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The Ethanolic Extract of Holy Basil Leaves (*Ocimum sanctum* L.) Attenuates Atherosclerosis in High Fat Diet Fed Rabbit

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Abstract. Atherosclerosis is the etiology of coronary heart disease (CHD) that contributes to the highest mortality rate of non-communicable disease groups in Indonesia. The early stage of this process is marked by the presence of fatty streaks lesion in the artery wall. This study aimed to investigate the effect of holy basil leaves (*Ocimum sanctum* L.) to suppress the formation of the fatty streak as previous study prove the reduced lipid levels, ROS, and inflammation after treated with this extract. 25 adult male New Zealand rabbits age 4-month weight 2500-3000 g fed with a high-fat diet (HFD) and 10 mg/kgBW/day Holy Basil Extract (HBE), 25 mg/kgBW/day HBE, 50 mg/kgBW/day HBE. The negative control group was received only HFD. For the experimental standard, one group provided with standard diet rabbits were included. The fatty streak was measured in aorta wall after 45 days of treatment by immunohistochemistry method and quantified using Image J software. The results showed that there was no significant difference in the fatty streak area between each group ($p > 0.05$). Still, there was a trend of decreasing fatty streak area dose dependent manner (negative control 5802.21 + 3690.41 μm^2, P1 5154.69 + 3990.79 μm^2, P2 4938.31 + 3690.18 μm^2, and P3 3611.68 + 4092.96 μm^2) even though it was not statistically significant ($p > 0.05$). This paper conclude that holy basil leaves extract may exert anti-atherosclerosis inhibition through attenuation of fatty streak formation.

INTRODUCTION

Mortality rates of non-communicable disease worldwide are expected to reach 52 million deaths by 2030, and currently, cardiovascular disease contributes to the highest mortality rates [1]. In 2008, World Health Organization (WHO) mentioned that there were 17.3 million deaths caused by cardiovascular disease, in which 7.3 million (42.2%) of them were caused by coronary heart disease (CHD) [2]. Currently, one of the research focus on cardiovascular is the prevention of CHD. Coronary heart disease is caused by inadequate blood and oxygen supply to the myocardium due to atherosclerotic lesions in the coronary arteries [3]. Atherosclerosis is a chronic inflammatory process that affects moderate and large blood vessels in the cardiovascular system. Atherosclerosis is initiated by increased blood lipid levels and high levels of ROS (Reactive Oxygen Species) in blood vessels that can cause endothelial dysfunction [2]. This process makes LDL (Low-Density Lipoprotein) easier to enter the intima tunica, and ROS will modify LDL into oxLDL (oxidized LDL) in the subendothelial space. This oxLDL will make endothelial activation that is characterized by the expression of selectin and integrin. The selectin and integrin expression will attract leukocytes to enter the intima tunica and will be converted into macrophages by GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor) [4].

Macrophages that phagocyte oxLDL will turn into foam cells can be microscopically seen as intimal macrophages that have died and contain cholesterol esters. The accumulation of foam cells can be seen as a fatty streak. Fatty streak consists of foam cells full of fat, but it does not disturb the blood flow. This lesion is started by the presence of flat yellow dots with a <1 mm diameter and then fused to form patches that extend to 1 cm or more. Fatty streak formation that can not be inhibited will turn into advanced atherosclerotic lesions, and when this lesion ruptures, it can be CHD

[4-6]. Coronary heart disease can be prevented by reducing atherosclerosis risk factors through lipid profile reduction, ROS inhibition, and inflammatory inhibition that can be achieved through lifestyle modification, such as not smoking, healthy diet, regular exercise, and the use of pharmacological agents such as statins. Statins are chemical drugs that can be used as inhibitory agents in CHD, but it have side effects including musculoskeletal complaints, gastro-intestinal discomfort, fatigue, liver enzyme elevation, peripheral neuropathy, insomnia, and neurocognitive symptoms [7]. To avoid these side effects, researchers made innovation through traditional medicine because it was easy to obtain, inexpensive, and had relatively lower side effects. WHO also recommends the use of traditional medicine for the maintenance of public health, prevention and treatment of diseases, especially in degenerative diseases, chronic diseases, and cancer [8]. The use of traditional medicine can be done by utilizing herbal plants.

One of the suspected plants to be beneficial in the prevention of CHD is holy basil (*Ocimum sanctum* L.). Holy basil is an important sacred medicinal plant from India and has the highly complex chemical composition, containing many biologically active phytochemicals. The volatile oil of the leaf contains eugenol (1-hydroxy-2-methoxy-4-alilbenzene), ursolic acid, carvacrol, limatrol, caryophyllene, and methyl carvicol [9]. Holy basil leaves also contains orientin (Ot) and vicenin (Vc) known to protect the body from the effects of radiation through its antioxidant activity [10]. Eugenol also acts as an anti-inflammatory through the activity of cyclooxygenase-1 inhibitors up to 97% at concentrations of 1000 μM [11].

Previous studies have reported that holy basil leaves extract plays a role in significantly reducing cholesterol and inhibiting lipid oxidation, causing atherosclerosis [12]. Another research showed that administration of holy basil leaf extract attenuated the high serum lipid profile and atherogenic index on hypercholesterolemia rats, also can slightly reduce the liver weight [13]. The study of holy basil leaves extract against atherosclerosis in this study was conducted on rabbit because it has the same lipoprotein and fat metabolism as humans. This equation includes major plasma lipoproteins such as LDL, high density lipoprotein (HDL), low excretion of bile acids, and being sensitive to dietary cholesterol [14]. The use of rabbits in similar studies is still rarely done, because the majority of experimental animals use as a model of atherosclerosis are mice. Based on the literature studies results, mice can not be used as animal models of atherosclerosis because they have significant differences in lipoprotein and human metabolism. This difference is like homogeneous mice HDL, and the ability of mice to excrete bile acids derived from cholesterol is much higher than humans and rabbits [14-16].

This study aims to prove the effect of holy basil leaves ethanolic extract in inhibiting atherosclerosis based on fatty streak area that has never been done in previous studies. Holy basil leaves in this study was dissolved using ethanol because it is a universal solvent that is good for extraction. Ethanol has lesser toxicity potential than other organic solvent and can dissolve all types of compounds such as nonpolar and polar [17].

EXPERIMENTAL DETAILS

The holy Basil leaves was retrieved from Materia Medica Batu Indonesia. The simplicial was diluted in ethanol 1:10. The extraction were performed using UAE (Ultrasonic Assisted Extraction) 3 serial with 2 minutes for each duration in 20 – 2000 kHz to increase the cell wall permeability, so that the internal material will come out [18]. The supernatant was filtered, collected and evaporated within a rotary evaporator at 45.4 °C. The extract was dried in the oven at 40°C and was kept at room temperature in laminated vacuum-sealed packaging until use [19].

This research was an experimental study with a post-test only control group design. The 25 males new zealand white rabbits, 4 months of age, and body weight 2.5-3 kg were obtained from UIN Laboratory Animal (Malang, Indonesia). The rabbits were provided with a standard diet and distilled water ad libitum and housed at 21-22 °C, 50% relative humidity, and exposed to 12 h light-12 h dark cycles [20]. After one week's acclimation, the rabbits were divided randomly into 5 groups (*n* = 5) as follows a) Group 1 (normal) was given normal diet; b) Group 2 (negative control) was received a high fat diet (HFD) only; c) Group 3 was administered a HFD and and holy basil leaves extract (HBE) 10 mg/kgBW/day; d) Group 4 was administered a HFD and 25 mg/kgBW/day of (HBE); and e) Group 5 was administered a HFD and and 50 mg/kgBW/day (HBE). The ingredient composition of HFD was as follows: Nova pellets containing 3% of fat and 2% of cholesterol obtained from cow brains [21]. The cow brain was prepared by weighing it first, then blending it and administering it to the rabbit orally once a day. The weighed (HBE) was put into a hard shell capsule and given to rabbits once a day every 8.30 am. On the 46th day, the rabbits were sacrificed with chloroform inhalation. All experiments were approved by the Institutional Animal Care and Use Committee (No. 006-KEP-UB-2020) of Brawijaya University, Malang, Indonesia.

The aortas were isolated after termination and were sliced into 8-μm-thick transverse sections. The section was stained with Hematoxylin Eosin and observed under a Nikon Eclipse E200 microscope using 400× magnification in

5 fields [22]. The results of observations (fatty streak area) were captured and measured using ImageJ software [23]. In this research, data collection methods were observation and quantitative data with ratio scale of fatty streak area of rabbit aortic arch preparations. The data obtained were tested for normality and homogeneity as a condition for conducting One Way ANOVA test. The results of One Way ANOVA test were not significantly different, so no further tests such as Post Hoc and Pearson correlation tests.

RESULTS AND DISCUSSION

The Effect of Atherogenic Diet and *Ocimum sanctum* L. to Rabbit Weight

The bodyweight of HFD fed rabbits in each group was weighed before and after being treated. The normality test of weight data was normally distributed. Furthermore, weight data were tested by paired sample *t*-test to compare the average body weight before and after treatment in each group. The test results can be seen in Figure 1.
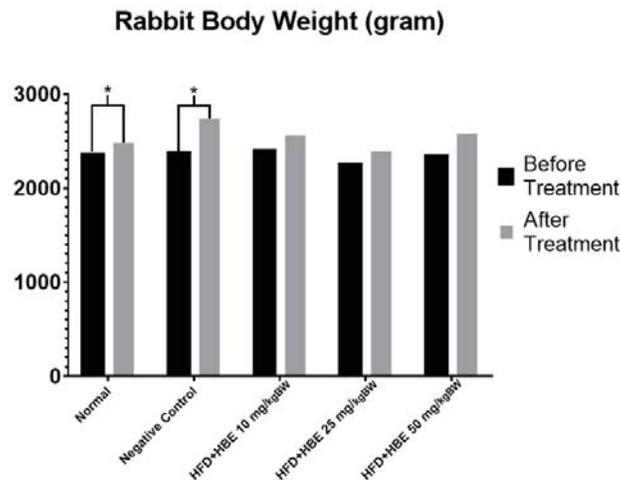

FIGURE 1. The average weight gain of rabbits in each group (before and after treatment). HFD: High Fat Diet; HBE: Holy Basil Extract. The * sign indicates p-value<0.05, and bar without * sign indicates p -value>0.05.

Based on Figure 1, there was a significant difference between body weight before and after treatment only in normal and negative control group. The results of this study indicated that the HFD can affect rabbit's body weight, as evidenced by the results of paired sample *t*-test which showed that there were differences in rabbit's body weight before and after treatment in group negative control with p-value 0.007 (significantly different). This findings clearly supports the sensitivity of rabbit to the HFD because rabbits can not increase sterol excretion, so that it increased the mobilization of lipoprotein-rich ester cholesterol from liver to circulation [14]. Thus, the excess of fat is stored mainly in adipocytes, subcutaneous tissue, and intraperitoneal cavity [24]. Interestingly, the increase of body weight tends to decrease with HBE administration though no significant difference between body weight before and after treatment in each group which can be seen from p-value 0.149, 0.465, and 0.090, respectively. This statement was suitable for other studies about clinical trials of holy basil leaves extract to obese patients, which showed that administering 250 mg of holy basil leaves extract twice a day for eight weeks significantly improved lipid profiles and BMI (Body Mass Index) [25]. The content of holy basil leaves extract, which was known to be associated with weight loss in rabbits, was an antioxidant that can reduce cholesterol, triglyceride, LDL levels, and increased HDL through the inhibition mechanism of 3-Hydroxy-3methylglutaryl Coenzyme A (HMG-CoA) reductase which acted as a catalyst in cholesterol formation. This process decreased the forming cholesterol process, triglycerides, very low density lipoprotein (VLDL), and significantly increase HDL and total fecal sterol contents, reducing the amount of subcutaneous or visceral adipose in the body [12]. The difference in weight results for rabbits can be influenced by several factors, such as rabbit's physiological condition due to saturation of atherogenic diets and organoleptic conditions of rabbits.

The Effect of Atherogenic Diet and *Ocimum sanctum* L. on Fatty Streak Area of Aortic Arch Rabbit

Fatty streak area of each rabbit was measured in 5 visual fields at a magnification of 400× using software ImageJ that has been calibrated according to the magnification of the microscope used. Results from the calculation of the average fatty streak area are shown in Table 1.

TABLE 1. Average of fatty streak area

Groups	Fatty Streak Area (μm^2)
Normal	2245.42 ± 1007.15
Negative control	5802.21 ± 3690.41
HFD + HBE 10 mg/kgBW/day	5154.69 ± 3990.79
HFD + HBE 25 mg/kgBW/day	4938.31 ± 3690.18
HFD + HBE 50 mg/kgBW/day	3611.68 ± 4092.96

Based on statistical calculation, the results showed that each group had p-value: 0.301, 0.746, 0.302, 0.969, 0.137 respectively, and the homogeneity test showed p-value 0.268 ($p > 0.05$) which indicates that the data was normally distributed and homogeneous. These results met the requirements for One Way ANOVA test. Based on One Way ANOVA test, *p*-value was 0.627 ($p > 0.05$), which indicated that there was no significant difference from the average area of fatty streaks for each group. The microscopic view of fatty streak area from each group can be seen in Figure 2.
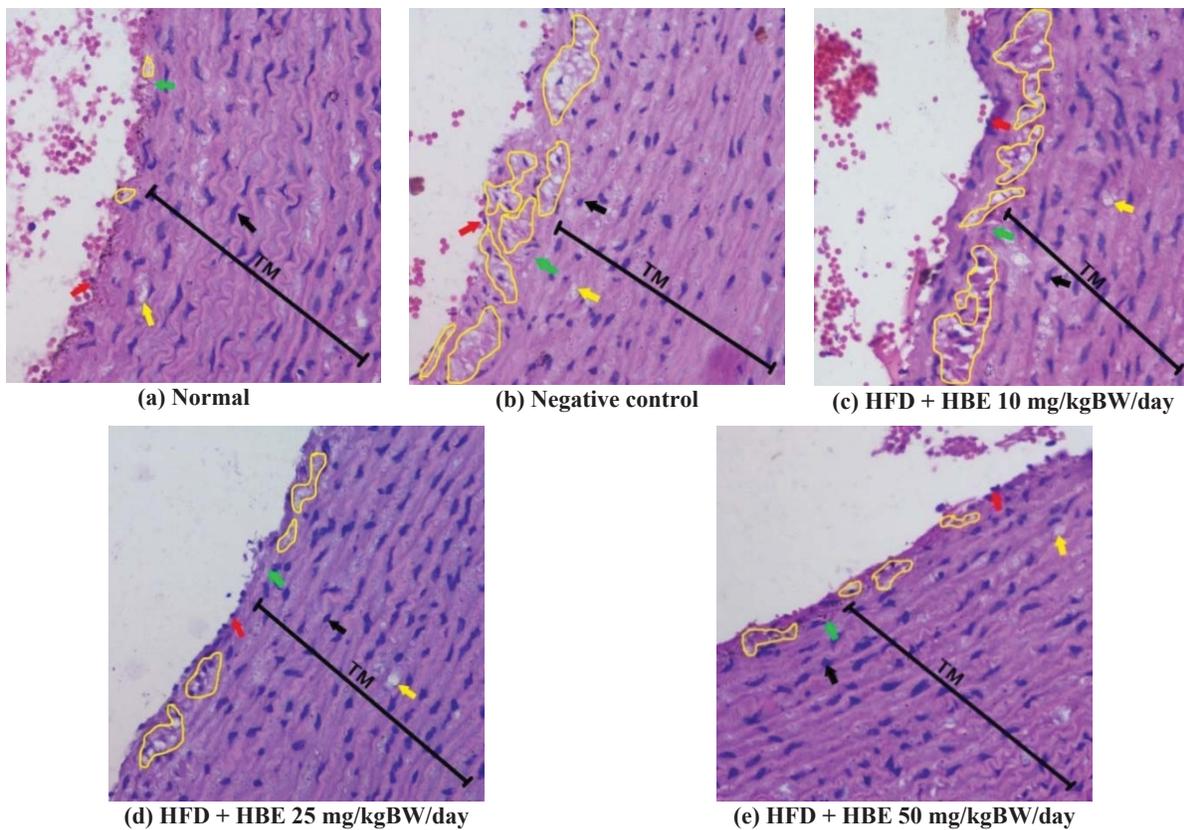

(a) Normal

(b) Negative control

(c) HFD + HBE 10 mg/kgBW/day

(d) HFD + HBE 25 mg/kgBW/day

(e) HFD + HBE 50 mg/kgBW/day

FIGURE 2. Histopathological overview of fatty streak area in rabbit aortic arch (400× magnification with 68 μm scale)

The images presented were one field from each group with H&E staining and 400× magnification. Red arrows indicated endothelial cells, yellow lines indicated fatty streaks, green arrows indicated internal elastic lamina separating tunica intima from tunica media (TM), yellow arrows indicated foam cells, and black arrows indicated smooth muscle cells.

Figure 2a showed endothelial cells and a thin fatty streak area in the tunica intima (separated by internal lamina elastica), accompanied by foam cells and smooth muscle cells in tunica media arranged horizontally towards the aortic lumen. Figure 2b showed endothelial cells and a large fatty streak area so that the intima was thickened and damaged, accompanied by foam cells and smooth muscle cells in the tunica media that were arranged horizontally towards the aortic lumen. Figure 2c showed the endothelial cells and a thinner fatty streak area with a slight thickening of the intima which was still intact, accompanied by foam cells and smooth muscle cells in the tunica media arranged horizontally towards the aortic lumen. Figure 2d showed endothelial cells and a thinner fatty streak area in the tunica intima, which had slight damage but showed progression to normal, accompanied by a small number of foam cells and smooth muscle cells in the tunica media arranged horizontally towards the aortic lumen. Figure 2e showed endothelial cells and a little fatty streak area in the tunica intima, which was still intact and shows a progression to normal, accompanied by foam cells and smooth muscle cells in the tunica media that are arranged horizontally towards the aortic lumen. Fatty streak consisted of many foam cells with have no significant elevation, so they did not interfere with blood flow. Microscopically, fatty streak consisted of accumulating foam cells, smooth muscle cells, or T lymphocytes in smaller numbers [6]. Figure 2 showed that the largest fatty streak area was Figure 2b (group negative control), while the smallest fatty streak area was Figure 2a (group normal). As for Figure 2c (group HFD + HBE 10 mg/kgBW/day), there was a decrease of fatty streak area compared to group 2b, but it was still larger than Figure 2d (group HFD + HBE 25 mg/kgBW/day) and Figure 2e (HFD + HBE 50 mg/kgBW/day).

Rabbits were sensitive to atherosclerosis induced by dietary cholesterol because rabbits could not increase sterol excretion, thus increasing the mobilization of lipoprotein-rich cholesterol esters from the liver to circulation. This condition will increase atherogenic lipoproteins and decrease lipoprotein receptors, which caused atherosclerotic lesions [26]. One of the atherogenic diet foods was cow brain (100 grams of cow brain contains 2 g cholesterol) and Nova pellets (3% fat) [21], which is proven to be used to induce atherogenic animal models in rabbit aorta which could be seen from our findings that demonstrated the average fatty streak area of the atherogenic diet group negative control was 5802.21 μm^2 which was higher than the average fatty streak area of normal group (2245.42 μm^2). Those results were suitable for another researcher who stated that an atherogenic diet of 0.3-2% cholesterol plus 4-8% fat could cause hypercholesterolemia and the formation of atherosclerotic lesions in aorta [14].

The formation of this fatty streak could be caused by high cholesterol and fat consumption, which can lead to hypercholesterolemia and hyperlipidemia. Hypercholesterolemia will lead to metabolic oxidative stress by increasing lipid profiles and decreasing endogenous antioxidants [27]. Hyperlipidemia can increase oxygen free radicals and inactivate NO (Nitrate Oxide). This condition caused the endothelial function to be disrupted and lead to chemical changes of fat, resulting in oxidation of LDL. LDL oxidation can also trigger an inflammatory response and stimulate vascular cells to produce MCP-1, IL-6, IL-8, VCAM-1, ICAM-1, and E-selectin, which can recruit monocytes into the intima, and cause monocyte adhesion and T lymphocytes. Monocytes will turn into macrophages due to the presence of M-CSF, which is secreted by endothelial cells and smooth muscle cells. Low-density lipoprotein, which has been wholly oxidized, will bind to the scavenger receptor macrophage so that if it occurs continuously, macrophages can turn into foam cells. The accumulation of foam cells with lipid-laden monocytes and T lymphocytes will form fatty streaks [28,29].

This study showed that holy basil leaves extract could reduce fatty streak area in rabbit aorta, which was observed histopathologically though not statistically significant. It was clear that there was a trend of decreasing fatty streak area with the increase of HBE administration. Previous research using a larger dose variation did produce better results in terms of triglyceride and total cholesterol parameters [30]. Drug consumption needs to be further explored until it reaches a steady-state dose, which widens the dosage range [31]. The holy basil leaves mechanism that decreases fatty streak area is based on its ability to reduce blood lipid levels, liver, and aorta. Holy basil leaves are also known to increase faecal sterol excretion and inhibit cholesterol biosynthesis [13]. The pharmacological effects of holy basil leaves are mainly due to the antioxidant content. There are three antioxidant groups in holy basil leaves, such as phenolic acids, flavonoids, and tannins. The main antioxidant component in holy basil leaves is essential oil containing eugenol (total 65.31%) [32].

Those compounds are cardioprotective antioxidants and can prevent atherosclerosis by inhibiting LDL oxidation, which plays an essential role in the atherosclerosis process. Apart from the inhibition of LDL oxidation, these antioxidants are known to inhibit smooth muscle cell proliferation, macrophage migration, and anti-inflammatory [33]. Previous research showed that giving holy basil leaves extract could decrease the total cholesterol levels in hypercholesterolemia rats model [12]. Another study also showed that supplementation of holy basil fresh leaves led to a significant decrease in homocysteine, total cholesterol, triglyceride, LDL, VLDL and a significant increase in HDL levels. This result was probably due to the essential oil content in holy basil leaves [34].

Research by Suanarunsawat showed that experimental mice fed a high cholesterol diet without holy basil leaves treatment could cause multifocal degeneration, necrosis, and disorientation of smooth muscle cells in aortic tissue. This result was inversely proportional to the group given holy basil leaves extract, which showed no lesions in the aortic tissue [13]. Previous studies have shown that some medicinal plants had an antioxidative activity that could reduce the production of lipid peroxide, so it will prevent atherosclerosis and protect organs at risk from hyperlipidemia [32]. The study results by Suanarunsawat also proved that holy basil leaves extract not only reduced serum and hepatic lipids but also decreased serum AST, ALT, LDH, and CK-MB. Besides, there was an emphasis on lipid peroxidation, where the activity of antioxidant enzymes was increased in mice liver and heart tissue with holy basil leaves treatment. This study was suitable for various previous studies, where holy basil leaves extract could inhibit the formation of the fatty streaks according to the dose given [13].

The limitations of the study were as follows (1) the weaknesses of treatment and handling of rabbits, (2) doses range of holy basil leaves extract given; (3) duration of the extract treatment. Making animal models of atherosclerosis rabbits requires special techniques and high hygiene. In this study, several obstacles such as cow brain feed that could not enter the rabbit's mouth correctly due to the difficulty administering to rabbits, and the difference in the number of Nova pellets eaten by every rabbit. The amount of rabbit feed consumption is highly dependent on body size, genetic characteristics (breed), environmental temperature, production levels, housing, quality, and quantity of feed and disease [35].

The extract doses given in this study may also undergo several processes during administration, absorption, distribution, metabolism, and excretion. The administration is carried out orally using capsules where the rabbit chews it, which can cause the amount of extract to be reduced, and the habit of rabbit who often vomits part of the capsule. Holy basil leaves extract during the distribution process may not be wholly absorbed because pills are given only 30 minutes after giving the cow's brain. The presence of food in the stomach will slow down the emptying time so that the drug becomes challenging to absorb [36]. However, there was no data regarding the metabolism and excretion of holy basil leaves extract so that these two factors may reduce the levels of active compounds from holy basil that will reduce its effectiveness. Holy basil leaves extract tends to decrease fatty streak area according to the addition of doses (10, 25, and 50 mg/kgBW/day).

SUMMARY

Collectively our findings indicate that administration of holy basil leaves extract may potentially inhibit the fatty streak formation in HFD fed Rabbit. Further studies need to be performed to provide strong evidence confirming these findings and also to explore the pathway involved.

ACKNOWLEDGMENTS

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