol level of dyslipidemic rats. All values are expressed as Mean±SD, n=5; #p<0.05 compared to normal; *p<0.05 compared to positive control. The statistical calculation was performed using Anova.

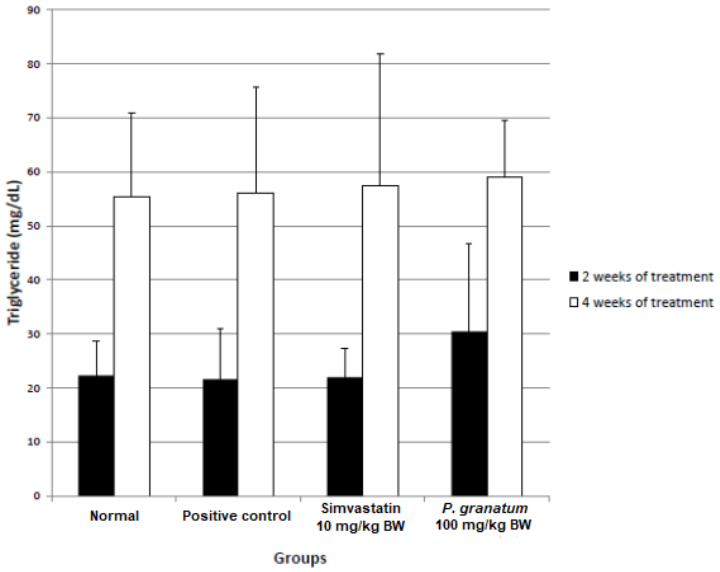

Figure 2. Effect of extract on triglyceride level of dyslipidemic rats. All values are expressed as Mean±SD, n=5; #p<0.05 compared to normal; *p<0.05 compared to positive control. The statistical calculation was performed using Anova.

it was put into 70% alcohol, alcohol 80%, 90% alcohol, 95% alcohol, absolute alcohol, and xylol for 2 minutes each. The object glass cleaned for the remaining xylol and covered with a cover glass. Furthermore, the slide was observed under a microscope.

Statistical Analysis

ANOVA in SPSS 21.0 was used to analyze statistically significant differences between the control and the experimental groups. $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

At the beginning of induction of dyslipidemia, the average total cholesterol level of all the test groups was 78.4 mg/dL which were not statistical different among other groups (Figure 1). After 12 weeks of induction, the total cholesterol level of the dyslipidemic rats increased significantly compared to normal group (Figure 1). *P. granatum* leaf extract at dose of 100 mg/kg bw given for a month on dyslipidemic rat could reduce total cholesterol and LDL-cholesterol level. Unpublished

data showed that an increase dose of the extract could increase its activity in reducing total cholesterol and LDL-cholesterol level.

In this study, the induction of dyslipidemia was performed exogenously for 3 months. The chow compositions for dyslipidemia induction were high cholesterol chow, 100 mg/kg bw pure cholesterol in vegetable oil, 200 mg/kg bw of cholic acid in vegetable oil, and propylthiouracil 12.5 mg/kg bw in aquadest. Cholic acid is one of the major bile acids in the body that was given to increase the absorption of exogenous cholesterol by inhibiting cholesterol conversion to bile acids (Hall, 2016). Meanwhile, propylthiouracil was given to inhibit metabolism of cholesterol, therefore it increased the levels of cholesterol serum (Rizos et al., 2011). In this study, we only used male rats as it has been shown in previous publication that there is no effect of gender in increasing of serum and hepatic cholesterol level upon cholesterol supplementation (Marounek et al., 2012).

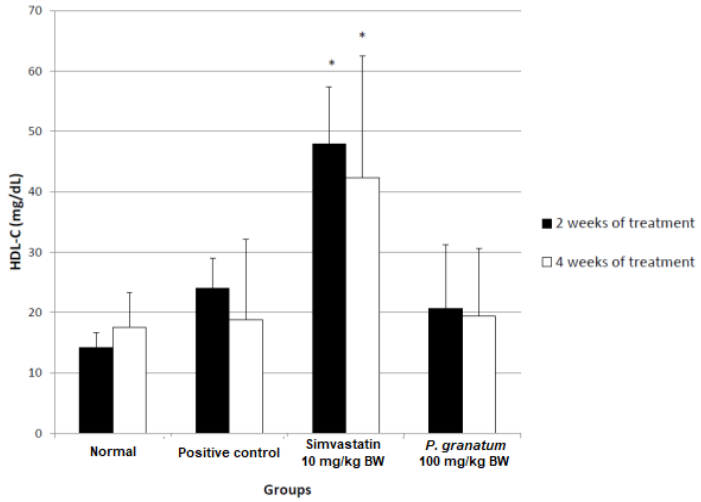

Figure 3. Effect of extract on HDL-cholesterol level of dyslipidemic rats. All values are expressed as Mean±SD, n=5; #p<0.05 compared to normal; *p<0.05 compared to positive control. The statistical calculation was performed using Anova.

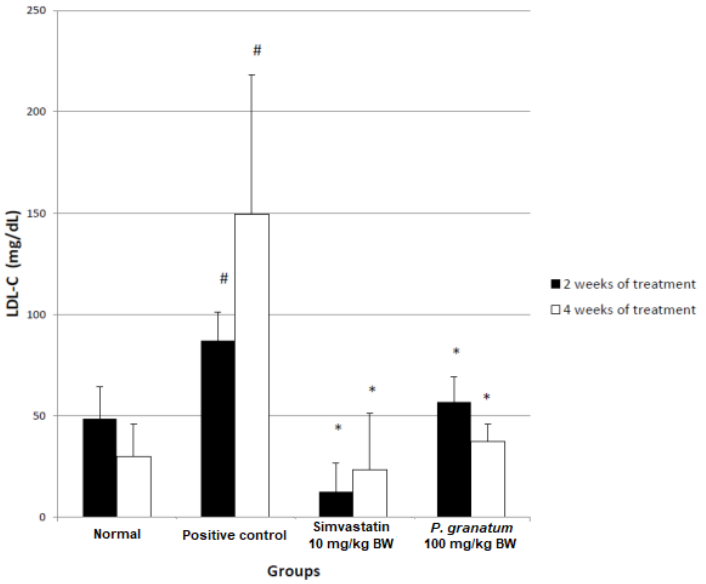

Figure 4. Effect of extract on LDL-cholesterol level of dyslipidemic rats. All values are expressed as Mean±SD, n=5; #p<0.05 compared to normal; *p<0.05 compared to positive control. The statistical calculation was performed using Anova.

Upon simvastatin treatment, it was found that simvastatin could reduce total cholesterol and LDL level significantly compared to positive control (Figure 1 and 4). Furthermore, it could increase HDL level significantly but had no effect on triglyceride level (Figure 2 and 3). Simillar to simvastatin, punica leaf extract treatment could reduce total cholesterol and LDL levels significantly compared to positive control after 2 and 4 weeks of treatment (Figure 1 and 4). However, it has no effect on triglyceride and HDL levels at any time of observation (Figure 2 and 3).

Histological study showed that high cholesterol induction exhibited a significant increased aortic wall thickness compared to normal group. In simvastatin and *P. granatum* leaf extract group, the treatments could reduce aortic wall thickness significantly compared to positive control (Figure 5).

The results of this study suggest that *Punica granatum* leaf extract has potential to be developed as antidyslipidemia drug. It has been reported before that *Punica granatum* leaf has antidyslipidemia effect, however the animal model used was alloxan or streptozotocin-induced non–insulin-dependent diabetes mellitus albino rats (Das & Barman, 2012; Salwe et al., 2015). Alloxan and streptozotocin are known as a substance that can damage ß-cell pancreas, leading to hyperglycemia. Although diabetic rats exhibit abnormalities in lipid metabolism as evidenced by the elevated levels of cholesterol, triglycerides, low-density lipoprotein cholesterol and very low-density lipoprotein, this model is not directly showed its effect on induction of dyslipidemia exogenously.

Besides the leaf, the antihyperlipidemia activity of the flower, fruit (aril and seed), and fruit peel of *Punica granatum* has also been studied (Abaas et al., 2014; Sadeghipour et al., 2014). All those parts of *Punica granatum* showed its activity in lowering the lipid profile. Compared to aril and seed, fruit peel of *Punica granatum* had better effect on lowering total cholesterol level (Mageid et al., 2016). It would be interesting to study further on its activity compared to leaf extract.

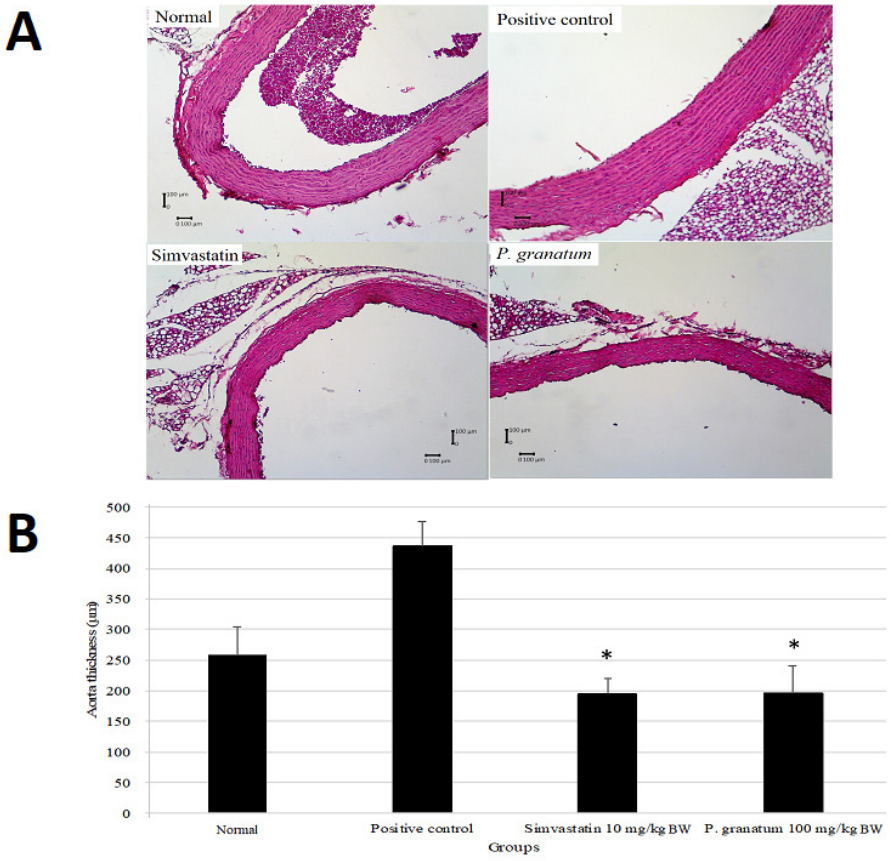

Figure 5. Histological examination of aortic hypertrophy in extract treated dyslipidemic rats. (A) Representative cross section of aortic wall stained with HE. Magnification 400x. (B) Bar graphs show the quantitative analysis of aortic wall thickness. N=3, *P<0.05 vs positive control. The statistical calculation was performed using Anova.

Punica granatum fruits, seeds, and peels contain numerous valuable ingredients such as flavonoids, ellagitannin, punicalagin, ellagic acid, vitamins, and minerals (Jasuja et al., 2012). Punicalagins and ellagitannin have been reported to be responsible for immeasurable health benefits due to its strong antioxidant activity (Suo et al., 2009). Antioxidant has been reported to play a role in protecting from oxidative damage by reactive oxygen species, thus it might reduce the peroxidation rate and prevent the progressive disease due to dyslipidemic condition (Yang et al., 2008). Recent evidences showed that *Punica granatum* fruits, peels, and seeds can inhibit free radical effect and modulation of enzymes activity linked with diseases development and progression (Rahmani et al., 2017). *P. granatum* leaves contains tannins (punicalin and punicalagin), flavonoid glycosides (luteolin and apigenin) (Jurenka, 2008). Tannins are able to decrease cholesterol and triglyceride serum level in high-fat diet-induced obese mice (Lei et al., 2007). For futher research, it would be interesting to find the major component that play a role in antidyslipidemia effect.

CONCLUSION

This study demonstrated that pomegranate leaf extract at dose of 100 mg/kg bw rat has potential to be developed as antidyslipidemia drug as it can reduce total cholesterol and LDL-cholesterol level in hypercholesterolemia chow-induced dyslipidemic rat model.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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