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Potential of phenolic compounds in persimmon fruit extract (*Diospyros Kaki* L.) against atherosclerosis in rats

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ABSTRACT

Persimmon fruits (*Diospyros kaki* L.) contain catechins and epigallocatechin gallate (EGCG) which have antioxidant properties. The study aimed to prove the antioxidant potential of phenolics in persimmon fruit (*D. kaki* L.) in reducing oxidation of LDL and as antiatherosclerotics. The research method included an anti-oxidant test using a UV-VIS spectrophotometer and measurement of polyphenol level using High-Performance Liquid Chromatography (HPLC). Also, blood serum levels were examined *in vitro* and *in vivo* based on the histopathology of the aorta of male Sprague-Dawley rats subjected to a cholesterol diet. There were five test groups: positive control (receiving α-tocopherol), negative control, dose I (administered with the extract at 450 mg/kg BW), dose II (extract 900 mg/kg BW), and dose III (extract 1800 mg/kg BW). The testing was carried out for eight weeks. Antioxidant test using DPPH method produced an IC_{50} of 44.07±15.06 mg/mL. The polyphenol contents identified in the persimmon extract were catechins 2.55% and EGCG 9.49%. The results showed that the persimmon extract exhibited antioxidant activity that prevented atherosclerosis through the mechanism of LDL oxidation. At a concentration of 1,800 mg/kg BW, the extract decreased the aortic wall thickness by 31.20% and showed activities that were not significantly different from the positive control (p value= 0.05).

Keywords: *Diospyros kaki* L., histology, aortic, lipid, antioxidant

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INTRODUCTION

Arterial vessels are channels that carry blood, oxygen, and nutrients from the heart to all parts of the body (Moore and Tabas, 2011). The characteristics of a healthy artery are flexible, strong, and elastic, and the inner surface layer is flat and very smooth, allowing blood to flow unhindered. One of the disorders of the arteries is called atherosclerosis. According to WHO (World Health Organization) (2008), atherosclerosis is the most common cause of coronary heart disease, which is 98%, and the rest is due to arterial spasm and abnormalities (2%). In the last decade, heart and blood vessel diseases like atherosclerosis have developed into a major killer in Indonesia. Management therapy is often used today, especially in the acute phase, in the form of fibrinolytic administration or intervention management that is followed by drug therapy (Aru *et al*., 2006). However, such treatment must also be supported by preventive measures to lower the risk of atherosclerosis.

Atherosclerosis is a change in the artery intima-media, which is the accumulation of fat (lipids), carbohydrate complexes, blood, and fibrous tissue (Gast *et al*., 2015). The risk factors are parameters that determine the presence of atherogenic lipoproteins, oxidized LDL (Low-Density Lipoprotein), endothelial dysfunction, plaque stability, and vascular inflammation, as well as the risk of thrombosis and fibrinolysis disorders and genetic factors (Trpkovic *et al*., 2015). LDL oxidation is one cause of atherosclerosis (Gast *et al*.,2015). The presence of fat accumulation for a long time, or hypercholesterolemia, will increase free radicals in the body. At the time of fat accumulation, NFκB (Nuclear Factor Kappa B) regulates the expression of cyclooxygenase, lipooxygenase, cytokines, chemokines, and molecular adhesion, causing an inflammatory response. In the next development, NFκB is estimated to express the M-CSF gene (macrophage colony-stimulating factor), i.e., a factor that stimulates the infiltration, differentiation, and transformation of monocyte and, as a result, oxidized LDL turns into foam cells. Herbal remedies are mostly picked as a preventive measure and in some cases are used for curative efforts. According to a herbal study of fig fruit (*Ficus carica* L.) in white male rats (*Rattus norvegicus* L.), the extract of the dried figs contains polyphenols with the highest concentration ranging between 1.090-1.110 mg/100 g and inhibits the activity of macrophages or endothelial cells in the arterial wall. The downside is figs must be imported from Libya (Putra *et al*., 2012) because they cannot grow well in the tropical climate of Indonesia, unlike persimmons.

Persimmon has the potential to be developed as an antioxidant against free radicals due to its phenolic contents (Kurniasari *et al*., 2018). Phenolics found in this fruit are catechins and their derivatives. Previous studies have reported its capacity as antiatherosclerotics (Park *et al*., 2006). It is also efficacious in generating laxative activity on plasma lipid (Gorinstein *et al*., 1998). Its antioxidant values have been reported to hover around lC_{50}= 43.36±1.78 mg/mL (Jang *et al*., 2010). This paper will present a further study aiming to find the value of the efficacy of persimmon extract (*Diospyros kaki* L.) in inhibiting plaque formation in blood vessels by observing the antioxidant activity of its phenolic groups *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plants collection

Samples of persimmon fruit were collected from Kuningan, West Java based on particular physical characteristics, namely at least 10±2 cm in diameter, yellowish green, and not rotten. These plants were microscopically identified at the Laboratory of Botany, Pusat Konservasi Tumbuhan Kebun Raya-LIPI, Bogor.

Preparation of extracts from persimmon fruits

A total of 5 kg of dried samples of persimmon were macerated using 7.5 L of ethanol 80% (Park *et al*., 2006). This maceration was then performed again using 5 L of the same solvent and followed by the fractionation stage.

Phytochemical screening was then carried out (Harborne 1987) to determine the alkaloids, terpenoids, saponins, tannins, and flavonoids and compare them with the standard. The total phenolic compounds of the persimmon extract were quantitatively tested with the Folin-Ciocalteu reagent. As for the antioxidant activity, it was identified using the DPPH method with the help of UV-VIS Spectrophotometer at a wavelength of 515 nm (Singh & Joshi, 2011). The extraction yield was calculated using the following formula:

Extraction yield (%) = [weight of dried extract /weight of dried sample] x 100 (Fereira, 2011)

Meanwhile, the radical scavenging activity (RSA) was calculated using the formula below and expressed as percent inhibition:

DPPH RSA (%) = [1 – (sample OD/control OD)] x 100 (Fereira, 2011)

***In vivo* test preparation**

This research began with the submission of an ethical clearance request to the Committee of Research Ethics of Universitas Indonesia. The research permit was granted after the issuance of a certificate of ethics No. 688/H2.F1/ETIK/2013.

The test animals were male Sprague Dawley rats aged 2-3 months and weighed 180-200 g. The test animals in this study were treated with a high-fat diet. The one-month hyperlipidemic diet containing cholesterol (3%), cholic acid (0.3%), and vegetable oils. Based on the preliminary test, this type of food intake can increase cholesterol in the blood. If cholesterol and LDL are accumulated in the blood, the risk of oxidation and inflammatory reactions will be higher. Before receiving the diet plan, they were allowed to adapt to the condition of the cage and the experimental environment. The adaptation was carried out for seven days during which the rats received regular feeding. During the treatment, the anti-atherosclerotic activity was determined *in vivo*. In the preliminary test, the histology of the aorta was performed to observe the manifestation of atherogenesis. The results showed that foam cells or plaques were formed in the aorta. After one month of hyperlipidemic diet, thirty (30) rats were divided into six (6) random groups. The negative control group was subjected to a high-fat diet only without receiving any antioxidants. For the positive control group, α-Tocopherol (13.5mg/kg BW) was used as a comparison. The other three groups were given persimmon extract with different doses, namely 450, 900, and 1,800 mg/kg BW. These treatments were conducted every day for eight weeks.

Examination of blood lipid levels

Blood was collected after eight weeks of the hyperlipidemic diet. The microhematocrit tube was inserted into the orbital sinus of the rats, which is located at the inner corner of the eye, towards the back of the eyeball. The tube was then rotated gently, allowing blood to flow through the capillary tube (Figure 1). The blood was collected in a vacuum tube that had been added with heparin (Hoff, 2000).

Figure 1. Sinus-orbital blood collection

The blood-filled tube was then centrifuged at 7000 rpm for 5 minutes to get a clear filtrate. Furthermore, reagents were added to test the levels of cholesterol, triglycerides, LDL, and HDL. The inspection reagents used in the procedure were DiaSys-Cholesterol FS and Human, and the blood was observed with a UV-VIS spectrophotometer. This analysis employed the CHOD-PAP and GHOD-PAP methods (CHOD= oxidase cholesterol; GOD= glucose oxidase; GPO= glycerol-3-phosphate oxidase; PAP= phenol + aminophenazone).

Histological check and characterization of aortic wall thickness

The test rats were sacrificed after blood collection, and the aorta was removed for histological examination of the formed foam cells (Figure 2).

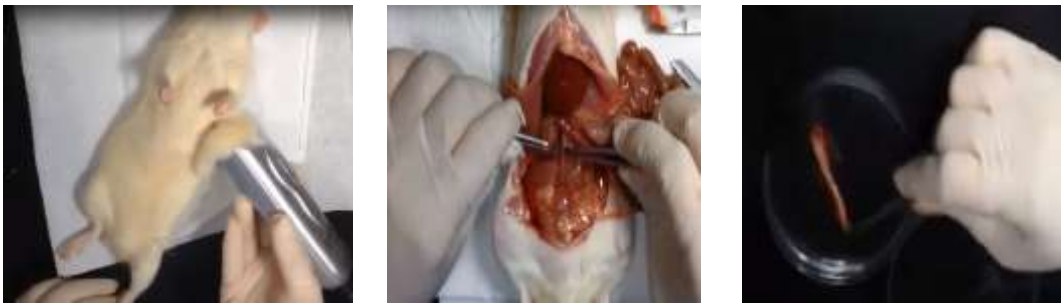

Figure 2. Aorta collection for histological examination of the foam cell

The tissue was stained with Oil Red-O (OR-O) and Hematoxylin-Eosin (HE). A slide observation was performed under a microscope or polarizing optical microscope. Foam cells are red colored cells found in the tunica of the aortic blood vessels. These cells can cause osmosis and space pressure. The number of foam cells was counted at a magnification of 100x to 400x from five points of view. After eight weeks of treatment, each of the test rats was subjected to surgery and aortic tissue retrieval. The aortic tissue was preserved with formalin stored in a paraffin slide. Then, the slide was examined to identify the thickness of the abdominal aortic wall under a microscope that was equipped with a Ken-A-Vision X-1000-1 projector. The aortic wall thickness was measured from five points of view, and the result was the mean values of the measurements.

RESULTS AND DISCUSSION

Extraction yields

Persimmon fruit weighing 10.5 kg was dried, and it produced 2,250 g of dry samples (21.24%). Then, it was processed with maceration using ethanol 80% and yielded 690.625 g of extract or 30.69%. The subsequent procedure was liquid-liquid fractionation using four (4) solvents, namely hexane, ethyl acetate, ethanol, and butanol in sequential order. The results obtained from the combination of the three polyphenol fractions were 601.9 grams, and the yield was 18.52%.

Total Phenolic Contents (TPC)

The phenolic compounds were classified using HPLC (High-Pressure Liquid Chromatography). HPLC is an instrument used to separate compounds. The separation was carried out with suitable solvents and supported by high pressures of up to 400 atm (Table I).

Table I. The total polyphenol contents of the persimmon fruit extract based on the HPLC analysis results

Sample	Analysis	Method	Result	Unit
persimmon fruit extract	Polyphenol (as catechins)	HPLC	2.55	%
	Polyphenol (as EGCG)		9.49	

Table I shows the results of the observations of persimmon extract using HPLC. The identified catechins and EGCG are polyphenol compounds. These secondary metabolite compounds are agents that induce antioxidant activity in the extract (Figure 3). The majority of the flavonoids/polyphenols found in the fruit consists of catechins like epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). Epicatechin (EC) and epigallocatechin (EGC) give a slightly bitter taste with a little sweetness after oral administration, whereas gallates (EGC and EGCG) accentuates the strong flavor of catechin ($C_{15}H_{14}O_6$), which is a polyphenol from a group of flavonoids. Catechins have antitumor and antioxidant properties. Catechin and EGCG are the main class of catechins derived from the secondary metabolites of plant polyphenols (Putra *et al.*, 2012).

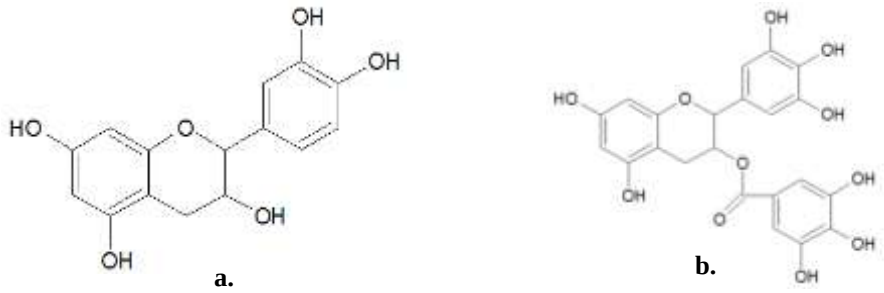

Figure 3. The chemical structure of polyphenols (a. Catechins and b. EGCG)

DPPH radical scavenging activity (RSA)

The antioxidant test method in this study used DPPH (1,1-diphenyl-2-picrylhydrazyl). DPPH is a compound that can trap radical groups that are relatively stable because it contains organic nitrogen. Its physical properties are dark purple with strong absorbance at a maximum wavelength of 517 nm. A standard curve was made by comparing it with the α-tocopherol standard. The IC_{50} of tocopherol was about 6.45 µg/mL. The data above the standard curve shows a close relationship between the concentration and percentage of inhibition. Testing on the polyphenol extracts of persimmon fruit also used the DPPH method. It identified the presence of secondary metabolites belonging to polyphenol groups; one of which is EGCG that has antioxidant potential. The antioxidant activity of this type of polyphenols is characterized by high reactivity as a hydrogen or electron donor. Also, the capacity of polyphenol derivatives can stabilize free radicals by removing unpaired electrons (functioning as chain-breakers) and chelate transition metals. Based on the curve and calculation, the IC_{50} was 44.07±15. 06 mg/mL. According to Jang *et al.* (2010), the antioxidant activity in Korean persimmon fruit reaches 43.36±1.78 mg/mL. Therefore, Indonesia's persimmon also has antioxidant activity that is almost the same as persimmons in Korea. Based on an *in vitro* study by Hartoyo (2002), catechin phenolic groups—both in extracts and single isomeric compounds—can protect LDL from oxidation that is triggered by Cu^{2+} ions. Catechins are a group of polyphenols that have antioxidant activity. However, the mechanism of the inhibition of LDL oxidation by extract or catechin isomer is not known yet (Moore and Tabas, 2011).

The body weight of test rats after hyperlipidemic diet

The average body weight of the rats before and after receiving a high-fat food intake and the administration of persimmon extract is different, as seen in Table II:

Table II. Different body weights in test rats before and after treatment

Groups	The difference in body weight (g)
Normal	59.2 ± 0.84*
Positive control (α-tocopherol)	64.6 ± 1.67*
Negative control	76.8 ± 3.96
Extract 450 mg/kg BW rat	68.6 ± 2.61*
Extract 900 mg/kg BW rat	63.6 ± 1.67*
Extract 1800 mg/kg BW rat	60.6 ± 2.61*

* shows a significant difference with the negative control

Giving α-tocopherol can reduce the weight of the test rats like in the normal group. It is because α-tocopherol has anti-oxidant and antihyperlipidemic potentials. The administration of persimmon extract has been shown to significantly reduce the weight of the rats ($P < 0.05$). Previous research using Korean persimmon juice also proves that this fruit can reduce body weight (Park *et al.*, 2006). In conclusion, the phenolic group contained in the extract of persimmon (*Diospyros kaki* L.) that grows in Indonesia has not only antioxidative properties but also efficacy in countering hyperlipidemia.

Blood lipid levels and characterization of the aorta

The examination of the lipid profile includes total levels, HDL, triglycerides, and LDL. Each of them has a correlation value to the atherosclerosis process. Catechins and EGCG can inhibit the oxidation of LDL and keep lymphoid cells save from the cytotoxic effects of oxidized LDL. Catechins can inhibit the oxidation of blood plasma and act as exogenous antioxidants, such as the antioxidant activity exhibited by α-tocopherol and β-carotene. Accordingly, α-tocopherol is used as the standard of comparison for positive control. The results of the study are described in Table III:

Table III. The lipid profiles (cholesterol, HDL, triglycerides, and LDL) of test rats after a fat diet and the administration of persimmon extract

Group	Cholesterol levels (µg/dL)	Triglycerides (µg/dL)	LDL(µg/dL)	HDL(µg/dL)
Normal	148.80 ± 1.13*	91.58 ± 1.12 *	42.35 ± 3.05 *	88.14 ± 2.09 *
Positive control (α-tocopherol)	176.75 ± 2.05*	129.55 ± 1.58*	75.91 ± 1.33 *	74..94 ±2.89 *
Negative control	206.14 ± 0.95	168.01 ±1.74	137.57 ± 2.18	35.12 ± 1.57
Extract 450 mg/kg BW	175.53 ± 2.31	138.29 ± 1.85*	102.15 ± 2.91 *	45.71 ± 3.00 *
Extract 900 mg/kg BW	161.68 ± 1.32*	114.58 ± 1.75*	72.58 ± 1.86 *	66.24 ± 1.36*
Extract 1800 mg/kg BW	146.68 ± 2.07 *	101.512 ±1.94 *	49.60 ±2.50 *	76.78 ± 2.84 *

* shows a significant difference with the negative control

The cross-sections of the aorta were measured and analyzed from five (5) points of view. The results of the microscopic observation are shown in Figure 4.

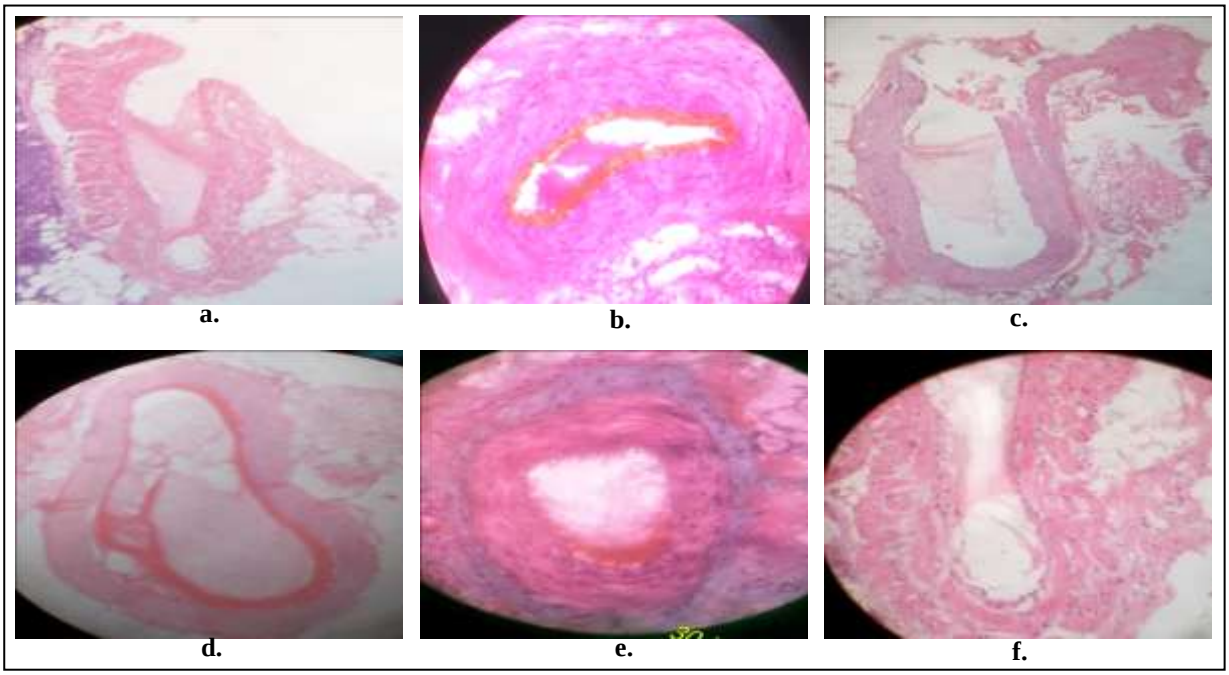

Figure 4. The cross-sections of the aorta belonging to six groups of rats after receiving a fat diet and extract of persimmon (viewed at 100x magnification): a. Normal group, b. Negative control, c. Positive control (*α*-tocopherol), d. Extract 450 mg/kg BW, e. Extract 900 mg/kg BW, and f. Extract 1,800 mg/kg BW

Table IV. The aortic wall thickness of the test rats

Groups	Aortic Wall Thickness (µm)
Normal	448.21 ± 37.03 *
Positive control (α-tocopherol)	541.07 ± 14. 94 *
Negative control	698.21 ± 15.97
Extract 450 mg/kg BW Rats	612.50 ± 23.23 *
Extract 900 mg/kg BW Rats	567.86 ± 14.94 *
Extract 1800 mg/kg BW Rats	480.36 ± 21.32 *

* shows a significant difference with the negative control

Based on the results of the microscopic observations presented in Table IV, the aortic wall of the test rats from the thinnest to the thickest is as follows: the normal group, extract 1,800 mg/kg BW, positive control, Extract 900 mg/kg BW, Extract 450 mg/kg BW, and negative control. When the phenolic extract of the persimmon fruit was given at a dose of 1,800 mg/kg BW, it could reduce the aortic wall thickness to the one found in the normal group ($P< 0.05$). Compared to the negative control group, at this dose, the extract decreased the aortic wall thickness by 31.20%. Meanwhile, the rats in the groups of Extract 450 mg/kg BW, Extract 900 mg/kg BW, and the positive control did not experience a significant thinning in the aortic wall like the normal groups. These groups reduced the aortic wall thickness by, respectively, 12.28%, 18.67, and 22.5%. According to Storey (2018), this finding reflects the cholesterol diet that causes hyperlipidemia in the blood profiles. This diet increases

the levels of LDL, triglycerides, and total cholesterol but decreases the HDL. Compared to the other groups, the thickest aorta was found in the negative control group ($p< 0.05$). LDL oxidation stimulates the occurrence of monocyte adhesion in the endothelium and its accumulation in macrophages. Macrophage cells will eventually manifest into foam cells, which are likely to develop into plaques that attach to the aorta.

The manifestations of these events will thicken the aorta and cause a blockage (embolism), as shown in Figure 5.

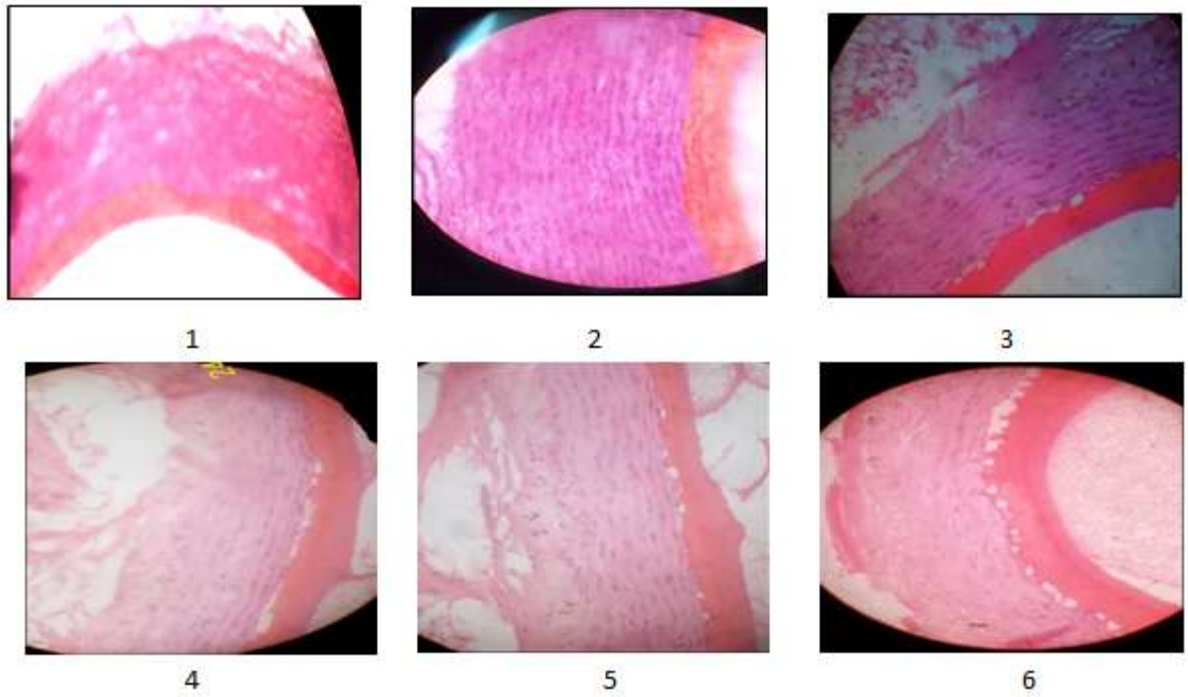

Figure 5. The cross-sections of the aorta at 400x magnification: (1) negative control, (2) normal, (3) positive control (α-tocopherol), (4) Extract 450 mg/kg BW, (5) Extract 900 mg/kg BW, and (6) Extract 1,800 mg/kg BW

Based on these data, there is a correlation between aortic wall thickness and lipid profile values. The highest levels of cholesterol, triglyceride, and LDL, as well as the lowest HDL, were found in the normal group. This finding also corresponds to the thickness of the aortic wall. Increased cholesterol levels in the circulation can cause accumulation of fat on the inner walls of blood vessels known as plaques (Liu *et al.*, 2016). The oxidation of LDL can be recognized by the Sc-R (scavenger receptor) macrophage. The oxidized LDL is removed from the blood through receptor-mediated endocytosis, then accumulates and forms the foam cells. LDL oxidation is cytotoxic to arterial cell walls, and this can induce an inflammatory process of thrombosis. The majority of cells in the arterial cell wall, including endothelial cells, smooth muscle cells, and monocytes (macrophages), can oxidize LDL. LDL oxidation stimulates endothelial cells to secrete monocyte chemotactic protein 1 (MCP-1), which is a monocytic chemotactic factor, and place and accumulate macrophages in one site. Macrophages attach to the endothelial layer through specific endothelial adhesion molecules and, then, migrate from endothelial to subendothelial cells. This process is the sign of the initial process of atherosclerosis (Moore and Tabas, 2011).

Tocopherol and the polyphenol extract of persimmon can inhibit LDL oxidation by chelating

delocalized chains in the compound (Duthie *et al*., 2016). Based on the data, there is no significant difference between the effects of Extract 1,800 mg/kg BW and the treatment received by the normal groups. The aortic wall thickness was observed under a microscope at 400x magnification. Macrophages in the form of foam cells were identified and compared among the groups. In the negative control, the cross sections of the aorta have numerous clearing islands. There is monocyte pressure on the image, which is caused by LDL buildup on the walls of the abdominal aorta. This finding is consistent with the lipid profile that shows the highest cholesterol level in the negative control group. The correlation formed between induced colic acid, palm oil, and cholesterol can increase LDL levels and reduce HDL levels. The positive control and normal groups, based on the histopathological observation, have a similar number of clearing islands (Figure 5), which proves the assumption that α-tocopherol has inhibitory effects on LDL formation, as seen from the few plaques on the endothelial wall of the arteries. It also demonstrates that there is a correlation between aortic thickness, lipid profile, and the risk of atherosclerosis. The histopathological observation showed that persimmon increased the number of clearing islands, which then confirms the previous data that show how a significant increase in the dosage of the extract of persimmon decreases the aortic wall thickness, LDL, and triglycerides but increases HDL. The outcome of the administration of persimmon extract is a lower risk of atherosclerosis. Catechin isomers function as primary antioxidants by reducing the formation of free radicals triggered by Cu^{2+} (Hartoyo, 2002). Catechins can act as chelating agents to activate Cu^{2+} and other metal ions that initiate free radicals. Other research has proved that tea catechins can protect polyunsaturated fatty acid in LDL. Antioxidant activity can be utilized in the prevention of several diseases; one of them is atherosclerosis (Fereira, 2011).

CONCLUSIONS

The phenols identified in persimmon fruit extracts (*Diospyros kaki* L.) are catechins (2.55%) and EGCG (9.49%). Based on the results of the DPPH method, the antioxidant activity of persimmon fruit extract (*Diospyros kaki* L.) is 44.07 ± 15.06 mg/mL. The persimmon extract (*D. kaki* L.) can significantly reduce the oxidation of LDL and prevent atherosclerosis. Also, it can substantially decrease thickening due to the accumulation of foam cells in the aorta.

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