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Liver Histological Response of Hyperlipidemic Male Rat (*Rattus norvegicus*) to Lakum Leaf Extract

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Abstract— *The leaf of lakum (Cayratia trifolia (L.) Domin.) contains compounds that are expectedly antihyperlipidemic. This study aims to determine the liver histological response to the lakum leaf extract and its effect on the levels of SGOT and SGPT in hyperlipidemic male rats (Rattus norvegicus). The male Wistar rats were randomly divided into 4 treatment groups. P_0 was a group of rats that were given standard feed, P_1 was a group that were fed with high fat diet, P_2 was a group that were fed high fat diet and lakum leaf extract with a dose of 40mg/200g BW (body weight)/day, and P_3 was a group were fed high fat diet and simvastatin at a dose of 0.18mg/200g BW/day. High fat diet was given for 30 days, whilst treatments were implemented for 28 days. The histological descriptions showed that the hepatocyte cell repair occurred in the treatment group that was given lakum leaf extract but there were no significant differences in liver weight as well as SGOT and SGPT levels. Lakum leaf extract can be used as an antihyperlipidemic agent whilst maintaining the histology of hepatocytes and would not interfere with the liver function of the hyperlipidemic male rats.*

Keywords— ***hyperlipidemic, lakum leaf extract, liver, SGOT, SGPT, white rats.***

I. INTRODUCTION

The leaf of lakum (*Cayratia trifolia* (L.) Domin.) contains alkaloids, flavonoids, saponins, tannins, phenols, amino acids and proteins, carbohydrates, cardio glycosides, terpenoids, and steroids (Sundaram et al., 2015b). Dichloromethane isolation of the compounds in the leaf extract of the lakum plant exhibits contents of β-sitosterol, stigmasterol, squalene and lutein (Ragasa, 2014).

Squalene is an intermediate agent in the biosynthesis of cholesterol and all steroid hormones. Squalene can affect the synthesis rate of HMG-CoA reductase in cells. Squalene inhibits the activity of HMG-CoA reductase and increases the activity of Acyl-CoA cholesterol acyltransferase. As a result, the cholesterol formation will be inhibited; a good implication for hyperlipidemic sufferers (Ronco and Stefani, 2013).

Various secondary metabolites found in the lakum plants, such as flavonoids, phenolics, terpenoids, steroids, vitamin C, and squalene, have antioxidant activity. Antioxidants can ward off free radicals that are formed due to the accumulation of lipids in the liver. The presence of these antioxidant properties can inhibit further damage to the liver caused by oxidative stress due to hyperlipidemia. (Brewer, 2011).

Liver damage due to hyperlipidemia can lead to increased release of metabolic enzymes into the blood such as glutamic oxaloacetic transaminase and glutamic pyruvate transaminase. Serum Glutamic Oxaloacetic Transaminase (SGOT) is an enzyme found in the liver, ren, heart, and muscles. Meanwhile, Serum Glutamic Pyruvate Transaminase (SGPT) is an enzyme that is mostly found in the liver – small amounts are also found in the heart muscle, ren, and skeletal muscle (Nasution et al., 2015). Between both, increase in SGPT enzyme secretion is considered to be in stronger relation to the presence of hepatocellular damage (Reza and Rachmawati, 2017). The normal SGOT level that can be found in mice is 45.7-80.8 IU/L and as for the SGPT, it is 17.5-30.2 IU/L (Gad, 2016).

Based on the research of Batra et al. (2013) the use of lakum plant root extract at a dose of 200 mg/kg body weight and 400 mg/kg body weight can significantly reduce triglyceride levels, total cholesterol, and LDL cholesterol,

as well as increase HDL cholesterol levels in diabetic rats (Batra et al., 2013). The results from the study of Kumar et al. (2011) showed that lakum ethanol extract administration at a dose of 200 mg/kg BW for 7 days increased endogenous antioxidant activity, and decreased serum aminotransferase levels to near normal values. Histological analysis showed that there were necrosis and fat infiltration in the group that was not given the extract, while the group that was given the extract showed hepatocyte regeneration.

Based on this description, it is apparent that lakum leaf has the potential to be used as herbal medicine to help overcome hyperlipidemia. Accordingly, investigation of the liver histological response of hyperlipidemic male rats *(Rattus norvegicus)* to the administration of lakum extract is, thus, necessary.

II. METHODS

Feed Preparation

The composition of triglyceride-induced hyperlipidemia feed per 1 kg consisted of 296.7 g cornstarch, 140 g casein, 250 g fructose, 214 g solid oil, 50 g alpha-cellulose, 35 g mineral mix, 10 g vitamin mix, 1.8 g methionine, and 2.5 g choline chloride. The standard feed used in this study was Paterung-B51. The standard feed per pack (500 g) was observed to consist of 13% moisture content, 18.5-20% protein, 4% fat, 6% crude fibre, 8% ash, 0.9% calcium, and 0.7% phosphorus.

Lakum Leaf Extract Preparation

Cleaned semi-aged lakum leaves (third to fifth row of leaves from the shoot) as much as 800 g were prepared, cut into pieces, and dried in an oven at 40^{o}C until a constant weight was obtained. The dried leaves were soaked in 96% ethanol solvent for 2 x 24 hours for maceration. The maceration product was then filtered and the filtrate was evaporated using a rotary evaporator at a temperature of 800^{o}C. The extract obtained from the evaporation was in the form of a paste. Preparation of lakum leaf extract stock solution at a dose of 40 mg/200 g BW was done by dissolving 10 grams of lakum leaf extract in 50 ml of distilled water. For each treatment involving the extract, 0.2 ml of the stock solution was used (Modification of Layli et al., 2016).

Animal Study

The present research on the liver histological response of male rats to the administration of grape bush leaf extract had received approval from the Health Research Ethics Commission (KEPK) from the Faculty of Medicine, Diponegoro University under Ethical Clearance No. 75/EC/H/KEPK/FK-UNDIP/V/2019. This research was conducted from February-May 2019 at the Animal Structure and Function Laboratory, Department of Biology, Faculty of Sciences and Mathematics, Diponegoro University Semarang.

As many as 20 male Wistar strain rats (*Rattus norvegicus*) were selected based on the criteria of 3 months old and weighing ± 200 g. The white rats were obtained from the Biology Laboratory, Faculty of Mathematics and Natural Sciences, UNNES.

The weighing of the rats was done every 3 days using digital scales. The ambient temperature used during the treatment ranged from 25-28^{o}C and the humidity 60-80%. Measurements of environmental temperature and humidity were carried out 2 times a day, at 0700-0800 and 1700-1800 hours.

The acclimation process was carried out for 7 days. After the acclimation process was complete, the rats were divided into 4 treatment groups. Group P_0 consisted of rats that were given standard feed, group P_1 were rats that were fed with hyperlipidemia-inducing feed without extract treatment, group P_2 were rats fed with hyperlipidemia-inducing feed as well as lakum extract at a dose of 40 mg/200 g BW, and group P_3 were rats fed with hyperlipidemia-inducing feed and simvastatin at a dose of 0.18 mg/200 g BW. Hyperlipidemia-inducing feed was given for 30 days. After 30 days, the first blood count was carried out to check that the mice had experienced hyperlipidemia. The treatment was continued with the administration of grape bush extract and simvastatin for 28 days. Grape bush extract and simvastatin were given orally using an orogastric tube. In this study, the observed variables were the histological description of the liver, hepatocyte diameter, AST and ALT levels, as well as the body and liver weights of the rats.

After 28 days, mouse dissection was conducted. The male white rats were put in a closed container and anaesthetized using a cotton ball soaked with chloroform until they passed out and they were operated on to remove the liver. The liver that had been taken was washed with saline solution and then weighed. An incision of the liver was performed transversely on the left lobule around the central vein with a size of 2x2 cm. The histological preparations of the liver were made using the paraffin method and the hematoxylin-eosin dye. Examination of the preparations was carried out at 40x, 100x and 400x magnifications. The analysis was performed using SPSS 22 software for windows.

III. RESULTS AND DISCUSSION

The histological structures of the livers of the male white rats after treatment are shown in Figure 1 and Figure 2. Histological description of the liver in the P_0 group, namely the group that was only given standard feed, indicated the normal condition of hepatocyte cells pinpointed by the prominent cell nucleus located in the middle of the cell, the clear boundaries between hepatocytes, and the regular

arrangement of the sinusoid parts (Surasa, 2014). Fat infiltration occurred in some hepatocyte cells. Sinusoid congestion was found in all treatment groups but with different severity. Congestion is a pathological reaction in which inflammation occurs due to injury. In acute congestion, the area that experiences the congestion is the central area and if the congestion lasts a long time, the entire lobule edge will experience blockage accompanied by enlargement of the liver sinusoid containing erythrocytes (Makiyah et al., 2018).

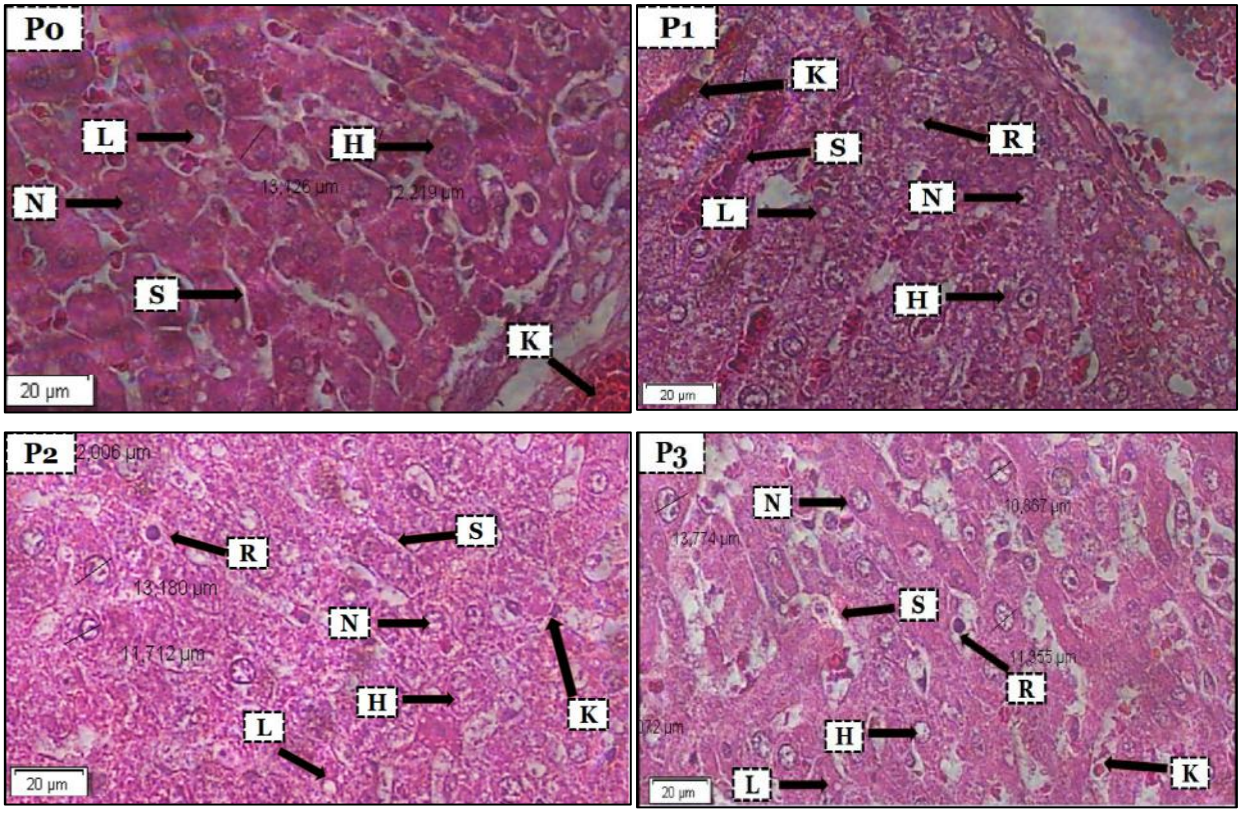

Fig.1: Liver Histology of Male White Rats Group P_0, P_1, P_2, and P_3 at 400x Microscope Magnification (H = hepatocytes, N = nucleus, S = sinusoid, L = fat infiltration, R = inflammatory cells, K = congestion)

In Figure 2, it can be seen that there were blood clottings (congestion) in the central vein in all treatment groups. Congestion in the central vein that occurred in the P_0 group was less severe than that in the P_1 and P_3 groups.

The histological description of the liver in the P_1 group, namely the group that was fed with hyperlipidemia-inducing feed alone, showed the occurrence of fat infiltration. This is indicated by the hepatocyte cells that pushed the nucleus to the edge, enlarged cell nucleus, inflamed cells, the edges of the hepatocytes that were not clearly visible, the sinusoids that were enlarged and filled with erythrocyte cells, as well as the central vein that were almost filled with erythrocytes due to congestion. Congestion shows a disturbance in circulation, which can indicate blockage or narrowing of blood vessels, even, failure in blood flow (Merdana, 2019).

The process of hepatocyte damage begins with the degeneration process where if it continues, it can cause necrosis (Utomo et al., 2012). Cell degeneration is characterized by an abnormal cell shape indicated by enlarged cells, enlarged or reduced cell nucleus, and the presence of non-nucleated (Hardiningtyas et al., 2014).

Hepatocyte cell damage can be caused by an increase in free radicals which can trigger changes in the properties of cell membranes and cytoplasmic membranes of cell elements such as mitochondria and lysosomes due to lipid peroxidation. Consequently, the edges of the hepatocytes can appear to be unclear in observations. Free radicals can also result in cell nucleus damage, in particular, abnormal cell structure and eventually necrosis. Cells that experience necrosis will release various mediators that can induce an inflammatory process and attract inflammatory cells (Andreas et al., 2012).

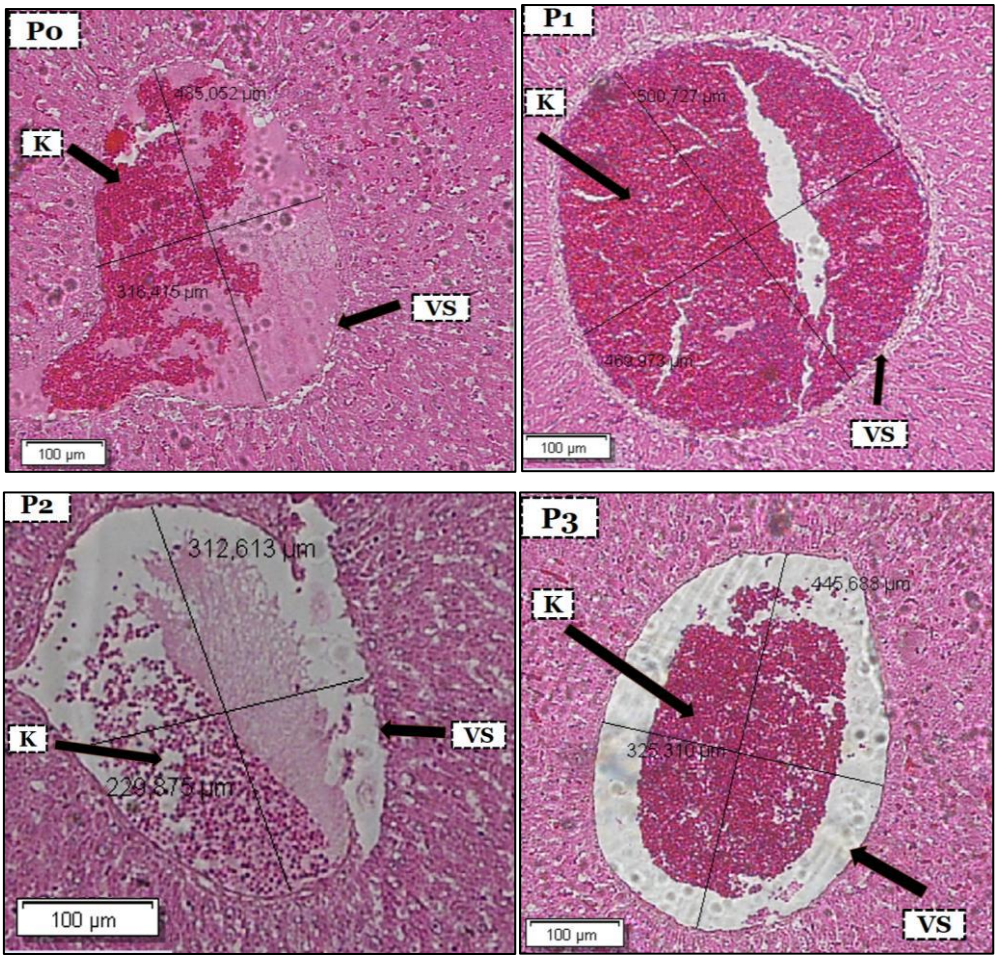

Fig.2: Histology of the liver central veins of male white rats in groups P_0, P_1, P_2, and P_3 at 100x microscope magnification (K = congestion, VS = central vein)

Inflammatory cells found in treatment P_1 were attracted by hyperlipidemic conditions. Hyperlipidemia can increase the production of free radicals. Excessive free radicals will attack cell macromolecules and can cause cell damage and death, leading to diapedesis which causes inflammatory cells to come out and infiltrate the tissue to remove damaged cells (Roslizawaty et al., 2016).

Fat infiltration in group P_1 occurred due to continuous hyperlipidemic feeding without discontinuation or extract treatment. An increase in the amount of free fatty acids into the liver due to hyperlipidemia will prompt the re-esterification of free fatty acids into triglycerides that will be excreted from the liver in the form of VLDL. Fat infiltration can occur when the amount of free fatty acids synthesized exceeds the capacity of the liver to oxidize or convert it into VLDL. The liver does not accumulate triglycerides under normal conditions, but the stress due to hyperlipidemic conditions will disrupt lipid metabolism which leads to lipid accumulation in the liver. Accumulation of lipids in the liver is also associated with lipotoxicity due to increased stress of the endoplasmic reticulum and mitochondria. Subsequently, free fatty acids will be oxidized through β-oxidation, esterified into triglycerides, and packed into lipoproteins to be secreted or stored as lipid droplets in the liver (Nassir et al., 2015).

The liver histological description of the male rats in group P_2, namely the treatment group that was given hyperlipidemia-inducing feed and lakum leaf extract at a dose of 40 mg/200 g BW/day for 28 days showed a reduction in congestion in the sinusoids and central veins. Though, the sinusoids did not appear to be regular; there were inflammatory cells, infiltration of fat, enlargement of the nucleus of cells, and the boundaries between hepatocyte cells that just begin to appear clearly. Administration of lakum leaf extract appeared to have resulted in the improvement of hepatic cells. This is in accordance with the research conducted by Kumar et al. (2011) on the liver response after the exposure to hepatotoxic substances that showed hepatocyte regeneration in the treatment group that was given lakum extract. This hepatocyte repair can be caused by the presence of antioxidants such as squalene.

The presence of flavonoids in lakum leaf also play role in reducing fatty liver (Castillo, 2009).

The histological description of the liver in the P_3 treatment group, namely the treatment group of that was given hyperlipidemia-inducing feed and simvastatin drug at a dose of 0.18 mg/200 g BW/day for 28 days showed the presence of fat infiltration in hepatocyte cells, enlarged cell nuclei, boundaries between hepatocytes that were not yet clear, the presence of inflammatory cells, and size of the sinusoid that had begun to decrease. However, severe congestion in the sinusoid and central veins were also observed. Simvastatin is an antihyperlipidemic drug that is widely used to reduce cholesterol formation. Its use, however, has contraindications for liver disease (Tripathi, 2013).

The P_3 treatment group displayed congestion of blood vessels in sinusoid, demonstrating the accumulation erythrocytes that filled the sinusoid. The widening of the sinusoids occurred because the toxins in the liver cells easily come into contact with the sinusoids. It is possible that simvastatin had a toxic effect on the liver cells. This can be due to the side effects associated with statin use, namely liver damage and myopathy (Karmayani, 2013). The widening of the sinusoids was caused by strong blood flow and damage to the liver cells caused by hyperlipidemia, where the sinusoid wall, consisted of endothelial cells, formed incomplete layers. The liver and sinusoid cells are bounded by the subendothelial cleft containing microvilli of the liver cells. This facilitates direct contact between the surface of the hepatic cells and the sinusoids, thereby facilitating the exchange of macromolecules. Based on the histological description of the liver, the use of lakum leaf extract in improving the histological structure of the liver in hyperlipidemic rats appeared to be more superior than simvastatin administration or no treatment at all (Surasa, 2014).

The effect of giving grape bush leaf extract on the liver histological response of male white rats was observed through the assessment of hepatocyte diameter, liver weight, and bodyweight of each treatment. Measurement results of the diameter of hepatocytes shown in Table 4.1. presented significantly different results ($P < 0.05$). This implied the effect of the grape bush leaf extract in the treatments.

Based on the Duncan test in Table 4.1, the P_1 treatment group was significantly different from the other treatment groups, while the control group (P_0) was not significantly different from the P_2 and P_3 treatment groups. Thus, it can be said that the hepatocyte diameter of hyperlipidemic rats that were not given lakum leaf or simvastatin had experienced a recognizable reduction in size. Moreover, the administration of lakum leaf extract or simvastatin was not thought to had caused hepatocyte damage, which was not significantly different from the control group (P_0).

Table 4.1. Average Hepatocyte Diameter, Liver Weight, and Body Weight of Male White Rats

Variable	Treatment Group			
	P0	P1	P2	P3
	$\bar{X}$ ± SD	$\bar{X}$ ± SD	$\bar{X}$ ± SD	$\bar{X}$ ± SD
Hepatocyte diameter (µm)	$12,29^{bc}$±0,65	$10,25^{a}$±0,51	$12,55^{c}$±0,96	$11,35^{ab}$±0,78
Liver mass (g)	9,93±1,14	9,24±1,12	8,32±2,01	7,66±0,61
Body mass (g)	$290,62^{c}$±15,43	$251,98^{b}$±20,86	$227,28^{ab}$±26,19	$208,55^{a}$±24,21

Description: Numbers followed by the different superscript on the same line indicate significant difference ($P < 0.05$)

P_0: Test animals fed only standard feed

P_1: Test animals fed only hyperlipidemia-inducing feed

P_2: Test animals fed with hyperlipidemia-inducing feed + lakum leaf extract at a dose of 40 mg/200 g BW/ day for 28 days

P3: Test animals fed with hyperlipidemia-inducing feed + simvastatin drug at 0.18mg/200 g BW/day for 28 days

According to Kumar's (2011) study, hepatocyte cells show regeneration from fat infiltration after the administration of the lakum leaf extract. A reduction in the diameter of the hepatocytes indicated that these cells were degenerated or had shown signs of necrosis (Hardiningtyas et al., 2014). The untreated hyperlipidemia condition can cause the nucleus and sinusoids to be enlarged, pressing onto the cell membrane. As a result, the cell membrane will experience a shrinkage in size. Hepatic cell necrosis is usually characterized by the presence of a hepatic cell nucleus that appears shrunken, irregular borders, and dark colouration. Necrosis is irreversible (Surasa, 2014). This phenomenon

had led to a reduction in the diameter of hepatocytes in the P_1 group.

The variation in hepatocyte size in the treatments did not affect the liver weight. Based on ANOVA analysis, the difference was determinedly not significant (P <0.05), indicating that there was no effect of treatment on liver weight. Fat infiltration in hepatocyte cells did not occur in all hepatocyte cells and only occurred in a small proportion of the hepatocyte cells affected. This condition caused the hepatic weight to not be affected by the infiltration of fat cells. Besides, the accumulation of triglycerides in lipid drops in the liver cells could also be released in the form of VLDL by the liver to the adipose tissue or other tissues that need it.

Based on ANOVA analysis, it appears that administration of lakum leaf extract had an effect on the bodyweight of the rats, indicated by a significant value of P <0.005, namely 0.003 <0.005. The control group (P_0) was significantly different from all treatment groups. Group P_2 was not significantly different from group P_1 but was different from group P_3.

The administration of lakum leaf extract not only affected the histological structure of the liver, but also the function of the organ. The indicators that were used to measure liver function after the administration of lakum leaf extract were the levels of SGOT and SGPT. AST is found in the liver, heart, and muscles, while ALT is found in the cytosol of hepatocytes and small amounts also in the heart, ren, and skeletal muscles. SGOT and SGPT will be released in large amounts into the bloodstream in the event of cellular injury (Kim et al., 2008; Nasution et al., 2015). The SGPT enzyme is considered to be much more specific in assessing hepatocellular destruction more effectively than the SGOT enzyme because the amount is higher in the liver (Reza and Rachmawati, 2017).

Based on the ANOVA test, there was no significant difference in the levels of SGOT and SGPT in the sample rats. This showed that there was no effect of treatment on the levels of SGOT and SGPT in the hyperlipidemic male rats. Measurement results of the levels of SGOT and SGPT are shown in Table 4.2.

Table 2. SGOT and SGPT Levels of Hyperlipidemic Male White Rats After Giving Lakum Leaf Extract

Variable	Treatment Group			
	P0	P1	P2	P3
	X ± SD	X ± SD	X ± SD	X ± SD
SGOT (UI/L)	147,50±43,31	141,50±46,54	167,00±53,24	199,50±32,83
SGPT (UI/L)	46,75±9,64	26,50±9,75	47,50±32,60	20,25±4,50

Description: Numbers without a superscript on the same line indicate no significant difference (P> 0.05)

P_0: Test animals fed only standard feed

P_1: Test animals fed only hyperlipidemia-inducing feed

P_2: Test animals fed with hyperlipidemia-inducing feed + lakum leaf extract at a dose of 40 mg/200 g BW/ day for 28 days

P3: Test animals fed with hyperlipidemia-inducing feed + simvastatin drug at 0.18mg/200 g BW/day for 28 days

IV. CONCLUSION

The use of lakum leaf extract at a dose of 40 mg / 200 g BW for 28 days can be used as an antihyperlipidemic which can repair hepatocyte cell damage due to hyperlipidemic conditions. The use of lakum leaf extract showed significant results on hepatocyte cell diameter and body weight, but there was no significant difference in the variable liver weight, as well as the levels of SGOT and SGPT in rats.

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