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Research Article

Antihyperlipidemic activity and mechanism test of cashew nut shell extract in male wistar rat induced by high-cholesterol foods

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ABSTRACT

Nutshell is a waste of cashew nuts product which is known to contain phytosterols was tested as an antihyperlipidemic agent with two methods: induction of hyperlipidemia and inhibition of cholesterol absorption method. In induction of hyperlipidemia, animals were induced using high cholesterol foods and induction agents (cholesterol, cholic acid, and propylthiouracil). After that were treated using simvastatin and cashew nut shell extract. Total cholesterol value was measured periodically after therapy, then aorta was isolated to examine the histology of aorta. In inhibition cholesterol absorption method, rats were given PTU and then were given ezetimibe and extracted orally. After that, all the animals were induced with exogenous cholesterol. Cholesterol level in the serum and feces was measured as well as to see the effects of inhibition of cholesterol absorption. For hyperlipidemia induction method, extract had different significance compared to the control group after 8 weeks of therapy. Atherogenic index of plasma (IAP) values of low-dose extract and high dose extract was significantly different compared to the control group. Histology of rats aorta for extract was significantly different in thickness compared to the control group. In the inhibition of cholesterol absorption method, extract 250mg/kg bw (group EKBKM 250) and 500 mg/kg bw (group EKBKM 500) inhibit the increase of total blood cholesterol within one hour after induction and significantly different compared to the control group. N-hexane extract cashew nut shells 500mg/kg have antihyperlipidemic effects, one of them is inhibition of cholesterol absorption.

1. INTRODUCTION

Cardiovascular disease is one of the leading causes of death in the world. In 2008, 17.3 million people died due to cardiovascular disease, of which 7.3 million died due to coronary heart disease and 6.2 million died due to stroke (World Health Organization [WHO], 2011). The results of the 2007 Riskesdas survey showed that disease did not run in the first position as a cause of death for all ages, namely 59.5% and stroke was a non-communicable disease that had the highest proportion of causes of death, namely 26.9%, followed by hypertension 12.3% and diabetes mellitus by 10.2%. WHO estimates that in 2030 nearly 23.3 million deaths worldwide will be due to cardiovascular disease (WHO, 2011).

One of the most deadly cardiovascular diseases is stroke. Stroke is caused by several factors, one of which is hyperlipidemia. Hyperlipidemia is a condition where cholesterol (lipid) levels in the blood are high. The lipids

then accumulate in the arteries and undergo oxidation. This triggers an inflammatory process. This inflammation causes the release of inflammatory agents that trigger plaque formation in the arteries. Plaque in the arteries will block blood flow from the heart to the organs that need it, so that the transport of nutrients and oxygen will be obstructed. This condition causes various complications of cardiovascular disease such as angina, coronary artery disease, heart attack and stroke. Hyperlipidemia is triggered by a diet that is high in cholesterol, a lifestyle, and the influence of certain medications (Wells, DiPiro, Scwinghammer & DiPiro, 2009).

This study aims to determine the effect of n-hexane extract of cashew nut shells by testing the hyperlipidemia model and inhibition of cholesterol absorption. To test the hyperlipidemia model, the parameters measured include measurement of total cholesterol, HDL, TG, and LDL levels in a certain period, as well as examination of the aortic thickening which is the initiation stage of atherosclerotic plaque formation. Meanwhile, the cholesterol absorption inhibition model was carried out to test the effectiveness of the extract against the absorption of cholesterol in the intestinal tract in vivo. We did this by measuring cholesterol in the blood and feces immediately after cholesterol induction was given. The comparative drug used is a drug known to work to inhibit cholesterol absorption in the digestive tract, namely ezetimibe.

2. MATERIALS AND METHODS

Research protocol had been approved by the Animal Research Ethic Committee with Ethical Approval number 03/KEPHP-ITB/02-2016. For the hypercholesterolemia induction method, an animal model of hyperlipidemia was obtained by inducing rats using cholesterol feed and oral administration of KKT (pure cholesterol, cholic acid, and propylthiouracil) every day. The tools used were oral sonde, syringe, surgical scissors, tweezers, restrainers, UV-vis spectrofotometry (Tecno 168), standard feed, cholesterol feed, cholic acid, pure cholesterol, propylthiouracil, total cholesterol reagent kit, triglycerides and HDL, simvastatin, ezetimibe, CMC sodium, cashew nut shells (*Anacardium occidentale*), n-hexane solvent, vegetable oil, two month old male Wistar rat with body weight 150-200 gr (Permatasari, 2014)

The test animals were grouped into five groups, namely the negative control group was given standard feed from the Laboratory of Pharmacy School ITB, the positive control group was given high cholesterol feed, the comparison group was given high cholesterol feed and the comparative drug simvastatin 25 mg/kg BW (group SIMV 25), the test group dose I and II was given high cholesterol feed and given the test extract, respectively, at a dose of 250 mg/kg BW (group EKBKM 250) and 500 mg/kg bw (group EKBKM 500).

Induction was carried out for three months. At the beginning of the fourth month, the animals were given tests and comparative drugs while continuously being induced by cholesterol. From time to time, blood is drawn to see the lipid profile. Blood draws were performed at the start of therapy, week 2, 4, 6, and 8 after therapy. The parameters measured were total cholesterol, triglycerides, LDL, and HDL. LDL levels, calculated using the Friedewald formula:

$$LDL = (total\ cholesterol) - (HDL) - (TG/5) \quad (1)$$

Blood and fecal cholesterol levels were measured using an enzymatic cholesterol reagent kit. Measurement data were processed using the IBM SPSS Statistics trial version 23 software (International Business Machine, Corp.). At the end of the treatment, the animals were sacrificed and autopsied, then the aorta was isolated and then stained to observe thickening in the aorta.

For the inhibition method of cholesterol absorption, an animal model of hyperlipidemia was obtained by inducing the animals using propylthiouracil at a dose of 9 mg/kg which was put into a rat drink. This method is done for seven days. Then, on the eighth day, the animals were induced using pure cholesterol and then given the comparative drug ezetimibe and the test extract, then their blood and feces were taken to measure their cholesterol levels. Blood sampling was carried out at 1 and 2 hours. Meanwhile, the stool was collected for 6 hours and 24 hours. Blood draws are made through a lateral vein. Cholesterol in feces must be prepared in advance, that is, the stool is extracted using the Folch method.

Statistical analysis was performed with the software IBM SPSS statistics trial version 23 (International Business Machine, Corp.). Statistical analysis between treatment groups was compared using one-way analysis of variance (ANOVA) accompanied by LSD (Least Significant Difference). Value with $p < 0.05$ indicates a significant difference.

3. RESULTS AND DISCUSSIONS

For the hyperlipidemia induction method, first male Wistar rats were induced for two months using cholesterol feed and oral administration of KKT (pure cholesterol, cholic acid, and propylthiouracil) every day. Cholesterol levels were measured at month 0 and at the end of month 2 as a successful parameter of hypercholesterolemia induction. The following table shows the increase in lipid profiles in mice that have been induced for 2 months.

The total cholesterol level after two months of induction was 335.2 ± 139.78 mg/dL. This proves that the successful induction is characterized by an increase in total cholesterol levels as a characteristic of hyperlipidemia. Another lipid profile that increased was triglyceride levels, which increased by 262.8 ± 106.62. LDL levels have increased significantly, namely 311.3 ± 131.52. High levels of LDL then enter the arteries and are then oxidized to endothelial cells which can cause macrophages to eliminate oxidized LDL and form foam cells. These foam cells are the precursor to atherosclerotic plaque formation. This increase in lipid profile occurs due to the synergistic effect of cholesterol feeding and the induction of KKT. Cholic acid in KKT functions to increase cholesterol by increasing cholesterol absorption and inhibiting the conversion of cholesterol to bile acids (Wang et al., 2005). Meanwhile, propylthiouracil inhibits thyroid hormone synthesis by blocking the oxidation of iodine in the thyroid gland resulting in a hypothyroid condition (Boelart, 2008). The thyroid hormone works to increase the secretion of cholesterol into the bile which is then excreted in the feces. With the inhibition of thyroid hormone, cholesterol secretion decreases and blood cholesterol levels increase. The combination of all of these results in high cholesterol levels. Even though animals are given therapy, cholesterol induction is still given to prevent a drastic reduction in cholesterol (Permatasari, 2014).

Cholesterol level after two weeks of therapy on average decreased significantly compared to the control group (**Figure 1**). The total cholesterol level of the control group was 276.25 ± 100.29 mg/dL, a slight decrease compared to two months of induction but still quite high. The cholesterol level in the SIMV 25 group, namely 144.75 ± 38.48 mg / dL, was significantly different from the control group. This also occurred in the group EKBKM 250 and the group EKBKM 500, respectively 195.29 ± 59.19 mg/dL and 196.4 ± 61.16 mg/dL. This suggests that cashew nut shell extract may have the same effect as HMG CoA reductase in lowering cholesterol. If the total cholesterol level is calculated against T_0 (**Figure 2**).

At weeks 4, 6, and 8 of therapy, cholesterol levels in the SIMV 25, EKBKM 250, and EKBKM 500 groups continued to decline and differed significantly from the controls. However, it has not reached normal cholesterol levels. This proves that cholesterol levels require longer therapy time to return to normal accompanied by non-pharmacological therapy. Statins take about 3 months to lower blood cholesterol levels (Rosolova et al., 2013).

Triglyceride levels were measured at the start and end of therapy (**Figure 3**). Triglyceride levels after two weeks of therapy for the SIM 25, EKBKM 250, and EKBKM 500 groups were 124.75 ± 50.61mg/dL, 116.57 ± 26.94 mg/dL, and 128 ± 41.13 mg/dL, respectively. This level did not have a significant difference in the control group. This means that simvastatin and extracts do not affect triglyceride levels. While the levels of triglycerides after eight weeks of therapy for the SIMV 25, EKBKM 250, and EKBKM 500 groups were 68.25 ± 4.99, 59.29 ± 13.02, and 57.4 ± 19 mg/dL, respectively. it has a significant difference compared to control.

HDL levels were also measured at the start of therapy and at the end of therapy (**Figure 4**). The HDL levels in the SIMV 25 group at two weeks of therapy were obtained at 10.75 ± 4.79 mg/dL, while for the HDL levels for the groups EKBKM 250 and EKBKM 500, respectively 8.29 ± 1.70 and 10.8 ± 3 , 03 mg/dL. This level value was not significantly different from the control group.

LDL levels were obtained from Friedewald's calculations. LDL levels in all induction groups increased after two months of induction (**Figure 5**). After two weeks of therapy, the LDL level of the SIMV 25 group decreased to be 111.85 ± 34.30 mg/dL. This level was statistically different from the control group. Meanwhile, the extract group did not show any significant changes in their LDL levels. In therapy after eight weeks, only the EKBKM 500

Table 1. Increase in Cholesterol, Triglyceride, and LDL Levels after two months of induction

Parameter	Increment (mg/dL)
Total cholesterol	335.2 ± 139.78
Triglycerides	262.8 ± 106.62
Low Density Lipoprotein (LDL)	311.3 ± 131.52

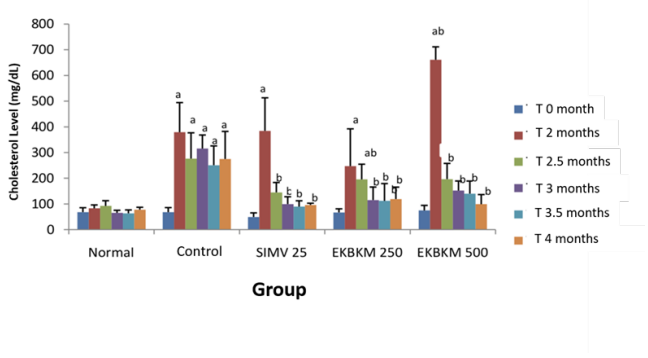

Figure 1. Average cholesterol levels in male wistar rat serum at induction and therapy for each treatment group

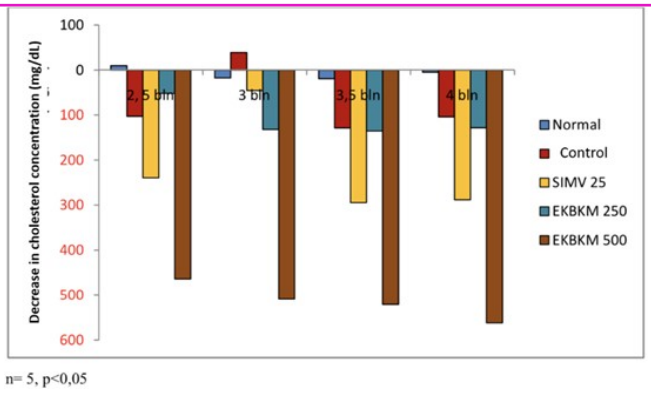

Figure 2. Changes in final cholesterol levels against t_0 in male wistar rat serum at induction and therapy for each treatment group

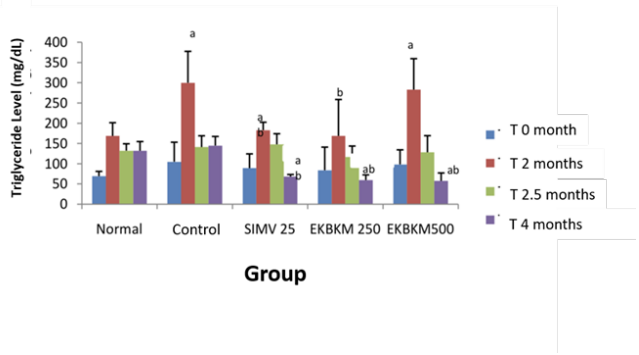

Figure 3. Average triglyceride levels in male wistar rat serum at induction and therapy for each treatment group

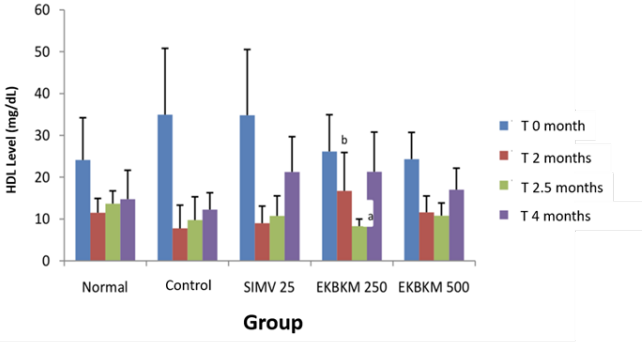

Figure 4. Average HDL levels in male wistar rat serum at the time of induction and therapy for each treatment group

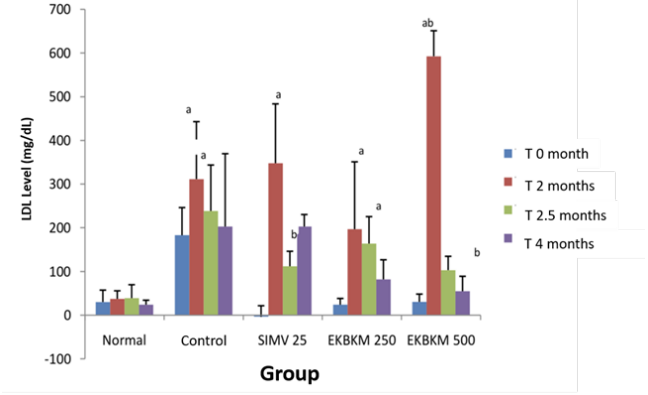

Figure 5. Average LDL levels in wistar rat serum at induction and therapy for each treatment group

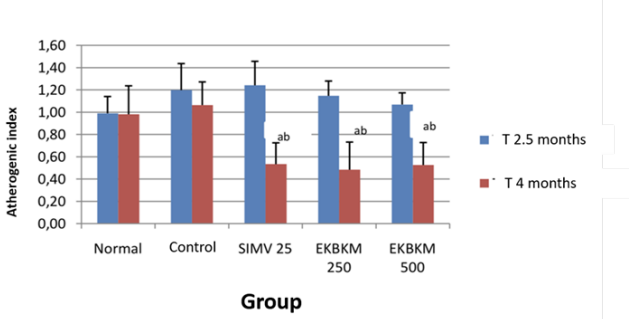

Figure 4. Plasma Atherogenic Index (PAI) value for each treatment group

group experienced a significant decrease compared to the control, namely 54.72 ± 34.22 mg/dL. This suggests that it is possible that the extract at a dose of 500 mg/kg BW has the same LDL-reducing effect as statins because of a fairly large decrease in LDL.

The TG and HDL lipid parameters were also measured to determine the Plasma Atherogenic Index (PAI) (**Figure 6**). High triglyceride levels are associated with an increase in the number of small particle size LDL which can lead to cardiovascular disease. PAI is calculated by using the logarithmic ratio of TG to HDL (Dobiášová and Frochlich, 2001). The greater the PAI value, the higher the risk of cardiovascular disease (Onat, Can, Kaya and Hergenç, 2010).

The IAP values of the SIMV 25, EKBKM 250 and EKBKM 500 groups showed significant differences against

Table 2. Animal aortic wall thickness after therapy

Group	Aortic Wall Thickness (µm)
Normal	59,09±0,88
Control	90,11±13,41
SIMV 25	68,91±9,51
EKBKM 250	54,18±16,20
EKBKM 500	67,78±15,14

the control group after eight weeks of therapy, 0.53 ± 0.19, 0.48 ± 0.25, and 0.53 ± 0.20 mg/dL, respectively (**Figure 6**). This value proves that the simvastatin drug and extract can reduce the PAI value so that it can reduce the risk of cardiovascular disease. Statins can reduce LDL levels by 29% and triglycerides by 40% during three months of therapy in patients who are given drugs with a fenofibrate combination (Rosolova et al., 2013).

The next parameter measured is the diameter of the aorta. Based on observations in the aorta, there are no plaques or foam cells that indicate the presence of oxidized LDL which is the precursor to atherosclerosis. However, there was thickening of the aorta in the simvastatin group and the test extract group (**Table 2**).

The mean aortic diameter of the SIMV 25 group rats was 68.91 ± 9.51 µm. This value has a significant difference compared to control. Likewise, the EKBKM 250 and EKBKM 500 groups showed significant differences to the control group, 54.18 ± 16.20µm and 67.78 ± 15.14 µm, respectively. These data prove that extract doses of 250 and doses of 500 can reduce thickening of the aorta even though it has not returned to normal. The picture of the rat aorta can be seen in the **Figure 7**.

Furthermore, the test for the absorption of cashew nut cholesterol gives the following results. In the early stages of testing, animals are given PTU 9 mg/kg BW for seven days which aims to disrupt cholesterol metabolism in mice, causing an increase in cholesterol levels in the blood. Then the mice were fasted for 18 hours to reduce the biological variation of the test animals. Blood sampling was carried out at three points, namely at 0, 1, and 2 hours after induction. This is because the increase in exogenous cholesterol absorption can be observed significantly within two hours after cholesterol induction (Dharsana, 2016). It can be concluded that at two hours after the induction of exogenous cholesterol, the inhibition of the increase in total cholesterol levels in the blood cannot be observed significantly in the control (**Figure 8**). Therefore, the inhibitory effect of the increase in total blood cholesterol levels can only be observed one hour after exogenous cholesterol (T_1) induction.

Stool collection was carried out for 6 hours and 24 hours using a metabolic cage. For 24 hours of fecal collection, animals are still given a controlled amount of food as a consideration in the code of ethics for experimental animals. The comparative drug used was ezetimibe (EZT 0.9) at a dose of 0.9 mg/kg BW. The dosage selection is based on previous studies, the effective dose of ezetimibe in mice is 0.1-3 mg/kg BW (Van Heek, 2002). Ezetimibe is given one hour before cholesterol induction. It is intended that ezetimibe works effectively and maximally in inhibiting the absorption of exogenous cholesterol when its onset has been reached. The onset of ezetimibe in mice was 90 minutes after oral administration. The test preparations used were n-hexane extract of cashew nut seed shells at a dose of 250 and 500 mg/kg bw. The extract dosage comes from previous studies and can have a lowering effect on the lipid profile.

From the graph (**Figure 9**), it shows that in the EKBKM 250 and EKBKM 500 groups there was a decrease in total cholesterol levels in the blood at the 1st hour, but there was an increase in the total cholesterol levels at the 2nd hour. The analysis was continued using one-way analysis of variance (ANOVA) accompanied by LSD. EZT 0.9, EKBKM 250, and EKBKM 500 groups could significantly inhibit the increase in total blood cholesterol levels to the control one hour after induction. whereas at the second hour there was no significant difference in cholesterol levels.

Apart from total cholesterol levels in the blood, other parameters measured were total cholesterol levels in feces that were collected for 6 hours and 24 hours (**Figure 10**). The analysis was continued using one-way analysis of variance (ANOVA) accompanied by LSD. EZT 0.9, EKBKM 250, and EKBKM 500 group could not significantly increase the total cholesterol excretion in feces for six hours compared to the control group. The results of statistical analysis of total cholesterol levels in feces for 24 hours showed a significant difference, which means that the EZT 0.9 and EKBKM 500 groups increased the total cholesterol excretion in 24 hours of

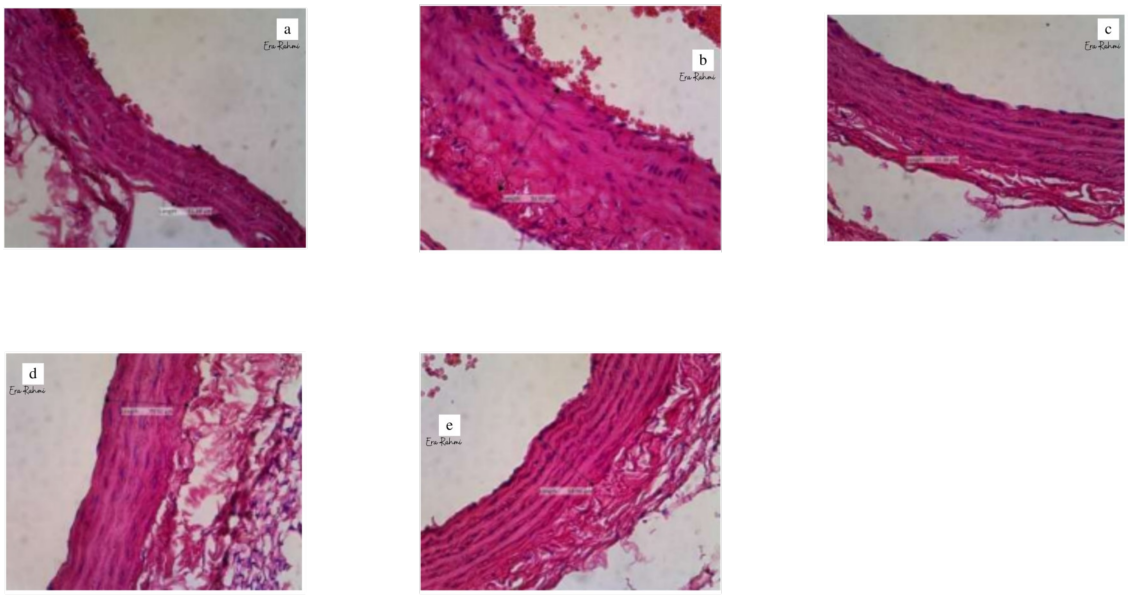

Figure 7. Observation results of rat aortic diameter (a) Normal group; (b) Control group; (c) SIMV 25 group; (d) EKBKM 250 group; and (e) EKBKM 500 group

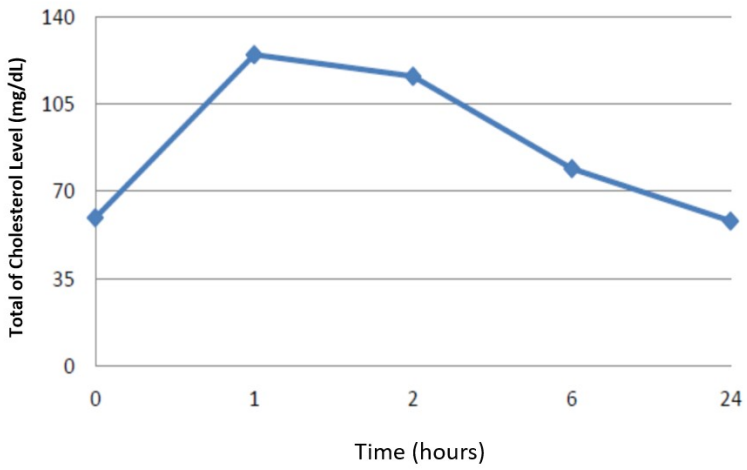

Figure 8. Results of measurement of blood cholesterol levels at 0, 1, and 2 hours which are the basis of measurement time

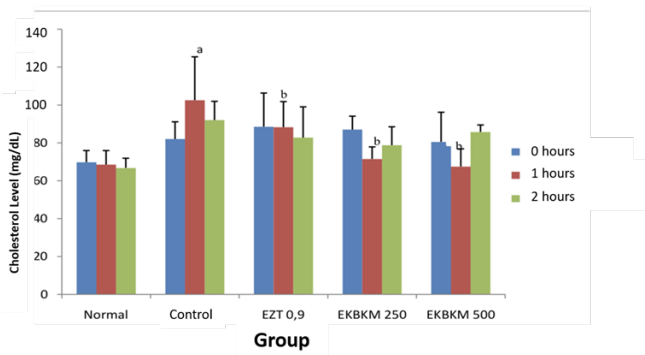

Figure 9. Average cholesterol levels in male wistar rats at 0, 1, and 2 hours in each treatment group

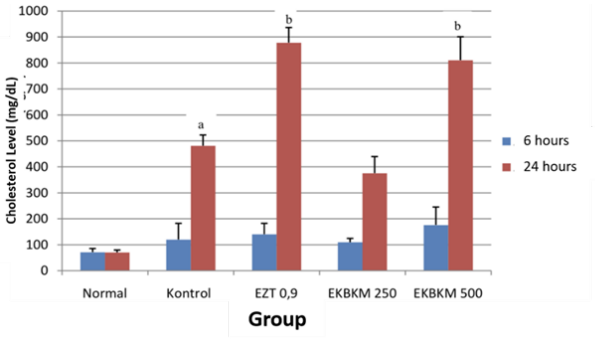

Figure 10. Average cholesterol levels in male Wistar rat feces at 0, 1, and 2 hours in each treatment group

feces significantly by 82.63%. and 68.64% for the positive control group. The extract at a dose of 500 mg/kg BW has an antihyperlipidemic effect comparable to ezetimibe 0.9 mg/kg BW in inhibiting the absorption of cholesterol in the intestine.

By reducing good cholesterol levels in the induction method and the inhibition of absorption, it will indirectly reduce LDL levels in the blood which is the main cause of cholesterol buildup in the aortic wall and subsequently becomes atherosclerosis.

4. CONCLUSIONS

The n-hexane extract of cashew nut seed shells at a dose of 500 mg/kg BW has an antihyperlipidemic effect, one of the mechanisms is to inhibit cholesterol absorption in the digestive tract which is comparable to ezetimibe 0.9 mg/kg BW rats. It is necessary to purify the extract of cashew nut kernel shell and be tested further to determine the possibility of another mechanism of action of the n-hexane extract of cashew nut seed shell as an antihyperlipidemic.

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