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Antihyperlipidemia Activity of Ethyl Acetate Fraction from Melinjo (*Gnetum gnemon* Linn.) Leaf in White Male Wistar Rats Induced by Propylthiouracil

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ABSTRACT

The antihyperlipidemia activity of ethyl acetate fraction from Melinjo leaf (*Gnetum gnemon L.)* against white male wistar rats induced by propylthiouracil has been carried out. Wistar male rats were divided into six groups, which are a normal, a positive control, a negative control, and the other three groups with a dose treatment of 10 mg/kg BW, 30 mg/kg BW, and 50 mg/kg BW. Experimental hyperlipidemic rats were induced by daily intake 0.01% PTU and high-fat for the duration of 2 weeks except the normal group was only given standard feed. Hyperlipidemic rats group were orally with three doses (10, 30, dan 50 mg/kg BW) of the ethyl acetate fraction for 2-weeks onward. The cholesterol and triglycerides levels were measured by using enzymatic method of CHOD-PAP and GPO-PAP. HDL levels were measured by using *Cholesterol HDL Analysis Kit,* and LDL levels were calculated by using indirect calculation. Decreased triglycerides levels at dose of 10 mg/kg BW and increased HDL levels at dose 30, 50 mg/kg BW were not significantly different with simvastatin ($p>0.05$), while decreased cholesterol and LDL levels at dose 10, 30, 50 mg/kg BW were significantly different with simvastatin ($p <0,05$). Based on the relationship between the percentage reduction in cholesterol, triglycerides, LDL and increased of HDL level, the effective ED_{50} of ethyl acetate fraction of Melinjo leaf is 34,74 mg/kgBW. Histopatological analyses of liver's rat showed that dose of 50 mg/kg BW was the same as simvastatin in its the percentage of necrosis and the percentage of fat degeneration of 20%. These results indicate that the ethyl acetate fraction of Melinjo leaf has an antihyperlipidemia, and there is a significant difference compared to the negative control ($p <0,05$).

Keywords: *Gnetum gnemon Linn., antihyperlipidemia, total cholesterol, triglyceride, HDL, LDL*

1. INTRODUCTION

Hyperlipidemia, also known as dyslipidemia, is a condition where there is an increasing in plasma cholesterol, *Low Density Lipoprotein* (LDL), triglycerides and a decreasing in *High-Density Lipoprotein* (HDL) levels or a combination of these abnormalities. Hyperlipidemia is a risk factor of pathogenesis cardiovascular disease [1,2]. World Health Organization (WHO) claimed that in 2018 there were 17.9 million people died because of cardiovascular disease. At least, 15 out of 1000 Indonesian people suffering from heart disease [3]. Hyperlipidemic disease can be treated pharmacologically by giving oral hypolipidemic. However, using oral hypolidemic has side effects, such as myositis, gastrointestinal, pruritus, urtikaria, impotensi, dysfunction liver and kidney. Alternative treatment to reducing the side effects of oral hypolipidemic can used Melinjo plant (*Gnetum gnemon L.)* which belongs to the Gnetaceae family traditionally can used as decreasing blood sugar, diuretic, act as antioxidant. and reducing hypertension [4]. Melinjo leaves based on the research contain flavonoids, saponins, tannins, phenolic compounds, and steroids [5]. Research showed that the phytochemical screening of ethyl acetate extract of melinjo leaves contained alkaloids, flavonoids, tannins, polyphenols, steroids and triterpenoids. Giving ethanol extract of melinjo leaves (*Gnetum gnemon L.*) at a dose of 250 mg /kgBW was effective in reducing total cholesterol

levels in Wistar rats induced by a diet high in fat and propyltiouracil [6].

Flavonoids work by reducing cholesterol synthesis by inhibiting the activity of the enzyme acyl-CoA cholesterol acyl transferase (ACAT) on HepG2 cells which plays a role in reducing cholesterol esterification in the intestine and liver, and inhibits the activity of the enzyme 3-hydroxy-3-methyl glutaril CoA which causes inhibition. cholesterol synthesis [7]. Flavonoids have unsubstituted hydroxy groups so they are polar, flavonoids can dissolve in semi-polar solvents such as ethyl acetate. The purpose of fractionation to separating the components of the active compound from an extract that has been produced. Ethyl acetate is a semipolar solvent so that it is able to attract compounds with a wide polarity range from polar to non-polar. The ethyl acetate fraction of Moringa family *Moringaceae* leaves with a dose of 7.44 mg/kgBW and mulberry leaves family *Moraceae* at a dose of 40 mg/kgBW can reducing total cholesterol and LDL levels [8].

2. METHODS

2.1. *Sample Preparation*

The sample used was the leaves of the melinjo plant (*Gnetum gnemon L.*) with a wet weight of 1.9 kilograms obtained from the Inderalaya region, South Sumatra. The melinjo plant has been determined at the Laboratory of the Purwodadi Botanical Garden Plant Conservation Center (LIPI), Purwodadi, Pasuruan, East Java. The melinjo leaves taken are green leaves. Melinjo leaf samples were sorted, weighed, and dried under the sun covered with a black cloth until dry and the weight was constant. The dried melinjo leaves are mashed using a blender and weighed in net weight.

2.2. *Making Melinjo Leaf Ethanol Extract*

1 kg of melinjo leaf simplicia powder was extracted by maceration method for 1 x 72 hours with 2 remacerations. 96% ethanol solvent is used until the melinjo leaf powder is submerged. The obtained maserate was then filtered with Whatman paper. The resulting filtrate was then concentrated with a rotary evaporator at a temperature of 50℃ until a thick extract was obtained. The extract period obtained was calculated the percent yield [9].

2.3. *Preparation Ethyl Acetate Fraction of Melinjo leaf*

The ethanol extract was fractionated by the liquid-liquid partition method using n-hexane and ethyl acetate solvents in a separating funnel. The first fractionation, the ethanol extract was fractionated using n-hexane solvent with a ratio of 1: 1 for 3 times. Obtained n-hexane fraction and ethanol fraction. The n-hexane fraction was separated, then the ethanol fraction was fractionated using ethyl acetate and water with a ratio of 2: 2: 1 for 3 times. Obtained ethyl acetate fraction and ethanol fraction. The fraction was concentrated using a rotary evaporator with a temperature of 50℃.

2.4. *Phytochemical Screening Test Fraction*

Phytochemical screening tests were carried out to determine the content of secondary metabolites and class of compounds contained in melinjo leaves. Secondary metabolites is flavonoid, alkaloid, saponin, phenolic, steroid and triterpenoid.

2.5. *Animals Preparation*

This study has obtained an ethical clearance from Research Ethics Committee of Ahmad Dahlan University, Ethical Approval Number 022011023 tanggal 29 Desember 2020.

Test animals used were male Wistar white rats weighing between 150-250 g and aged 2-3 months. The Rats used are healthy, not deformed, and behave normally. These test animals were acclimatized to be able to adapt to the laboratory environment for seven days. The division of the number of each group of rats for testing the levels of triglycerides, total cholesterol, LDL, and HDL. The group of test animals used can be seen in Table 1.

Table 1. Antihyperlipidemia Activity Test Group for Melinjo Leaf Ethanol Extract

Group	Treatment
Normal	Na CMC 0.5% + Standard feed
Negative Control	Propyltiouracil 0.01% + Na CMC 0.5% + High fat feed
Positive Control	Propyltiouracil 0.01% + Simvastatin 0,987 mg/kgBW + high fat feed
Group 1	Propyltiouracil 0.01% + suspension of ethyl acetate fraction of melinjo leaves 10 mg/KgBW + high fat feed
Group 2	Propyltiouracil 0.01% + suspension of ethyl acetate fraction of melinjo leaves 30 mg/KgBW + high fat feed
Group 3	Propyltiouracil 0.01% + suspension of ethyl acetate fraction of melinjo leaves 50 mg/KgBW + high fat feed

2.6. *Antihyperlipidemia Testing Procedure*

Measurement of triglyceride levels using an enzymatic colorimetric assay using *glycerol phosphate oxidase p-aminophenazone* (GPO-PAP). Blood serum was taken using a micro pipette of 0.01 mL inserted in a test tube, then added a solution of triglyceride reagents as much as 1 mL and 0.01 mL of distilled water. The absorption is measured at a wavelength of 500 nm with respect to the blank. Measurement of standard triglyceride uptake [10].

Measurement of total cholesterol levels using an enzymatic colorimetric test using *Cholesterol Oxidase p Amino Antipyrine* (CHOD-PAP). Blood is drawn using a hematocrit pipette of 2 mL on the retroobital plexus, allowed to stand for 15 minutes and centrifuged for 20 minutes at 3000rpm. Blood serum is pipetted with a 10µL micro pipette, put in a test tube and placed in a biosystem analyzer. Then a 1000 µL CHOD-PAP reagent kit was added. The absorption is measured at a wavelength of 500 nm with respect to the blank [11].

Total cholesterol, triglycerides, and HDL cholesterol were measured, then LDL cholesterol was calculated using the fridewald formula .

$$LDL = \text{total cholesterol} - \left(HDL + \frac{Triglyceride}{5}\right)$$

Measurement of HDL levels was carried out with the HDL Cholesterol Analysis Kit [12].

2.7. *Effective Dose Value (ED_{50})*

The effective dose value (ED_{50}) can be calculated using a linear regression equation based on the relationship between the decrease in triglyceride levels, total cholesterol, LDL (Low Density Lipoprotein), and an increase in HDL (High Density Lipoprotein) concentration on the extract concentration analyzed using the formula in equation regresi linier.

2.8. *Macroscopic Observation of the Liver*

One rat from each treatment group was anesthetized using diethyl ether, then surgery was performed. Test animals dissected by making incisions along the thorax to the pubis, then the liver is taken. The liver that has been taken is observed macroscopically by looking at the color, texture, weight, and fatty liver which is characterized by the presence of fat on the surface of the liver. After dissection the test animals are sacrificed by neck dislocation. Then the sacrificed animal is properly buried in the soil.

2.9. *Liver Histopathology Observations*

The embedding process is done by embedding the tissue into the mold using pure paraffin media. The cutting was carried out using a rotary microtome with a thickness of 5 µm and attached to a slide that had previously been given Mayer's albumin reagent. The preparations were stained with hemactocillin - eosin (HE) for 2 minutes, then covered with a glass cover and labeled. Histopathological observations of the liver were carried out under a microscope with a magnification of 100x. Furthermore, the results of the examination of the preparations were carried out by observing the morphological shape of the rat liver tissue structure [11].

3. RESULT AND DISCUSSION

3.1. *Extraction Process*

1 kg of melinjo leaves implicity powder was obtained, it was extracted by maceration method using a solvent in the form of 96% ethanol. The maceration process is carried out by soaking the simplicia powder of melinjo leaves with 96% ethanol filter liquid in a closed container to prevent solvent evaporation during the extraction period and is protected from light at room temperature carried out 1 x 72 hours and remacerated 2 times [13].

The filtrate is then filtered and evaporated with a rotary evaporator at a temperature of 50 ° C with a rotation of 40 rpm to get a thick extract). The solid extract of melinjo leaves obtained from the evaporation process was 174.095 g with a yield percentage of 17.4095%. The yield states the share of raw materials obtained from the total raw material in the extraction process. The size of the yield shows the effectiveness of the extraction process carried out [14].

3.2. *Fractionation Process*

The ethanol extract of the melinjo leaves obtained was then carried out by liquid-liquid fractionation. Fractionation aims to separate groups of compounds based on their solubility in solvents with different levels of polarity. The fractionation of the ethanol extract of melinjo leaves was carried out in stages starting from a non-polar solvent in the form of n-hexane to attract non-polar compounds and also dissolve the pigment contained in the melinjo leaf sample in the form of chlorophyll.

Chlorophyll is a non-polar pigment so it will dissolve in n-hexane [15]. The ethanol extract of melinjo leaves was fractionated using n-hexane with a ratio of 1: 1 ethanol and n-hexane extract. Fractionation was carried out in three repetitions, in order to obtain the n-hexane fraction and the ethanol fraction.

The ethanol fraction obtained was fractionated again using ethyl acetate with the addition of water (2: 2: 1). The addition of water to the fractionation process aims to clarify the boundary between ethanol and ethyl acetate. The adjacent polarity index between ethanol solvent (polarity index 5.2) and ethyl acetate (polarity index 4.4) causes no visible boundary plane. Water that is polar will be more likely to mix with ethanol, so that the ethanol polarity index increases which causes the boundary fields of ethanol and ethyl acetate to be visible. The addition of water will also increase the density of ethanol which causes the ethanol fraction to be at the bottom of the separating funnel. This fractionation was carried out three times. The ethyl acetate fraction obtained was evaporated using a rotary evaporator at a temperature of 50 °C, so that the ethyl acetate fraction of melinjo leaves was obtained as much as 33.55 g.

3.3. *Phytochemical Fraction Screening Test*

Phytochemical screening used a sample of ethyl acetate viscous fraction of melinjo leaves. Phytochemical screening aims to determine the content of secondary metabolite compounds contained in the viscous fraction. Phytochemical screening is carried out by reacting the fractions with certain reagents. The profile of secondary metabolites of ethyl acetate viscous fraction can be seen in Table 2.

Table 2. Phytochemical Extract Screening Test Result

Secondary Metabolites	Result
Flavonoid	+
Alkaloid	-
Saponin	+
Tannin	+
Phenolic	+
Steroid	-
Triterpenoid	-

Screening the ethyl acetate fraction of melinjo leaves in the table above shows that the positive ethyl acetate fraction contains flavonoids, saponins, tannins, and phenolic compounds. As a comparison, the results of phytochemical screening of the thick extract of melinjo leaves by taking samples at the same place showed that they were positive for flavonoids, tannins, phenolics, steroids, and saponins [5]. Steroid and triterpenoid compounds, not contained in the ethyl acetate fraction. This is because steroid compounds tend to be non-polar, therefore steroid compounds will readily and dissolve a lot in non-polar solvents . Flavonoid compounds were identified by the Shinoda test (Mg-HCl). Positive results from the flavonoid test were the formation of a brownish red color solution. Brownish red color indicates the formation of flavilium salts due to the reduction of concentrated Mg and HCl. Reduction with concentrated Mg and HCL produces complex compounds that are red or orange in color [7]. Tannin and phenolic tests showed positive results if a green-black color was formed when the addition of 1% FeCl3 solution. The addition of FeCl3 will form condensed tannins, the formation of a blackish green color because tannins or phenolic compounds will form complex compounds with Fe3+. Saponins were carried out by using a foam test, namely by adding water and shaking them. The fraction screening results showed positive saponin results indicated by the emergence of foam that lasted for 10 minutes. Saponins are glycosides with a hydroxy group on the molecule and contain both hydrophilic and hydrophobic groups. Foam can be formed because saponins have the property to reduce the surface tension of water.

3.4. *Antihyperlipidemia Activity Test*

Measurement of body weight of test animals was carried out before induction or after acclimatization, after induction, and after treatment. This was done to see the differences in group body weight between treatments and to see the effect of acclimatization, high-fat feeding and PTU as an inducer as well as simvastatin and fractions as a treatment of rat body weight. The data on the body weight of the tested animals were obtained by weighing the body weight using animal scales. The results of measuring the body weight of test animals can be seen in Figure 1.

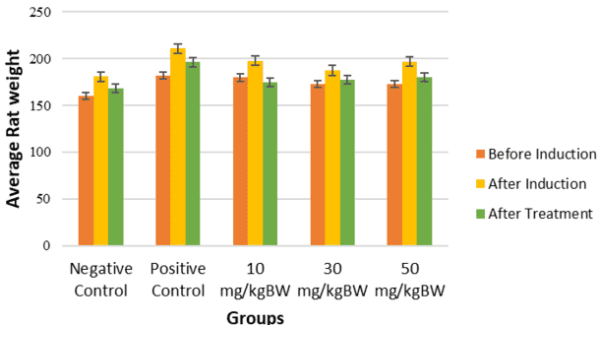

Figure 1. Graph of average body weight of rats

During acclimatization the rats' body weight may experience a decrease of no more than 10%, and an increase in body weight of no more than 20% [16]. Based on Figure 1 the graph of the results of the average body weight of the rats above, after induction there was an increase in the body weight of the rats in all test groups but not more than 20%. Provision of propylthiouracil (PTU) induction has an effect in reducing thyroid activity, especially in suppressing the secretion of T3 and T4 hormones which play a role in body metabolism, resulting in hypothyroid conditions. PTU causes an increase in TSH levels through a negative feedback mechanism due to inhibition of the formation / synthesis of thyroid hormones (T3 and T4) by the thyroid gland and conversion of T4 to T3 in tissues. Thyroid hormone affects lipid metabolism, supports lipolysis, and provides fatty acids as fuel to induce the release of energy so that thyroid dysfunction leads to a significant change in body weight. Low thyroid hormone levels cause a decrease in the release of basal energy, so that hypothyroidism causes weight gain [17].

High-fat feed will cause an increase in the amount of fat deposited in the adipose tissue [2]. Any stored fat is not directly used as an energy source but is stored in the form of triglycerides. This is what causes weight gain in all groups. Paired t test of body weight measurement, namely that there is a significant difference between the body weight of the test animals before induction or after acclimatization with the body weight of the test animals after high-fat feed induction and PTU ($p < 0.05$). This means that the induction of high-fat feed and PTU affects the weight gain of the tested animals. This is in line with research which shows that hypothyrodism by giving PTU 54 mg/kgBW / day orally for 14 days increases the body weight of male Wistar rats [17].

3.5 *Measurement of Triglyceride Levels, Total Cholesterol, HDL and LDL*

Measurement of triglyceride levels, total cholesterol, HDL and LDL was carried out three times, namely before induction, after induction, and after treatment.

Normal total cholesterol levels in mice are 10 - 54 mg/dL [18]. Figure 2 shows a graph of the results of the average total cholesterol levels of mice after acclimatization or before induction which have an average total cholesterol level exceeding the normal limit, this indicates that the rats have high total cholesterol before induction High levels of total cholesterol in mice can be caused by feeding with high fat content prior to acclimatization. In addition, stress can also trigger an increase in cholesterol, causing high levels of total cholesterol in the test rats before being induced by high-fat supplements and PTU.

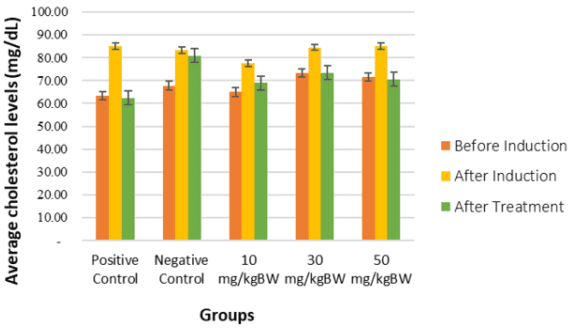

Figure 2. Graph of the average cholesterol levels of rats

Based on Figure 2, after the rats were treated, the largest average reduction in total cholesterol levels was in the positive control group of 22.56 ± 12.48 mg/dL. The positive control group experienced the greatest reduction in cholesterol levels due to the administration of simvastatin which works by competitively inhibiting HMG CoA reductase in the cholesterol synthesis process in the liver. Meanwhile, the negative control group experienced the opposite, where the reduction in total cholesterol was only slightly 2.28 ± 1.71 mg/dL. This is because the negative control group was only given 0.5% Na CMC induction which was inert and had no effect on reducing total cholesterol levels. A decrease in total cholesterol levels occurred in each treatment.

The mechanism of flavonoids in reducing total cholesterol levels is by reducing the activity of HMG-CoA reductase, reducing the activity of the Acyl-CoA Cholesterol Acyltransferase (ACAT) enzyme, and reducing cholesterol absorption in the digestive tract [19]. Saponins can reduce serum cholesterol levels by the possibility of binding saponins with cholesterol. Meanwhile, according to other studies, saponins also work by depositing cholesterol and interfering in the enterohepatic circulation of bile acids which makes cholesterol absorption in the intestine disturbed [13]. While the tannins in the body will bind to body proteins and will coat the intestinal wall so that fat absorption will be inhibited.

Triglyceride levels were measured using the GOD-PAP method.Triglycerides will be enzymatically hydrolyzed into glycerol and free acids with a special lipase to form a color complex that can be measured using a spectrophotometer [12].

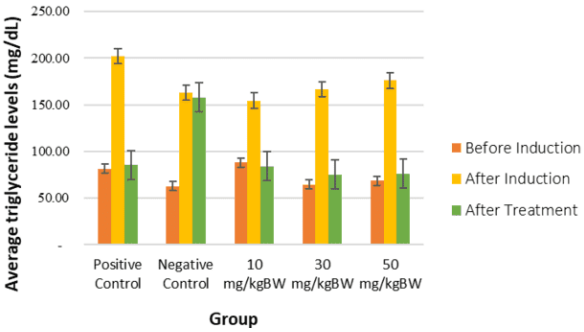

Figure 3. Graph of average triglyceride levels in rats

Based on the literature, normal triglyceride levels in mice are 26-145 mg/dL [18]. The results of the measurement of rat triglyceride levels can be seen in figure 3. In Figure 3 shows the average triglyceride levels of rats above, the results of the rats' triglyceride levels after acclimatization or before induction have an average triglyceride level in the normal range. After the induction of PTU and high-fat supplements, there was an increase in triglyceride levels in the mice, this was caused by the metabolism of excess fat consumed by the rats into triglycerides [17].

The decrease in triglyceride levels was in the positive control group given simvastatin, which was 116.85 ± 64.48 mg/dL. Based on the research which states that a decrease in triglyceride and LDL levels is associated with a decrease in total cholesterol [11]. This relationship is unidirectional, namely if the total cholesterol level decreases, the triglycerides and serum LDL will also decrease. The decrease in triglyceride levels in the negative group is because the body can naturally degrade fat into fatty acids and glycerol which can then form micelles [21].

The decrease in triglyceride levels in each group was due to the content of secondary metabolites contained in the ethyl acetate fraction of melinjo leaves. Flavonoid compounds reduce cholesterol levels by increasing the density of LDL receptors in the liver, binding to apolipoprotein B, reducing triglycerides (Tg) and increasing HDL and reducing cholesterol levels in the blood [21].

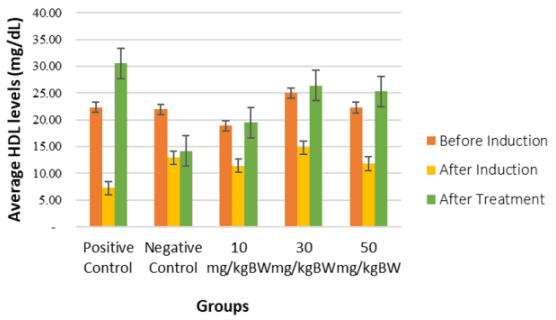

Figure 4. Graph of average HDL levels of rats

Normal rat blood plasma HDL cholesterol levels are ≥35 mg/dL [18]. The results of measurements of rat HDL cholesterol levels can be seen in Figure 4. In Figure 4, the average HDL levels of mice after acclimatization or before induction have an average less than the normal limit, this is because the rats already have high total cholesterol so that levels HDL in mice is low. After being induced high fat feed and PTU, there was a decrease in HDL levels in all groups. This is because feeding high in fat, where feeding high in saturated fatty acids can suppress HDL synthesis by decreasing levels of apolipoprotein A-1, which is a precursor for the formation of HDL [22].

The highest increase in HDL was in the positive control group given simvastatin, which was 23.25 ± 12.99 mg/dL. The mechanism of simvastatin in increasing HDL levels is by inhibiting HMG-CoA reductase which will increase the activity of the Proliferator Activated Receptors (PPARα) which regulates gene transcription so that it can reduce triglycerides, LDL. Simvastatin can also increase HDL by synthesizing apo AI in the liver which can increase the formation of HDL particles precursors, and reduce Cholesterol Ester Transfer Protein (CETP) activity. Inhibition of this activity will increase HDL levels by 3-15% compared to the use of other drugs [11].

The increase in HDL levels in the test group was due to the secondary metabolites contained in the ethyl acetate fraction of melinjo leaves. Flavonoid compounds increase HDL by increasing the activity of lecithin cholesterol acyl transferase (LCAT). LCAT is an enzyme that can convert free cholesterol into cholesterol esters that are more hydrophobic, so that cholesterol esters can bind to lipoprotein core particles to form new HDL [22].

LDL levels were measured using the indirect method with the Friedewald formula. LDL levels are measured based on the results of reducing total cholesterol with HDL cholesterol levels and triglyceride levels, so that LDL levels are strongly influenced by these three measurements. Based on the literature, normal LDL levels in mice are 7-27.2 mg/dL [22]. The results of measuring LDL cholesterol levels in rats can be seen in Figure 5.

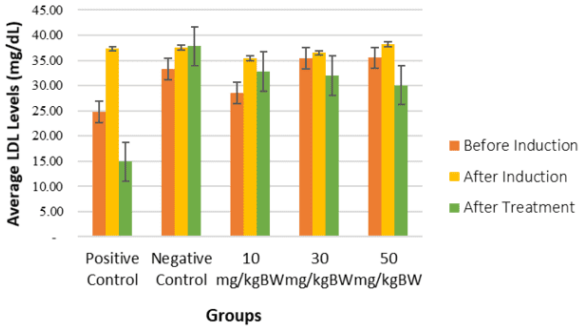

Figure 5. Graph of average LDL levels in rats

Based on Figure 5 which shows the graph of LDL levels in rats, the results of LDL levels in the negative group rats and the test group with the treatment dose of 10 mg/kgBW and 30 mg/kgBW before induction have an average LDL level exceeding the normal limit, this shows that the mice already have High LDL before induction. High levels of LDL in mice can occur due to feeding containing high fat before acclimatization, resulting in an increase in LDL of the test rats before induction.

All groups had increased LDL after PTU induction and high fat feed. This is because PTU is an antithyroid substance that can damage the thyroid gland and cause a hypothyroid condition. This condition causes a decrease in the synthesis and expression of LDL receptors in the tissues, so that LDL circulates a lot in the blood and causes hyperlipidemia [37]. In addition, some cholesterol is transported by VLDL and is hydrolyzed by lipoprotein lipases to form LDL [24]. Based on the graph above, after treatment, the highest reduction in LDL levels was in the positive control group given simvastatin, namely 22.43 ± 12.54 mg / dL.

One of the compounds that play a role in reducing LDL is flavonoids, where flavonoids inhibit modification of LDL oxidation. Inhibition of LDL oxidation can reduce LDL cholesterol levels [7].

The Friedewald method used in the indirect calculation of LDL levels has a weakness, namely when the chylomochron increases, the calculation error becomes large. In measuring LDL levels, the Friedewald method uses the assumption of the mass ratio of triglycerides to cholesterol in the form of VLDL which is relatively constant at a ratio of 5: 1. In fact, the ratio of triglycerides to cholesterol is not always constant at a ratio of 5: 1, so this can affect the results of checking LDL levels [26].

3.6 *Effective dose 50 (ED_{50})*

Effective dose 50 (ED_{50}) is the dose that is pharmacologically effective or effective in 50% of the population exposed to the drug. Based on the calculations obtained, the ED_{50} cholesterol value from the ethyl acetate fraction of melinjo leaves was 37.74 mg/kgBW, ED_{50} for triglycerides obtained is 21.51 mg/kgBW, ED_{50} for HDL obtained is 42.565 mg/kgBW, ED_{50} for LDL obtained is 37.14 mg/kgBW. Score *Effective dose*50 (ED50) ethyl acetate fraction of melinjo leaves as an antihyperlipidemia was taken from the mean value of Effective Dose (ED_{50}) for the four lipid parameters, namely 34.74 mg/kgBW. The data obtained from the Effective Dose (ED_{50}) are between the doses of 10 mg/kgBW and 50 mg/kgBW.

3.7 *Liver Macroscopic Observations*

Macroscopic observations of rat liver were performed by surgery with one rat in each representative group of animals (normal group, positive control, negative control, and 3 test groups). Macroscopic observations of the liver can be seen in Table 3.

Fatty liver can occur due to increased lipids in liver cells (hepatocytes). This is due to the failure of normal liver fat metabolism either due to a damage in the liver cells or the delivery of fat, fatty acids or carbohydrates to the fat secretion capacity of liver cells. Most of the fat in the liver is in the form of triglycerides, phospholipids, fatty acids, cholesterol, and cholesterol esters. In heavy fatty liver, liver fat can reach 50-60% of total liver weight [25].

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Table 3. Macroscopic Observation of Rat Liver

Group of Rats	Color	Texture	Weigh t (g)	*Balloni ng*
Normal	Brownish red	Chewy	9.67	-
Positive control	Brownish red	Chewy	11.97	-
Negative control	Blackish red	It's a little harsh	12.53	+
Group 1	Purple red	It's a little harsh	12.13	+
Group 2	Brownish red	Chewy	11.39	+
Group 3	Brownish red	Chewy	11.26	-

Macroscopically positive control rat liver showed the result that it had brownish red color, chewy texture, weight 11.97 g and there was no fat on the surface of the rat liver. This is due to giving simvastatin to positive control mice. Simvastatin is a statin class that can be used as an antioxidant. The mechanism of this statin class is by inhibiting oxidant formation by influencing NADPH oxidase, blocking the effects of ROS (reactive oxygen species) as an antioxidant, or increasing nitric oxidants that neutralize ROS radicals, including free radicals

Macroscopic observation of the liver of rats in the negative control group found the liver was blackish red, had a slightly hard texture, weighed 12.53 g and there was fat on the surface of the liver. This is because after induction of the test animal and it was declared hyperlipid, the negative control mice were only given 0.5% NaCMC which had no activity in reducing lipid levels and the effect of giving PTU which could cause a decrease in the synthesis and expression of LDL receptors in the tissue. Therefore, LDL circulates a lot in the blood and causes hyperlipidemia which can lead to fatty liver [17].

Macroscopic observation results in the liver of the test group rats, both the ethyl acetate fraction of melinjo leaves with a dose of 10 mg/kg, 30 mg/kg, and a dose of 50 mg/kg, showed that the fraction had activity in reducing fatty liver. Flavonoid compounds that function as antioxidants play a role in delaying the process of lipid oxidation so that they can prevent an increase in LDL (Low Density Lipoprotein) and total cholesterol levels. Tannin compounds can also reduce cholesterol levels in the body by binding bile acids into the small intestine and excreted through feces [26].

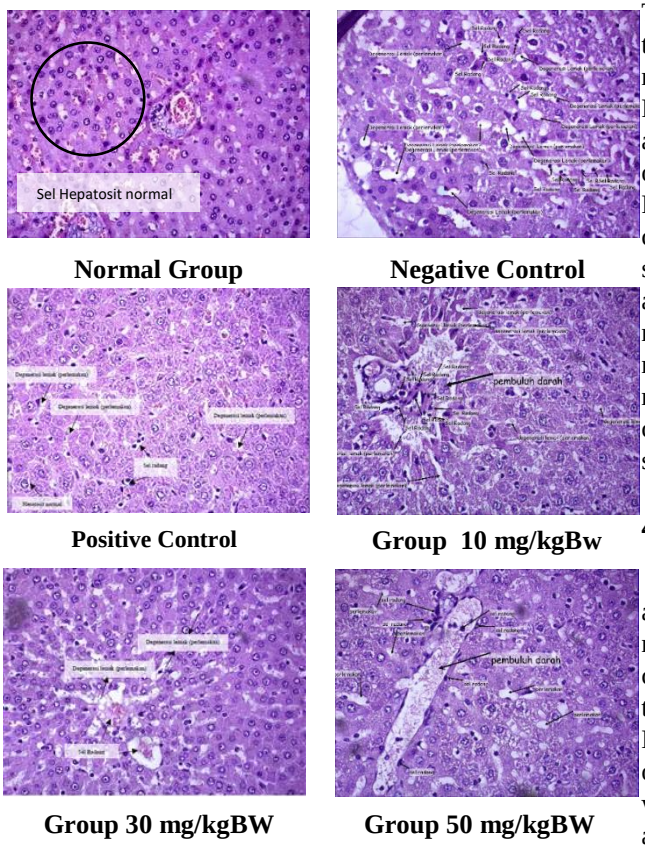
3.8 *Liver Histopathology Observations*

Histopathological evaluation to assess hepatocyte necrosis in this study used a grading system or a score based on the percentage of cells that were damaged. Cell necrosis in the normal liver can occur in about 0-10% of all cells in the centrilobular zone [11]. Based on Table 4, it shows that the microscopic condition of the liver in the normal group did not have damage to the tissue and there was no degeneration of fat or the liver in a healthy state. The negative group that was only given Na-CMC without any treatment showed that there was liver damage and the highest fat degeneration than the other groups, which was 80%. This is in line with the macroscopic results of the liver where the negative control group has abnormal liver color, texture, and weight

Table 4. Necrosis and Fat Degeneration Histopathology of Rat Liver

Group of Rats	Necrosis (%)	Fat Degeneration (%)
Normal	0	0
Positive Control	20	20
Negative Control	80	80
Group 1	60	60
Group 2	40	40
Group 3	20	20

Hepatocytes that experience necrosis appear without streaking of the cell nucleus so that they appear pale. Fatty liver is indicated by empty vacuoles of various sizes in the cytoplasm of the liver. Fat degeneration or steatosis is an intracitoplasmic accumulation of triglycerides that can occur due to an increase in free fatty acids, a reduction in free fatty acid oxidation, and a decrease in triglyceride exports due to deficiency of fat-binding apoproteins. Hepatocytes that undergo fatty degeneration appear as cells that have small (microvesicular) vacuoles in the cytoplasm at an early stage and will develop into larger vacuoles (macrovesicular) so that they press the nucleus to the edge [11].



Normal Group **Negative Control**

Positive Control **Group 10 mg/kgBw**

Group 30 mg/kgBW **Group 50 mg/kgBW**

Figure 6. Histopathology of the liver, enlargement of 40 x 100

Based on Table 4, the positive group given simvastatin experienced the lowest necrosis and fat degeneration, namely 20%. This is because Simvastatin acts as an antioxidant and against lipid peroxidation. Simvastatin antioxidant activity is associated with the presence of a carbonyl group. The carbonyl group in simvastatin can bind to free electrons from reactive radicals or as a scavenger agent [28]. The carbonyl group found in simvastatin is thought to react directly with reactive radicals due to exposure to PTU and high-fat feed.

The results of the histological microscopic examination in this study, which can be seen in Figure 6, show the dilation of blood vessels and bleeding in the sinusoids of the liver so that the tissue appears redder. Congestion is a pathological reaction as a manifestation of inflammation due to injury. After injury, capillary dilatation occurs due to vasodilator stimulation so that the vascularization at the site of the injury widened and filled with stagnant blood (congestion). In acute congestion, the area experiencing a dam occurs in the central area and if the congestion lasts a long time, the entire edge of the lobule experiences a dam accompanied by enlargement of the liver sinusoids containing erythrocytes.

The histological changes in the test group were due to the administration of the ethyl acetate fraction of melinjo leaves which contained flavonoids. Flavonoid compoundThese have antioxidant and anti-inflammatory effects by inhibiting xanthine oxidase which is responsible for the formation of ROS (Reactive Oxygen Species). ROS inhibition can reduce liver cell injury characterized by sinusoid dilation, necrosis, and steatosis. After administration of the ethyl acetate fraction of melinjo leaves showed repair of liver tissue microstructure damage in groups I, II and III with a radier image of the hepatocyte plate surrounding the central vein, found steatotic cells with dilated sinusoids.

4. CONCLUSION

The ethyl acetate fraction of melinjo leaves was able to reduce lipid levels the best at a dose of 50 mg/kgBW with a percent reduction in total cholesterol of 52.16%, LDL of 56.32%, triglycerides of 56.69% and a percent increase in HDL levels of 53, 34%. The effective dose (ED_{50}) of ethyl acetate fraction as an antihyperlipidemia was 34.74 mg/kgBW. The effect of giving ethyl acetate fraction of melinjo leaves (*Gnetum gnemon L.*) on the liver of male white rats Wistar strain is macroscopically at a dose of 10 mg/kgBW and 30 mg/kgBW there is ballooning while at a dose of 50 mg/kgBW there is no ballooning like the liver in the normal control group . The histopathological structure of the liver cells at a dose of 50 mg/kgBW was the same as the simvastatin group with% necrosis and the smallest% fat by 20% compared to other test groups.

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