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Histopathological of White Rats Aorta Induced by High-Fat Feed After Administered by Neem Leaf Ethanolic Extract

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Abstract. Neem (*Azadirachta indica* A. Juss) is one of the traditional medicines used by local people as antioxidants, anti-hyperlipidemia, anti-inflammatory, and treatment for other diseases such as heart disease. This study aimed to analyze the effect of neem leaf ethanolic extract on aortic wall thickness and aortic lumen diameter of white rats induced by high-fat feed. The male Wistar rats (*Rattus norvegicus* L.) with 2 months of age were used. This research was an experimental study with a Randomized Complete Block Design consisted of 6 treatments (P0: commercial feed, P1: high-fat feed and duck egg yolk 2.5 ml/200 g BW per oral, P2: P1 + 8 mg/200 g BW simvastatin, P3-P5: P1+ neem leaf extract of 75, 100, and 125 mg/200 g BW respectively) and 4 replications. Aortic preparations were made by the paraffin method and Hematoxylin-Eosin staining. Data were analyzed using ANOVA followed by Duncan Multiple Range Test with 95% confidence level. The result showed that the decrease in wall thickening and lumen narrowing was getting higher along with the increasing doses of ethanol extract of neem leaves. Based on the result of this research, it was found that the high doses of neem leaf ethanolic extract has the same ability with simvastatin to reduce aortic wall thickning and aortic lumen narrowing of white rats. This study is give a new information that the neem leaves can be used as an alternative medicine for cardiovascular disease.

Key words: Aortic Lumen Diameter; Aortic Wall Thickness; High-Fat Feed; Neem; White rats

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INTRODUCTION

Atherosclerosis is the most common cause of ischaemic heart disease and stroke, and accounted for one in four deaths in the whole world (Lozano et al., 2012). The prevalence of atherosclerosis is caused by several factors related to the lifestyle and habits of people who tend to consume high-fat foods (Maryani et al., 2016). Rahmiati et al. (2020) reported that the main source of fat intake comes from fried foods. Cooking oil containing unsaturated fatty acids will be easily damaged if heated repeatedly at high temperatures. This is due to a contact with oxygen from the outside air which facilitates the oxidation reaction with the formation of peroxide and hydroperoxide in oil during the frying process (Ayu et al., 2015).

One of the indicators of oil damage due to prolonged heating can be seen from the formation of free radicals (Zhang et al., 2012). Free radicals that are formed with excess capacity cause oxidative stress conditions (Widayati, 2012). Oxidative stress that occurs due to free radicals is reported to be involved in the emergence of various diseases, one of which is cardiovascular disease (Phaniendra et al., 2015). The initial stage in the pathogenesis of cardiovascular disease is endothelial dysfunction (Lee et al., 2012).

Endothelial dysfunction due to oxidative stress can also increase macrophage cell production. Accumulation of fat, macrophages, and platelets that occur in tunica intima and tunica media causes the walls of blood vessels to become thickened so that the diameter of the lumen will be narrower (Zhou et al., 2016). This is consistent with the research of Subermaniam et al. (2015) that rats fed standard feed with a mixture of 15% cooking oil (5 times frying) experience an increase in the thickness of the tunica intima and media in the aorta compared to the other treatment groups.

The induction of high-fat diets has an adverse effect on blood vessels. Ifora et al. (2016) stated that mice induced with a high-fat feed for 28 days had a narrower aortic lumen diameter due to the thickening of the blood vessel walls. This is because large amounts of fat intake into the body can increase total cholesterol and blood LDL (Low-Density Lipoprotein) (Setiawati et al., 2016). Rats fed a high-fat diet for 30 days had low HDL (High-Density Lipoprotein), while LDL and triglycerides had high levels (Sopandi et al., 2019). Fatimatuzzahro & Prasetya (2018) also proved that rats fed a high-fat diet (3 g pork oil/200 g BW and 2 g duck egg yolk/200 g BW) for four weeks had high levels of total cholesterol and triglycerides. High triglyceride levels cause an increase in LDL cholesterol formation (Tomkin & Owens, 2012).

High-fat levels, especially cholesterol can be reduced using cholesterol-lowering drugs, one of which is simvastatin (Hayatunnufus et al., 2019). Simvastatin is one of the four most important drugs used in the treatment of hyperlipidemia. The mechanism of action of simvastatin is by inhibiting the enzyme HMG-CoA reductase which will competitively inhibit the process of cholesterol biosynthesis in the body (Alakhali et al., 2013). The use of synthetic drugs in the long term can cause side effects such as nausea, itching, headaches, psychological disorders, hair loss, tachycardia, hyperuricemia, and even impaired liver function (Na'i et al., 2019).

Efforts to reduce the side effects of the use of synthetic drugs is by using natural medicines or traditional medicines from plants, one of which is neem. Research conducted by Bisht & Sisodia (2010) proved the anti-hyperglycemic and anti-dyslipidemic potential of neem leaves which can prevent an increase in total cholesterol, triglyceride, LDL, VLDL (Very-Low-Density Lipoprotein), and significantly increase HDL cholesterol levels. This is because neem contains bioactive compounds such as alkaloids, saponins, flavonoid, ascorbic acid, and tannins (Aathira & Suganthi, 2019). This research aimed to analyze the effect of neem leaf ethanolic extract on histopathological of white rats aortic induced by high-fat feed. This study can provide information to the society that the neem leaves with different dosages will not cause toxicity effect on the heart and can be used as an alternative to prevent the cardiovascular disease.

METHODS

High-Fat Feed

High-fat feed consisted of commercial feed and reused cooking oil. The heating process involved using 1 L of the fresh oil to fry 450 g of tofu in a metal pan with deep fat frying technique. The oil was heated 9 times at a temperature of 150-165° C and the cooking process lasted for about 10 minutes (Hanung et al., 2019; Muhartono et al., 2018). High-fat feed were formulated by mixing 30 g of commercial feed with 3 ml reused cooking oil.

Ethanol Extract of Neem Leaf

The neem leaf were obtained from the campus area of the Faculty of Science and Mathematics, Diponegoro University. The leaves were dried in an oven at 40-50º C for 10 days. The extraction of neem leaves had been carried out by maceration method using 70% ethanol as described by Sitasiwi et al. (2017).

Animal Care and Treatment

Twenty-four healthy male Wistar rats, 2 months of age (mean weight of 200 g) were obtained from the Department of Biology Laboratory of Semarang State University, Indonesia. Acclimation was done for one week, commercial feed and water were provided *ad libitum*. This research was an experimental study with a Randomized Complete Block Design consisted of 6 treatments and 4 replications, namely P0: as negative control was given commercial feed, P1: as positive control was given high-fat feed and duck egg yolk 2.5 ml/200 g BW per oral, P2: P1 + 8 mg/200 g BW simvastatin in 1 ml of distilled water, P3 to P5: P1 + neem leaf ethanolic extract with different dosages in 1 ml of distilled water (75 mg/200 g BW, 100 mg/200 g BW, 125 mg/200 g BW). High-fat feed and drinking water were given every morning for forty-five days as much as ± 30 g and 75 ml. Duck egg yolk was given once every two days (morning), while simvastatin and ethanol extract of neem leaf were given every evening orally for forty-four days using a disposable syringe.

Preparations and Staining of Paraffin Sections

The heart of white rats were prepared for histological observation using paraffin method. They were fixed in 10% buffered formalin, processed for paraffin sectioning, and sectioned at ±4 µm. The next following step was dehydration using graded alcohol (80%, 90%, and 95%) for 2 hours of each concentration. Then, the tissues were cleared using xylol I and II for 60 minutes and followed with the infiltration process using the paraffin. The next process was deparaffinization using xylol I and II for 5 minutes of each, and graded alcohol (95%, 90%, and 80%) for 2 minutes of each and rinsed with distilled water. The staining process was using Hematoxylin-Eosin. Slides were put into Hematoxylin for 2 minutes and were rinsed using water. The next step was put the slides into eosin for 5 minutes, then graded alcohol (80%, 90%, and 95%) for 2 minutes and xylol I and II for 2 minutes of each. The last step was the mounting process, namely by applying adhesive to the preparation and covering it with a cover glass.

Observation of Preparations

The thickness of the aortic wall was measured from the tunica intima to tunica adventitia, while the diameter of the aortic lumen was measured from one tunica intima to another tunica intima. All slides were observed under an Olympus BX51 microscope equipped with a photomicrograph at x40 magnification. Measurements of wall thickness and diameter of the aortic lumen were carried out at 2 points which represented wall thickness and overall lumen diameter and the data obtained were then averaged. Scoring

parameters of atherosclerosis were based on Sakamoto et al. (2018) (Tabel 1).

Statistical Analysis

The results of measurements of wall thickness and diameter of the aortic lumen were analyzed by the Shapiro-Wilk normality test (sample size <50) and homogeneity test. Normally distributed and homogeneous data were analyzed using one-way ANOVA at a 95% confidence level using SPSS 16.0 software, then followed by Duncan Multiple Range Test if there were significant differences.

RESULTS AND DISCUSSION

The results of the average analysis of wall thickness and aortic lumen diameter of white rats fed high-fat feed after administered by neem leaf ethanol extract can be seen in Table 2.

Table 1. Type of Atherosclerosis

Parameters	Type
Isolated macrophage, foam cells	I (Initial lesion)
Intracellular lipid accumulation	II (*Fatty streak*)
Grade 2 + small extracellular lipid pools	III (Intermediate lesion)
Grade 2 + core of extracellular lipid	IV (Atheroma lesion)
Lipid core and fibrotic layer, or multiple lipid cores and fibrotic layers, or mainly calcific, or mainly fibrotic	V (Fibroatheroma lesion)
Surface defect, hematoma-hemorrhage, and thrombus	VI (Complicated lesion)

Tabel 2. The average of wall thickness and aortic lumen diameter of white rats

Treatments	Variable	
	The average of wall thickness (µm) ± SD	The average of lumen diameter (µm) ± SD
P0	153.93^{a} ± 16.62	1360.61^{b} ± 75.33
P1	206.72^{c} ± 27.38	1180.22^{a} ± 95.62
P2	167.24^{ab} ± 11.60	1360.51^{b} ± 99.70
P3	196.96^{bc} ± 28.18	1214.85^{ab} ± 114.94
P4	178.12^{abc} ± 22.74	1312.58^{ab} ± 63.92
P5	173.77^{abc} ± 16.78	1340.23^{b} ± 99.70

Note: Numbers with different superscripts in the same column show significant differences between treatments at the level of 95% ($P < 0.05$). P0= negative control was given commercial feed, P1= positive control was given high-fat feed and duck egg yolk 2.5 ml/200 g BW per oral, P2= P1 + 8 mg/200 g BW simvastatin in 1 ml of distilled water, P3= P1 + 75 mg/200 g BW ethanol extract of neem leaf in 1 ml of distilled water, P4= P1 + 100 mg/200 g BW ethanol extract of neem leaf in 1 ml of distilled water, P5= P1 + 125 mg/200 g BW ethanol extract of neem leaf in 1 ml of distilled water.

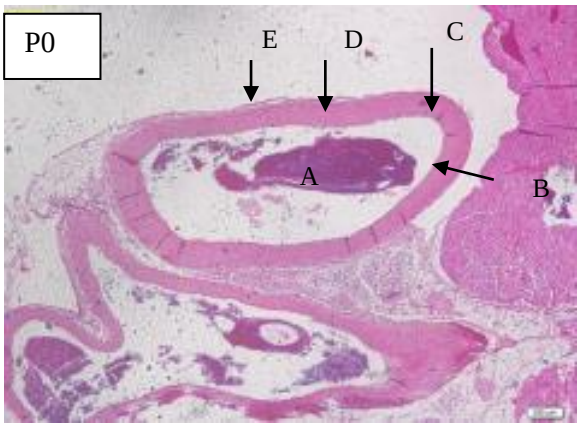

Figure 1. Histological structure of P0 with a magnification of 4x10. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia.

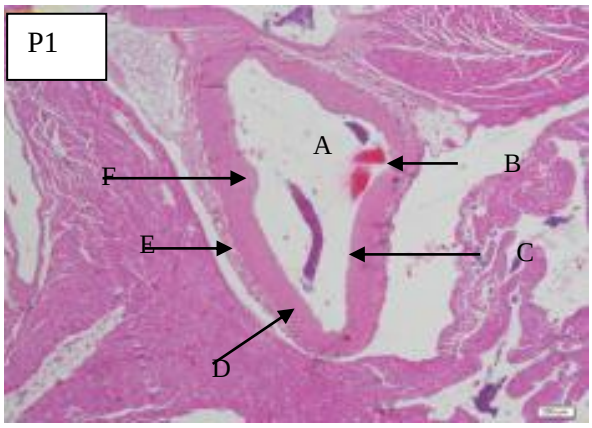

Figure 2. Histological structure of P1 with a magnification of 4x10. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia, F= plaque.

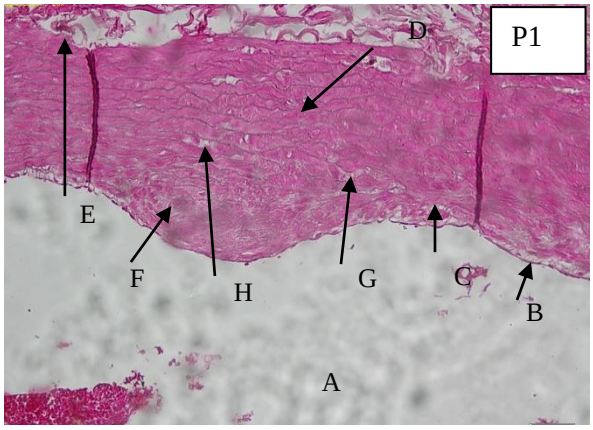

Figure 3. Histological structure of P1 with a magnification of 10x20. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia, F= plaque, G= foam cell, H= accumulation of lipid intracellular.

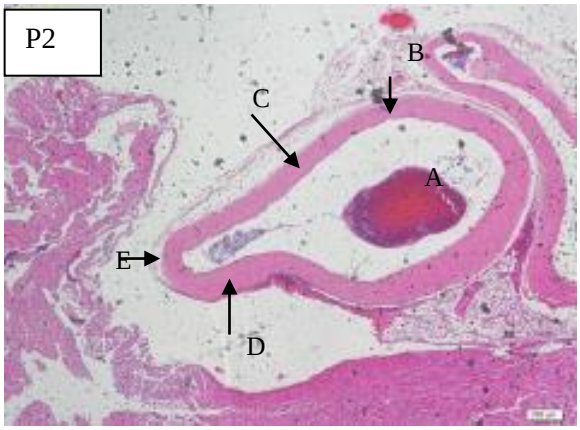

Figure 4. Histological structure of P2 with a magnification of 4x10. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia.

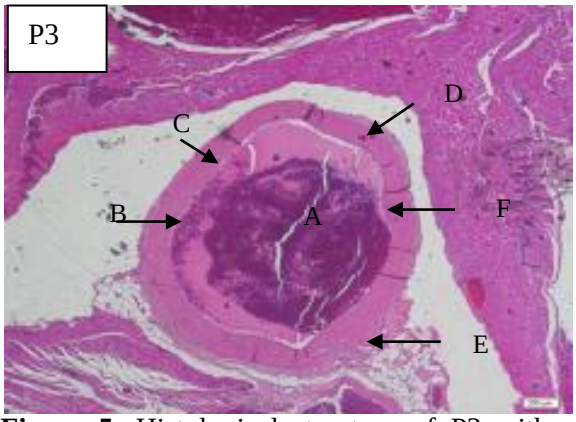

Figure 5. Histological structure of P3 with a magnification of 4x10. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia, F= plaque.

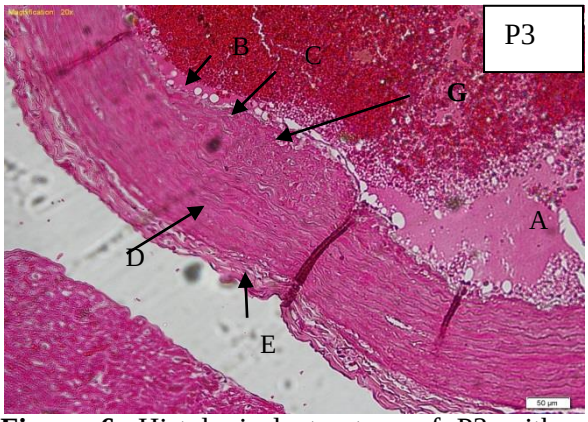

Figure 6. Histological structure of P3 with a magnification of 10x20. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia, G= foam cell.

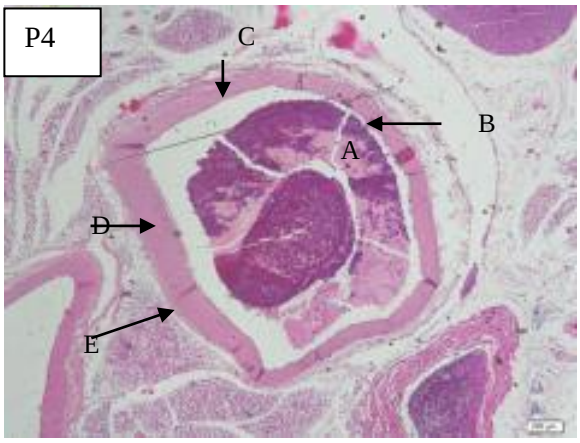

Figure 7. Histological structure of P4 with a magnification of 4x10. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia.

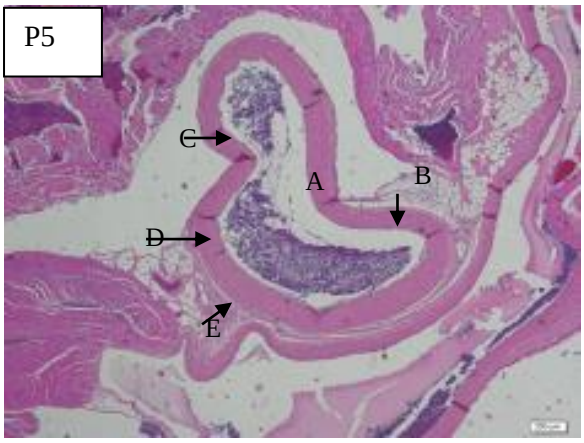

Figure 8. Histological structure of P5 with a magnification of 4x10. A= aortic lumen, B= endothelial cell, C= tunica intima, D= tunica media, E= tunica adventitia.

Results of the analysis showed a significant difference ($P < 0.05$) on the wall thickness and aortic lumen diameter of white rats fed high-fat feed after administered by ethanol extracts of neem leaf. Table 2 shows that the aortic of white rats in the negative control had a lower mean wall thickness and wider lumen diameter, that is 153.93 ± 16.62 µm and 1360.61 ± 75.33 µm compared to positive control groups and other treatments. Histological description of aortic wall of P0 (Figure 1) shows normal histology, meaning there is no accumulation of fat and macrophages and platelets which cause thickening of the walls and narrowing of the lumen of blood vessels. Based on the criteria adopted from previous studies (Sakamoto et al., 2018), the aortic histology of negative P0 was not found in the grading of atherosclerosis. This shows that the standard feeding and drinking water for 45 days has no influence on the aortic histopathology of white rats.

Based on Table 2, the positive control obtained the highest average wall thickness (206.72 ± 27.38 µm) and the narrowest average lumen diameter (1180.22 ± 95.62 µm). Microscopic observation of the aorta in the positive control group (Figure 3) showed the presence of foam cells and intracellular lipid accumulation of smooth muscle. Based on the criteria adopted from previous studies (Sakamoto et al., 2018), mice in group P1 can be categorized into Type II atherosclerosis because there is accumulation of smooth muscle intracellular lipids. This shows that the feeding of high-fat feed and duck egg yolk of 2.5 ml/200 g BW in rats for 45 days is thought to cause thickening of the wall and narrowing of the aortic lumen.

The changes in the aortic wall and lumen diameter in this study were thought to be due to the induction of high-fat feed and reused cooking oil. Christianty et al. (2020) stated that in aortic rats induced with a high-fat diet from a mixture of duck eggs and reused cooking oil with a ratio of 7:3 for 21 days showed the presence of foam cells and endothelial layers that are not neatly arranged. Similar results (irregular endothelial layer, the presence of foam cells, and intracellular lipid accumulation) were also found in the P1 group.

Repeatedly heating the oils at high temperatures will cause the amount of trans fatty acids to increase (Brühl, 2014). Sartika (2011) proved that trans fatty acids from fried foods caused an increase in triglycerides and LDL. Atherosclerosis has an association with cholesterol metabolic disorders, an increase in total cholesterol, triglycerides, and LDL cholesterol (Nirosha et al., 2014; Yu et al., 2013). Nelson (2013) stated that an increase in blood lipid levels such as total cholesterol, LDL, and triglycerides is a condition of hyperlipidemia. Induction of high-fat feed and duck egg yolk as much as 2.5 ml/200 g BW in the P1 group is thought to be the cause of high cholesterol. Isdadiyanto et al. (2020) proved that mice induced with high-fat feed and 2.5 ml of duck egg yolk/200 g BW for 45 days had higher total cholesterol, LDL, and triglyceride.

High LDL levels can also cause an increase in the number of LDL particles that enter the sub intima of blood vessels in predilection areas. LDL is then captured by macrophages by binding to LDL receptors, because the capacity of macrophages to capture LDL is limited, the number of sub intima LDL particles increases. This condition results in several remaining LDL particles which will be oxidized by macrophages and smooth muscle to produce mo-LDL (mildly oxidized LDL) or LDL-ox ions (Sakakura et al., 2013). LDL-ox is then captured by macrophages through the ScR receptor continuously and turns into foam cells (Linna, 2014). In the next process, proinflammatory cytokines such as Tumor Necrosis Fac-

tor-α (TNF-α) will be secreted in large quantities (Oguntibeju, 2019). TNF-α induces a decrease in Nitric Oxide (NO) resulting in vasoconstriction, increased proliferation of Vascular Smooth Muscle Cells (VSMCs), and platelet aggregation (Urschel & Cicha, 2015). The final process that occurs is the formation of a fibrous cap that can occasionally rupture and cause blockages in blood vessels (Esenwa & Elkind, 2016).

Simvastatin used in this study was an HMG-CoA reductase inhibitor which is more effective in reducing total cholesterol and blood LDL (Gravatt et al., 2017), can reduce triglyceride levels (Ontawong et al., 2019), and is used to prevent cardiovascular disease (Reiter-Brennan et al., 2020). The results in Table 2 show that the aorta of P2 had the second-lowest average wall thickness (167.24 ± 11.60 μm) and the second most extensive lumen diameter (1360.51 ± 99.70 μm) after negative control (P0) compared to group P1. Histological description of aortic wall of P2 (Figure 4) shows normal histology, no macrophages or foam cells were found, and no histological preparations included in the grading atherosclerosis. This shows that feeding high-fat feed and 2.5 ml duck egg yolk together with simvastatin for 44 days in mice can reduce the risk of atherosclerosis.

The treatment of P3, P4, and P5 can prevent wall thickening and aortic lumen narrowing compared to P1 group. The decrease in wall thickening and lumen narrowing occur along with the increased dose of ethanol extract of neem leaf. Histological description of aortic rats in the treatment P3, P4, and P5 presented in Figure 6, Figure 7, and Figure 8 sequentially experiencing a better development. Histological of aortic wall of P3 and P4 showed thickening, but not as bad as the histological of aortic wall of P1. The presence of thickening of the aortic wall in P3 and P4 is suspected because the administration of ethanol extract of neem leaves at doses of 75 and 100 mg/200 g BW for 44 days has not been able to prevent the accumulation of fat, macrophages, and platelets in the tunica intima and tunica media. However, the histological of aortic wall of P5 (Figure 8) did not show any changes in blood vessel walls. This means that the neem leaf ethanol extract 125 mg/200 g BW has almost the same effectiveness as simvastatin, which can reduce wall thickening and the aortic lumen narrowing.

Tembe-Fokunang et al. (2019) stated that the occurrence of heart and blood vessel disease which is triggered by high cholesterol levels can be lowered using neem leaves. Isdadiyanto et al. (2020) reported that rats induced by high-fat feed after administered by the ethanol extract of neem leaves had high HDL cholesterol levels and low triglyceride and LDL cholesterol levels. This is thought to be due to the role of phytochemicals in neem leaves.

The results of phytochemical screening of neem leaves with 70% ethanol solvent conducted at the Diponegoro University Integrated Laboratory showed that the neem leaves contain bioactive compounds such as tannins, saponins, alkaloids, triterpenoids, flavonoids, and ascorbic acid (vitamin C). Islam et al. (2012) also reported the presence of quercetin-3-o-beta-d-glucopyranoside in neem leaves. The quercetin content in neem leaves can increase reverse cholesterol transport (Cui et al., 2017) which transports excess cholesterol from peripheral tissues to the liver and small intestine for excretion so that blood cholesterol levels decrease (Fisher et al., 2012).

Ahmad et al. (2019) reported that flavonoids as natural antioxidants have an important role in cleaning free radicals and preventing degenerative diseases such as cardiovascular disease. The content of other compounds of neem leaves which have activities related to cardiovascular disease is ascorbic acid or better known as vitamin C. Vitamin C has a function to increase endothelial vasodilation so that it can prevent endothelial dysfunction which is one of the triggers for atherosclerosis (Airaodion et al., 2019).

Neem leaf as one of the traditional medicine, needs to be researched and optimized for its use. Future studies should be carried out to find the concentration of each phytochemical in neem leaves so that the compounds that have an active role in preventing atherosclerosis can be identified. In the laboratory, ethanol extract of neem leaves has been shown to reduce aortic wall thickening and aortic lumen narrowing. Therefore, this study provides the information regarding the potency of local plant sources such as neem leaves for the treatment of cardiovascular disease.

CONCLUSION

Ethanol extract of neem leaf had a good effect on the histopathology of white rats aortic which were fed high-fat feed. The results of the present study showed that the administration of ethanol extract of neem leaf on white rats induced by high-fat feed can reduce aortic wall thickening and aortic lumen narrowing. Doses 125 mg of neem leaf ethanolic extract have the same ability with simvastatin to reduce aortic wall thickening and aortic lumen narrowing.

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