

RESEARCH STUDY

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Apo-B Levels and Abdominal Aortic Wall Thickness in Hypercholesterolemic Rats Treated with Red Guava Fruit

Kadar Apo-B dan Ketebalan Dinding Aorta Abdominalis Tikus Hiperkolesterolemia dengan Perlakuan Buah Jambu Biji Merah

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ABSTRACT

Background: Coronary heart disease (CHD) is mainly related to hypercholesterolemia. Sometimes CHD occurs in people with normal cholesterol. Therefore, it is necessary to study other factors that cause CHD: apolipoprotein B (apo-B). Atherosclerosis is a marker of CHD, characterized by the thickening of the walls of blood vessels and the narrowing of blood vessels. Non-pharmacologically, CHD can be managed by consuming foods with high fiber and antioxidants, such as red guava fruit.

Objectives: This research aimed to analyze the consequence of red guava fruit on the Apo-B levels and the thickness of the abdominal aortic wall in hypercholesterolemic rats.

Methods: The research design was a pre and post-test and a randomized control group. Fifty hypercholesterolemic adults male Sprague Dawley rats were given red guava fruit. The primary treatment used red guava fruit as a treatment group, referring to the fiber requirement of 38 g/day, and simvastatin as a positive control group based on a human dose of 10 mg/day, the conversion of human to mouse dose, according to Laurence-Bacarach is 0.018. The negative control group was hypercholesterolemic rats. The standard feed for the rats was based on AIN93. The Enzyme-linked Immunosorbent Assay method measures apo-B levels, Kit For apo-B *Rattus norvegicus*, Catalog No. E92003Ra, produced by Uscn Life Science Inc. The aorta was taken after going through general anesthesia and thoracotomy. Furthermore, the aorta was processed for making histological preparations with hematoxylin-eosin (HE) staining to observe histopathological changes.

Results: Observation of aortic wall thickness using a microscope with a magnification of 400X. Simvastatin and red guava fruit have reduced Apo-B by 7% and 6%, respectively.

Conclusions: The performance of red guava fruit in reducing Apo-B concentration and the thickness of the abdominal aortic wall was equal to the simvastatin.

INTRODUCTION

Cardiovascular health is a set of risk factors for myocardial infarction and stroke, the physiological function of the cardiac, artery, and vein. Cardiovascular disease (CVD) is the primary basis of death globally. More than 75% of deaths due to heart disease are in the under and developing countries¹. CVD2 caused thirty-five percent of mortality in Indonesia. Cardiovascular disease included: (1) coronary heart disease (CHD) showed by angina pectoris or myocardial infarction (MI), (2) cerebrovascular disease revealed by ischemic attack and stroke, (3) high blood pressure, (4) peripheral artery disease, and (5) death by any of the above causes³. Hypertension, cigarette smoking, diabetes mellitus, elevated cholesterol levels, and obesity are the top six causes of death globally due to CHD⁴ being the primary cause of mortality (37%) in Indonesia, particularly in poor communities⁵. The highest cases are in North Kalimantan⁶.

Most cases of stroke and MI are related to the fatty deposits on the inner walls of the blood vessels that supply oxygen to the brain or heart. Usually, strokes and MI are caused by a combination of risk factors, such as obesity⁷; smoke⁸; alcohol; hypertension⁹; diabetes¹⁰; and hyperlipidemia¹¹. The leading cause of heart disease and stroke is cholesterol plaque which has developed into atherosclerosis¹². Early detection has to be applied to develop the best management to reduce the risk of CHD. Usually, this is done by cholesterol test, the measurement of the fats in the blood that can show the risk of heart diseases. People with CHD have higher triglycerides, total cholesterol, and LDL cholesterol level but lower HDL cholesterol levels than normal people¹³. However, many heart attacks occur in people with an average cholesterol level. The risk of CVD was expressively high for people with an LDL-C level of ≥ 70 mg/d L who are treated with statins¹⁴.

Another marker that can be used to diagnose and diagnose several diseases' prognosis is apolipoproteins. Apolipoproteins regulate the metabolism of lipoproteins and their uptake in tissues through enzyme cofactors, receptor ligands, and lipid transfer carriers¹⁵. There are many significant groups of Apolipoproteins: Apo-A, Apo-B, Apo-C, Apo-D, Apo-E, Apo-H, and Apo-J. This grouping is based on the characteristics, functional, and chromosomal location. The variation at the Apo-Aa locus is correlated with the susceptibility of CHD due to lipoprotein, and Lpa concentration is related to the genesis, progression, and complication of atherosclerosis and thrombosis¹⁶.

Apolipoprotein B (Apo-B) is the primary apolipoprotein that carries chylomicrons, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), lipoprotein a. Apo-B is not detected in high-density lipoproteins (HDL), Apo-B is a marker for early detection of the incidence of CHD too¹⁷. AMORIS Apolipoprotein-related Mortality Risk Study reports that as an early marker of heart attack, Apo-B is better than LDL cholesterol, HDL cholesterol, and triglycerides; therefore, the examination of Apo-B can replace the lipid profile examination standard as a target in therapy for the risk of CHD¹⁸.

Another examination of the risk of CHD is an atherosclerotic progression characterized by the thickening of the abdominal aorta vessel due to the formation of foam cells in continuous microscopic lesions. This condition occurs most frequently in the prone places of atherosclerotic lesions, especially in arterial branches or nearby. Therefore, the thickness of the aortic wall can be interpreted by examining the number of foam cells formed in the intima and media¹⁹.

The risk of CHD is done by tackling hypercholesterolemia both pharmacologically and non-pharmacologically. Pharmacologically it is done by administering drugs and chemicals that are normolipidemic. The standard drug commonly used in treating hypercholesterolemia is the simvastatin group. Simvastatin is a statin drug that acts as an HMG-CoA reductase inhibitor. The principle of action of simvastatin is to inhibit the formation of mevalonate, which at a later stage inhibits the formation of lanosterol. The last process is inhibiting lanosterol changes through the NADPH pathway to cholesterol so that the cholesterol produced is reduced²⁰. Statins have been speculated to stabilize plaque by decreasing smooth muscle cell migration and proliferation and modifying endothelial function. This plaque stabilization, independent of the cholesterol-lowering effect of statins, may lead to an inhibition of the shower embolization associated with extensive atheromas²¹. Tremblay reports that administering simvastatin 40 mg can reduce apo-B levels by decreasing Cholesterol LDL²². The use of this drug for an extended period needs to be considered related to the possibility of the emergence of unwanted side effects such as rhabdomyolysis, with general symptoms of muscle weakness and easy feeling tired²³.

The recommended diet to prevent hypercholesterolemia is to reduce the consumption of total and saturated fat and increase fiber consumption. Food as the largest source of fiber is vegetables and

fruits²⁴. Fruit containing fiber, especially with high pectin and antioxidants, and polyphenol, for example, red guava, has the highest fiber among local fruits²⁵. The nutritional content of guava fruit is moderate levels of folic acid, high vitamin C, and dietary fiber. Vitamin C in red guava is 257% higher than in white guava, but the calorie content is lower. The adequacy of daily intake of vitamin C from red guava is five times higher than from oranges²⁶. The antioxidant content of red guava is very high, included of beta-carotene, lycopene, beta-cryptoxanthin, and polyphenols that are good as an antioxidant. β -carotene captures free radicals, particularly peroxy radicals and hydroxyl. β -carotene interacts synergistically with vitamins C and E²⁷. Other antioxidant content in red guava is quercetin, guajaverin, leucocyanidin, and elagic acid. The antioxidant activity of quercetin, the flavonoid, is more vital than vitamin C and vitamin E. These antioxidants can prevent LDL oxidation¹³. Consumption of 250 ml of red guava juices for 21 days can decrease malondialdehyde MDA concentrations of toll collectors²⁸. These antioxidants can prevent LDL oxidation, which is characterized by an increase in MDA levels. This reduction in the LDL oxidation process impacts the inhibition of atherosclerosis progression²⁹. The main content of guava causes the ability of guava fruit to improve its lipid profile is soluble fiber (pectin). In theory, food fiber reduces cholesterol through several mechanisms, including (1) food fiber inhibits cholesterol absorption, (2) food fiber decreases cholesterol availability so that the transfer to the bloodstream is reduced, (3) food fibers able to prevent cholesterol synthesis, (4) Food fibers able to reduce food energy density thereby reducing cholesterol synthesis and (5) Food fibers able to increase bile excretion³⁰.

Based on the background mentioned above, this research aimed to analyze the consequence of red guava fruit on the Apo-B levels and the abdominal aortic wall thickness in hypercholesterolemic rats compared to simvastatin.

METHODS

This research used 50 adult Sprague Dawley male rats aged two months 150-160 g from LPPT UGM. Rats were adapted in the UGM PSPG laboratory for three days, given standard AIN 93 feed, followed by a standard high cholesterol diet for 14 days until the weight reached 200 g. On the 14th day, the rats had hypercholesterolemia (173.25 mg/dL), while the cholesterol levels of the rats fed normal AIN were 103.9 mg/dL, so there was a difference of 67% increase in cholesterol, then randomization and grouping was carried out in individual cages. The sample size was determined based on the Federer formula (1966), which was 9.65, fulfilled to become ten individuals³¹. Male hypercholesterolemic rats *Sprague Dawley* were treated with red guava fruit compared to those treated with the normolipidemic drug simvastatin. The dose of simvastatin was administered based on the conversion of the human dose to a rat dose of 200 g. A randomized pre-test and post-test control group design was developed for this research. K₀ was the rats fed with an AIN 93 as a standard feed composed of L-Cystine, Corn Starch,

Maltodextrin, Sucrose, Soybean Oil, Cellulose, Mineral Mix S10022M, Vitamin Mix V10037, Choline Bitartrate, TBHQ, antioxidant, as a negative control. K₁ was rats fed with high cholesterol as a positive control. The high-cholesterol diet used AIN 93 standard feed plus 1% cholesterol crystals and sodium cholate. This study's novelty was finding out the effectiveness of guava fruit in reducing the level of Apo-B.

P₁ was the rats treated with simvastatin 10 mg/day. The treatment with red guava fruit compared to the rats treated with the normolipidemic drug simvastatin. A randomized pre-test and post-test control group design was developed for this research. K₀ was the rats fed with a standard feed as a negative control. K₁, K₂, and K₃ groups were fed high cholesterol based on AIN93. The rats were fed a high-cholesterol diet for four weeks, after which cholesterol was checked to ensure that the rats were indeed hypercholesterolemic³². The novelty of this study was to find out the effectiveness of guava fruit in reducing the level of Apo-B. P₁ was the rats treated with simvastatin 10 mg/day. P₂ was the rats treated with red guava fruit flour 38 g/day based on ADA (American Dietetic Association)³³. The feeding was carried out ad libitum, while the dose is for humans according to the ADA reference, while for rats, it must be converted to rats weighing 200 g. the treatment of guava and simvastatin has been given by sonde³⁴. The levels of simvastatin and guava fruit are converted according to Laurence-Bacharach by 0.018 for rats. The treatment was run for eight weeks.

The analysis of Apo-B levels was measured from blood samples taken through the retroorbital sinus of rats using a hematocrit pipette. The analysis was carried out enzymatically based on the principle of immunoturbidimetry using Enzyme-linked Immunosorbent Assay Kit For apo-B *Rattus norvegicus*, Catalog No. E92003Ra, product by *Uscn Life Science Inc.* This enzymatic examination was conducted in the Biochemistry Laboratory, Faculty of Medicine, UGM.

Table 1. Apo-b t-test results of rats after treatment

Group	Mean (mg/dl)	SD	p Anova
K ₀	13.98 ±	1.22 ^a	< 0,001
K ₁	18.68 ±	1.68 ^b	
P ₁	16.58 ±	1.17 ^c	
P ₂	16.63 ±	1.54 ^c	

Note: The numbers followed by the same superscript letters show no difference between treatments

K₀: a group of standard feed negative control; K₁: a group of high cholesterol feed positive control

P₁: a group of drug treatment simvastatin; P₂: a group of guava treatment

For negative control (K₀), in the rats with a standard feed for eight weeks, the Apo-B concentration (K₀) had increased by 2 mg/dl but still in the normal value of 11-14 mg/dl. However, the positive control (K₁), the Apo-B concentration at the beginning of the experiment, was 17.8 mg/dl and increased to 18.7 mg/dl for eight weeks. The concentration of Apo-B for the rats that treatment with simvastatin (P₁) had reduced by 1.2 mg/dl, whereas treatment with red guava fruit (P₂) had reduced by 1.1 mg/dl (Figure 1). This result indicated that consuming simvastatin and red guava fruit reduced the concentration of Apo-B.

The termination was carried out by sedation using chloroform, put in a closed container until it died, then surgery was done to take the abdominal aorta. Abdominal aortic tissue was taken for their anatomy preparations. Observations were made by measuring rats' abdominal aortic wall thickness using a light microscope Olympus BX51 through a magnification of 400. This anatomy examination was conducted in the Micro-Anatomy laboratory, Faculty of Veterinary Medicine UGM. Data analysis was performed qualitatively on visual abdominal aortic wall thickness. The result was then statistically analyzed using paired t-test and ANOVA for the different tests between groups³⁵.

This research was conducted after obtaining ethical clearance from the Bioethics Commission for Medicine / Health Faculty of Medicine, Islamic University Sultan Agung Semarang, No. 395 / XII / 2020 / Bioethics Commission.

RESULTS AND DISCUSSION

The Consequence of Red Guava Fruit Treatment on Apo-B Levels

Based on this research, the hypercholesterolemic rats, when consumed red guava fruit for eight weeks, had an Apo-B concentration (16.63 mg/dl) was lower than the rats that did not consume red guava fruit (positive control was 16.68) (Table 1). A significant difference in Apo-B level was shown between the rats fed a standard feed (K₀) and the hypercholesterolemic rats (K₁). These significant differences also occurred in Apo-B concentration between K₁ with P₁ and P₂ (Table 1). However, P₁ and P₂ were statistically not significantly different. This result means that the treatment with red guava fruit was equal to the simvastatin on decreasing Apo-B concentration in hypercholesterolemic rats.

The results showed that the drug simvastatin P₁ and red guava fruit P₂ significantly reduced serum Apo-B levels of hypercholesterolemic rats with p <0.001 $\delta=1.2$ and $\delta=1.1$ (Figure 1). This decrease is above the normal Apo-B level but lower than the control. The hypercholesterolemic increases LDL, indicated by an increase of Apo-B³⁶. The results of different tests between groups showed no difference between P₁ and P₂, but both P₁ and P₂ differed from groups K₀ and K₁ (Table 1). Based on the statistical analysis, red guava fruit had the same effect as simvastatin on reducing the Apo-B of hypercholesterolemic rats. The mechanism of

decreasing Apo-B by soluble fiber (pectin) in red guava fruit occurs indirectly by cholesterol synthesis inhibition³⁷. The soluble fiber in the colon will be fermented by bacteria, producing short-chain fatty acids (SCFA) such as acetic, propionic, and butyric. Propionic acid plays a role in inhibiting cholesterol synthesis and occurs at the stage of inhibiting the activity of the HMG-coA. Enzyme³⁸. This inhibition of cholesterol synthesis decreases the synthesis of pro-atherogenic particles,

which is strongly related to the Fractional Esterification Rate (FER). The FER was strongly associated with changes in HDL, triglycerides, and Apo-B. A high-triglyceride concentration was correlated with VLDL particles and small dense LDL. An increase in plasma triglyceride concentrations was correlated with a rise incidence of CHD, accompanied by a rise in the small dense LDL population and an increased mass transfer of HDL cholesterol esters to Apo-B³⁹.

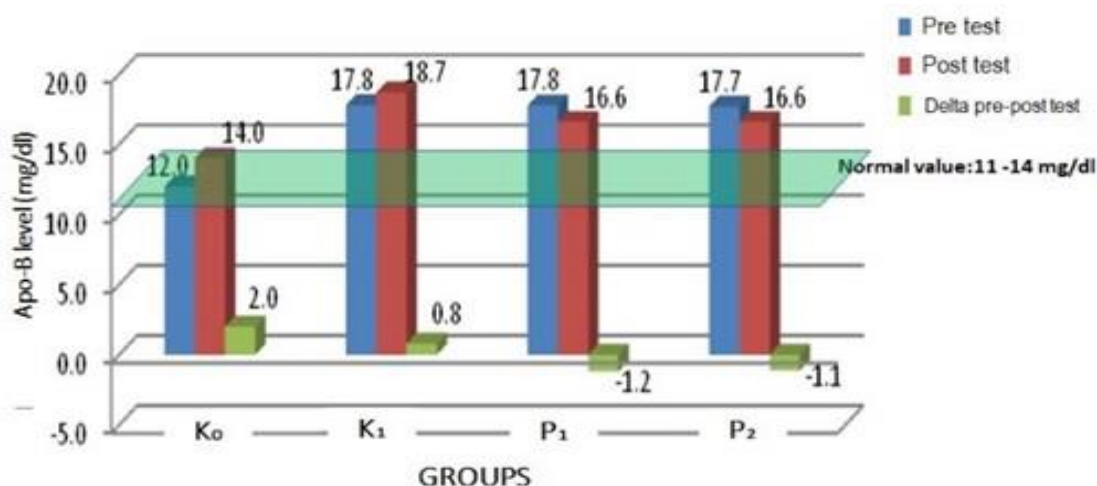


Figure 1. Apo-B concentration (P₁: simvastatin, P₂: red guava, K₀: standard feed, K₁: high cholesterol feed)

Apo-B is a large glycoprotein that plays a role in lipoprotein metabolism and human lipid transport. The human gene that plays an essential role in the synthesis of Apo-B is cited on chromosome 2 in the short arm p23 to p24. The gene commands triglycerides into triglyceride-rich lipoprotein, which is then transcribed into two forms of Apo-B particles. In the transcription process of triglycerides to Apo-B, only 48% in the intestine, so-called Apo-B-48, while 100% in the liver, so-called Apo-B-100⁴⁰. An earlier Apo-B examination was required for people indicated to have CHD risk because an increase in Apo-B in the body can potentially increase atherogenic particle formation through increased stimulation of cytokine production and inflammatory reactions¹⁸. The advantage of Apo-B in lipid-reducing treatments is that the antisense oligonucleotides target the mRNA of proteins in cholesterol metabolism. Initial trials of Apo-B antisense oligos have shown promise in decreasing 50% Apo-B levels, 30% LDL levels, and cardiovascular risk¹⁷. A new goal of the 2018 NCEP (National Cholesterol Education Program) guidelines is that Apo-B > 130 mg/dL is a risk-enhancing factor and needs a valuation in primary prevention treatment protocols. This result is an essential step toward analyzing

Apo-B for cardiovascular risk⁴¹.

The Consequence of Red Guava Fruit Treatment on Abdominal Aortic

There was a difference in abdominal aortic structure between normal rats, hypercholesterolemic rats, and rats with simvastatin and guava treatments. The normal histo-anatomy of the aorta abdominal with a standard feed of AIN93 is shown in Figure 2. Tunica intima (TI) and tunica media (TM) layering are in good order. No inflammatory cells were found on the wall (Figure 2), the shape of the TM tissue looks normal, and no fat is seen in the aorta of normal rats. In the hypercholesterolemic rats (K₁), many foam cells are almost in the TM, as shown with an arrow (Figure 3).

Infiltration of inflammatory cells is due to the oxidation of LDL, which initiates the acute inflammatory process, followed by vasodilatation. TM in the hypercholesterolemic rats treated with simvastatin (P₁), there were foam cells in TM, but smaller size than hypercholesterolemic without treatment (K₁) (Figure 4). This condition was similar to the hypercholesterolemic rats treated with guava fruits (Figure 5).

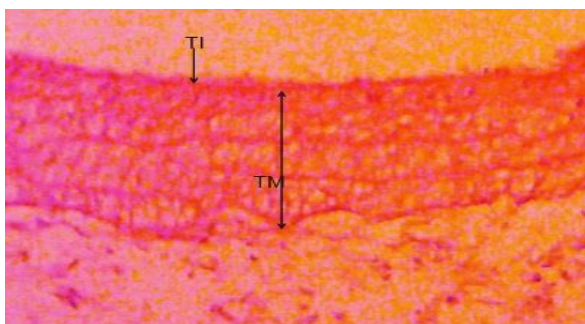


Figure 2. Histoanatomy of normal aorta rats (K_0) magnification of 400x, TI = tunica intima, TM= tunica media

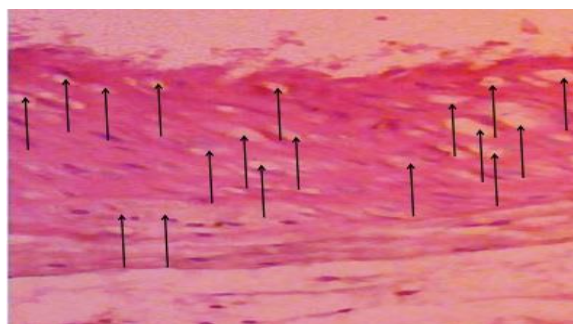


Figure 3. Histoanatomy of hypercholesterolemic aorta rats (K_1) magnification of 400x

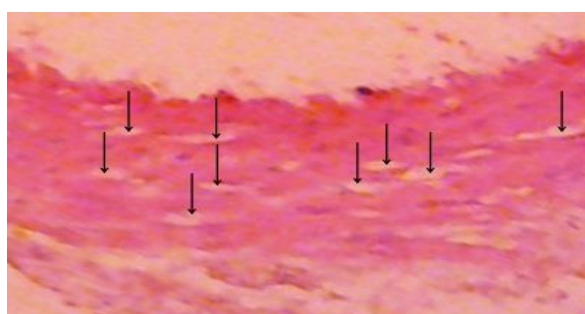


Figure 4. Histoanatomy of hypercholesterolemic aorta rats treated with simvastatin (P_1), magnification of 400x.

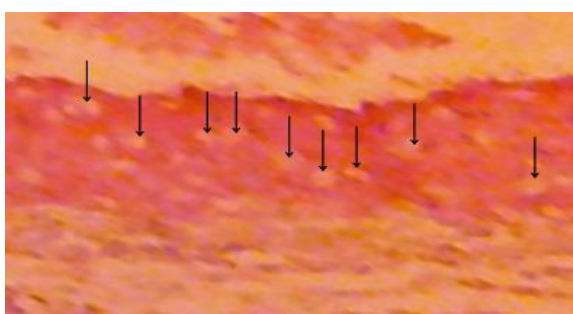


Figure 5. Histoanatomy of hypercholesterolemic aorta rats treated with red guava (P_2), magnification of 400x.

Increased LDL levels can trigger LDL oxidation and cause inflammatory reactions in blood vessel walls. Inflammatory reactions initiate immunocompetent cells such as lymphocytes, neutrophils, monocytes, and macrophages. The most precise picture of aortic histoanatomy from hypercholesterolemia is infiltration by macrophages and lymphocytes. Monocytes in the blood enter through the endothelial gap and enter the tunica media, followed by the formation of fat deposition. This damage is caused by hypercholesterolemia, which triggers free radicals, causes an inflammatory reaction, and causes fatty on tunica media⁴².

Immunological reactions and endothelial injuries cause vasodilation. As a result, the endothelial cells are disrupted, and the permeability of endothelial cells against various materials in the plasma increases. These induced materials to have access to the arteries. Wounds that occur in endothelial cells result in an inflammatory and immune reaction. The endothelial injury will trigger Reactive Oxygen Species (ROS), which then bind to LDL in the blood, and the oxidation process occurs. The endothelial wounds increase vasoactive peptides, which increase endothelial permeability, thus forming inter-cell cavities, and infiltration of fat and inflammatory cells occurs in the tunica media. Inflammatory reactions were responded to the body by removing inflammatory cells in the form of macrophages, neutrophils, and lymphocytes, where macrophages are the most obvious marker of the occurrence of hypercholesterolemia. Inflammatory cells that first appear are neutrophils because the inflammation that

occurs is acute inflammation. Neutrophils were inhibiting the presence of infection by releasing prostaglandin. The presence of prostaglandin causes increased vasodilation and vascular permeability. This causes fat deposition in the endothelium that enters the endothelial gap into the tunica media⁴³.

Giving red guava fruit which contains high antioxidants, shows a good effect on aortic histoanatomy. Hypercholesterolemia increased LDL and Apo-B, which induced an increase of fatty streak and development of foam cells in TI and TM¹⁹ and increased the thickening of the walls of blood vessels (Figure 3)⁴⁴. Simvastatin, the statin group, inhibits the enzyme HMG co-A reductase, reducing cholesterol synthesis that affects decreasing cholesterol. Another effect of statin is the reduction of LDL concentration and Apo-B, indicated by reducing the foam cells and decreasing the thickening of the walls of blood vessels (Figure 4)⁴⁵. It was seen that inflammatory cells are reduced, followed by reduced fat in the TI and TM. The aortic state is better with loss of fatty tissue and decreases in inflammatory cells in the TI for hypercholesterolemic rats given simvastatin (Figure 4) and hypercholesterolemic rats given red guava (Figure 5). Compounds in guava that play a role in inhibiting the progression of aortic wall thickness are pectin and antioxidants. The antioxidants in red guava fruit inhibit LDL oxidation by reducing ROS and increasing HDL by increasing Apoprotein A1 so that an inflammatory process and LDL oxidation are reduced. Antioxidants in red guavas, such as vitamin C, E, and flavonoids, have been speculated to stabilize plaque by decreasing smooth muscle cell migration and proliferation and modifying

endothelial function. This plaque stabilization, independent of the cholesterol-lowering effect of antioxidants⁴⁶, may lead to an inhibition of the shower embolization associated with extensive atheromas⁴⁷. The insoluble fiber in red guava fruit was able to bind fat in the intestine, and removed through feces, so there is no fat absorption in the intestine. Therefore, the fat in the blood decreases³⁷, resulting in a decrease in fat infiltration in the TM.

The advantage of this study is that giving red guava has been shown to reduce the risk of coronary heart disease by reducing apo-B levels and improving the performance of tunica media. This study was still limited to the wall of the abdominal aorta and not to the heart's blood vessels, so it only indicates the risk of CHD and does not directly describe the actual CHD incident.

CONCLUSIONS

Red guava fruit could decrease the risk of CHD by reducing Apo-B levels and thickness of the abdominal aorta wall equal to the simvastatin. Red guava fruit had good performance on the repairment the damage of TM and simvastatin. Therefore, red guava fruit can be applied to lower the risk of CHD.

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Conflict of Interest and Funding Disclosure

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