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The Effect of Patin Fish Oil Extract on LDL Cholesterol Levels *Rattus norvegicus* was Induced by Alloxan

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Abstract

Diabetes mellitus is a chronic hyperglycemic syndrome that can be accompanied by lipid metabolism disorders. Alloxan is a diabetogenic agent that is associated with an increase in LDL cholesterol levels. Patin fish contain omega-3 and oleic acid, which can potentially reduce or control LDL cholesterol levels. This study aims to determine the effect of Patin fish (*Pangasius hypophthalmus*) oil extract on LDL cholesterol levels of alloxan-induced *Rattus norvegicus*. The method of this study is the post-test-only control group design. Twenty-seven experimental animals were divided into 3 groups; (1) the 1st group was without treatment, (2) 2nd group was induced with 150 mg/kg BW alloxan on the 7th day; (3) 3rd group was induced with 150 mg/kg BW alloxan on the 7th day and given the Patin fish oil extract at a dose of 73 mg/kg BW on the 10th day for 14 days. Blood samples were taken on the 24th day to determine LDL cholesterol levels. The results of the One-Way ANOVA test showed a significant mean difference, $p=0,001$ ($p<\alpha$). The results of the Post-Hoc LSD test showed a meant difference between the 1st group and the 2nd control group $p=0,001$ ($p<\alpha$); 2nd group and 3rd group $p=0,026$ ($p<\alpha$); between the 1st group and the 3rd group $p=0,015$ ($p<\alpha$). Giving alloxan 150 mg/kg BW can significantly increase LDL cholesterol levels of *Rattus norvegicus* and administration of Patin fish oil extract 73 mg/kg BW can significantly reduce LDL cholesterol levels of *Rattus norvegicus*.

Keywords: Alloxan, LDL, *Pangasius hypophthalmus*.

Original Research Article

Pengaruh Ekstrak Minyak Ikan Patin terhadap Kadar Kolesterol LDL *Rattus norvegicus* yang Diinduksi Aloksan

Abstrak

Diabetes melitus merupakan sindroma hiperglikemia kronis yang dapat disertai dengan kelainan metabolisme lipid. Aloksan merupakan agen diabetogenik yang berkaitan dengan peningkatan kadar kolesterol LDL. Ikan Patin mengandung omega-3 dan asam oleat yang dapat berpotensi untuk menurunkan atau mengontrol kadar kolesterol LDL. Penelitian ini bertujuan untuk mengetahui pengaruh ekstrak minyak Ikan Patin (*Pangasius hypophthalmus*) terhadap kadar kolesterol LDL *Rattus norvegicus* yang diinduksi aloksan. Penelitian ini termasuk penelitian

eksperimental laboratoris dengan metode post test-only control group design. Hewan coba sebesar 27 ekor dibagi menjadi 3 kelompok; (1) kelompok kontrol negatif tanpa perlakuan, (2) kelompok kontrol positif yang diinduksi aloksan dosis 150 mg/kgBB pada hari ke-7; (3) kelompok perlakuan yang diinduksi aloksan dosis 150 mg/kgBB pada hari ke-7 dan diberi ekstrak minyak Ikan Patin dosis 73 mg/kgBB pada hari ke-10 setiap hari selama 14 hari. Pengambilan sampel darah dilakukan pada hari ke-24 untuk menentukan kadar kolesterol LDL. Hasil uji One-Way ANOVA menunjukkan perbedaan rerata kadar kolesterol LDL yang signifikan dengan nilai $p=0,001$ ($p<\alpha$). Hasil uji Post-Hoc LSD menunjukkan perbedaan rerata yang signifikan antara kelompok kontrol negatif dan kelompok kontrol positif $p=0,001$ ($p<\alpha$); kelompok kontrol positif dan kelompok perlakuan $p=0,026$ ($p<\alpha$);

*juga antara kelompok kontrol negatif dan kelompok perlakuan $p=0,015$ ($p<\alpha$). Pemberian aloksan 150 mg/kgBB dapat meningkatkan kadar kolesterol LDL *Rattus norvegicus* secara signifikan dan pemberian ekstrak minyak Ikan Patin*

*(*Pangasius hypophthalmus*) 73 mg/kgBB dapat menurunkan kadar kolesterol LDL *Rattus norvegicus* secara signifikan.*

Kata kunci: *Aloksan, LDL, *Pangasius hypophthalmus**

INTRODUCTION

Diabetes mellitus is defined as a hyperglycemic syndrome. Usually accompanied by associated metabolic disorders (lipids and proteins) (Tjokroprawiro & Murtiwi, 2015). The global prevalence of diabetes mellitus worldwide is 9.3% with a total of 463 million sufferers in 2019 (International Diabetes Federation, 2019). Without good intervention, it is estimated that in 2030 it can reach 578 million and in 2045 it will reach an increase of 51% so that it can reach 700 million sufferers (Saeedi et al., 2019). The Southeast Asia region was one of the regions with the highest number of diabetes mellitus patients in 2014 and occupied the second-highest number of deaths in the world in 2019 (International Diabetes Federation, 2019; World Health Organization, 2016).

Indonesia in 2019 was included as a country with a high number of people with diabetes, which was ranked 7th with a total of 10.7 million in the adult category aged 20-79 years (International Diabetes Federation, 2019). According to RISKESDAS, diabetes mellitus in Indonesia shows a prevalence rate of 1.5%, and the prevalence in East Java Province is 2%, these two prevalences are at all ages, while based on the age of 15 years, the prevalence in Indonesia is 2% and in East Java Province is 2.6% (RISKESDAS, 2018).

Low insulin levels, so that they cannot respond adequately or the occurrence of insulin resistance in target tissues, can cause metabolic abnormalities in carbohydrates, proteins, and lipids. Insulin resistance that causes diabetes, can also cause various other manifestations, such as obesity, nephropathy, essential hypertension, and dyslipidemia (Kharroubi, 2015). Lipid metabolism disorders with signs of abnormalities in the lipid fraction, namely increased levels of LDL cholesterol, total cholesterol, with or without triglycerides, as well as a decrease in HDL cholesterol, are referred to as dyslipidemia (Tedhy & Wisnu, 2020). Dyslipidemia is usually accompanied by a decrease in LDL particle diameter, where the small LDL cholesterol particle

diameter is more susceptible to oxidation, and chronic hyperglycemia can promote LDL cholesterol glycation, both of these processes are believed to increase LDL cholesterol atherogenicity (Elzubair, 2011; Kharroubi, 2015). LDL cholesterol in the management of dyslipidemia is the main target because LDL has a relationship with cardiovascular events (Erwinanto et al., 2017).

Insulin deficiency can lead to activation of the hormone-sensitive lipase enzyme found in adipose cells. Normally, insulin can inhibit this enzyme. In the absence of insulin, hormone-sensitive lipase enzymes can cause triglycerides to hydrolyze and release large amounts of fatty acids into the blood circulation and cause free fatty acid levels to become too much (Hall, 2016). Hypercholesterolemia in diabetes mellitus is also caused by an increase in VLDL because insulin cannot inhibit lipoprotein lipase which in turn will increase LDL production, as well as reduce LDL receptor affinity. This situation causes the clearance of LDL and VLDL remnant to decrease and causes the LDL concentration to increase (Hanum, 2013). A large number of patients with diabetes mellitus and its relation to LDL cholesterol can potentially harm the sufferer. Poor glycemic control coupled with the presence of lipid disorders such as increased cholesterol, LDL, triglycerides (TG), and decreased HDL greatly affects mortality and morbidity (Driyah et al., 2019). Proper management needs to be done in order to control LDL cholesterol properly so that it can avoid its dangerous risks.

Alloxan is a derivative of urea or an oxygenated pyrimidine derivative, which in aqueous solution forms as an alloxan hydrate (Rohilla & Ali, 2012). Alloxan itself is a glucose analog because it has structural similarities or molecular shape with glucose which causes alloxan to penetrate the plasma membrane through GLUT2 and enter the cytosol. The characteristics possessed by alloxan are hydrophilic with a partition coefficient of 1.8 (Handajani, 2021). Alloxan causes selective necrosis of pancreatic cells which have a role to

produce insulin. The diabetogenic action of alloxan is obtained when injected parenterally such as subcutaneously, intraperitoneally, or intraperitoneally (Rohilla & Ali, 2012). Alloxan is not only used in the induction of diabetes mellitus but can also be used in the induction of hyperlipidemia (Handajani, 2021).

Catfish are fish that live in freshwater, where they are found in many of Indonesia's major rivers, especially in Kalimantan, Sumatra, and parts of Java. There are various types of catfish, one of which is the Siamese catfish (*Pangasius hypophthalmus*) and the Jambal catfish (*Pangasius djambal*) (Harmain & Dali, 2017).

According to research conducted by Soltan, (2012), omega-3 in fish oil can control LDL cholesterol levels (Soltan, 2012). Omega-3 has an atheroprotective function by reducing LDL synthesis. This effect is carried out by inhibiting the SREBP-1 (sterol regulatory element-binding protein-1)-mediated pathway, besides that omega-3s can also inhibit VLDL synthesis which will reduce the production of LDL cholesterol (Pizzini et al., 2017; Sinulingga et al., 2019). The potential for lowering cholesterol levels can also be provided by the content of other catfish, which is known as oleic acid. Oleic acid works by maintaining the function of LDL receptors on cell membranes, which in turn reduces LDL cholesterol levels in the circulation (Sinulingga et al., 2019).

Based on the description that has been written, the purpose of this study was to determine the effect of catfish oil extract (*Pangasius hypophthalmus*) on alloxan-induced *Rattus norvegicus* LDL cholesterol levels which is expected to help the health sector in the future and can be used as an adjuvant therapy

MATERIALS AND METHODS

This experimental laboratory research was carried out at the Biochemistry Laboratory, Faculty of Medicine, Hang Tuah University, Surabaya. The method used is the post-test-only control group design method. Probability sampling is a sampling technique used in this study, with the type used being simple random sampling.

Making Catfish Oil Extract

The method for making catfish oil extract in this study used the wet rendering method according to previous research conducted by Julaikha (2014), where the use of the wet rendering method could produce more extraction

than using the dry rendering and Bligh and Dryer methods (Hidayaturrahmah et al., 2016; Julaikha, 2014).

Catfish weighing 750 grams, washed and drained first, then cut into pieces and placed into a stainless container containing 500 ml of distilled water, then boiled until boiling, then let stand for 30 minutes while stirring slowly. After settling, strain the catfish stew so that the crude oil can be separated from the solids (Panagan et al., 2011).

Purification of catfish oil is carried out on crude oil to obtain pure catfish oil, namely by adding 2.5% NaCl to crude oil and heating for 5 minutes at 50°C (Hastarini et al., 2012; Panagan et al., 2011). After heating, the oil is separated from the water using a separatory funnel and stored in an Erlenmeyer. Then, add bentonite at 1% by weight of the oil and then heated again at 80°C for 30 minutes (Hastarini et al., 2012). The last step is to centrifuge for 10 minutes at a speed of 10,000 rpm and the oil is separated from the sediment (Julaikha, 2014).

The calculation of the dose of catfish oil extract 73 mg/kg BW with an average body weight of 176.44 grams was 12.9 mg. The catfish oil extract was dissolved in 1 ml of 0.5% CMC Na solution and administered by the intragastric sonde.

Experimental animal treatment stage

Adaptation of experimental animals was carried out for 7 days, then the treatment stage was carried out for 17 days. Alloxan induction at a dose of 150 mg/kg BW was carried out by dissolving in 0.9% NaCl solution which would be injected intraperitoneally (i.p.) on the 7th day (Miaffo et al., 2019). The dose of alloxan given to experimental animals with an average body weight of 173.8525 grams was 26.078 mg dissolved in 0.1 ml of 0.9% normal saline solution.

Treatment Group 1 is a group that is not treated. Group 2 was the group induced by alloxan at a dose of 150 mg/kg BW dissolved in 0.9% NaCl solution and injected intraperitoneally (i.p.) on the 7th day and then waited for 3 days until the 10th day. Group 3 was the group that was induced by alloxan at a dose of 150 mg/kgBW as was done in group 2 and was given catfish oil extract at a dose of 73 mg/kg BW dissolved in 0.5% CMC Na solution on the 10th day by intragastric sonde every day for 14 days.

At the end of the study, the experimental animals were sacrificed under anesthesia with the method of intramuscular injection of ketamine at

a dose of 50 mg/kg BW (Struck et al., 2011). The intramuscular injection method is an injection technique through muscle tissue, generally in the thigh muscle, or can be done on a thick muscle, namely the bicep femoris (Nugroho, 2018).

Blood samples were taken from the rat heart or intracardiac (Putri & Pralitasari, 2018). Intracardial blood sampling is done when large amounts are needed, so the experimental animals will be dissected (Nugroho, 2018). Before taking blood samples, experimental animals were given anesthesia. When he fainted, surgery was performed on the thoracic part of the needle, and it was inserted directly into the heart (Arief et al., 2012; Nugroho, 2018). The syringe used is a 3 cc syringe, then the blood that has been taken is transferred into a vacutainer blood tube without the addition of additives with a red cap. Centrifugation of test tubes containing experimental animal blood for 10 minutes at a speed of 12,000, then calculate LDL cholesterol levels (Arief et al., 2012).

Determination of LDL Cholesterol Levels

The homogenous enzymatic colorimetric assay method was used to analyze LDL cholesterol based on the breakdown of cholesterol esters by cholesterol esterase into cholesterol and free fatty acids. The presence of oxygen causes oxidation by cholesterol oxidase from cholesterol to 4-cholestenin and hydrogen peroxide. The hydrogen

peroxide then reacts with HSDA and 4-amino antipyrine due to the presence of peroxidase, resulting in a purple-blue coloration. The color of this purple-blue dye is directly proportional to the concentration of cholesterol in LDL. Then photometric measurements were taken with absorbance at 583 nm to determine the value of LDL cholesterol (Elzubair, 2011; Singh, 2016).

Data analysis

The data was carried out by the Shapiro-Wilk test as a normality test accompanied by a significance level of which was 0.05. Levene's test was carried out as a homogeneity test (Barton & Peat, 2014). If the data is normally distributed and homogeneous, then a comparative hypothesis test is carried out from the three groups, namely the One-Way Anova test parametric statistics (Sugiyono, 2012). The results of the One-Way Anova Test were significant, so it was continued with the LSD (Least Significance Difference) posthoc test (Barton & Peat, 2014).

RESULT

Blood Glucose Level

The results of the average blood glucose level measurement from group 1 were 105.33 mg/dl and group 2 was 249 mg/dl as can be seen in Figure 1.

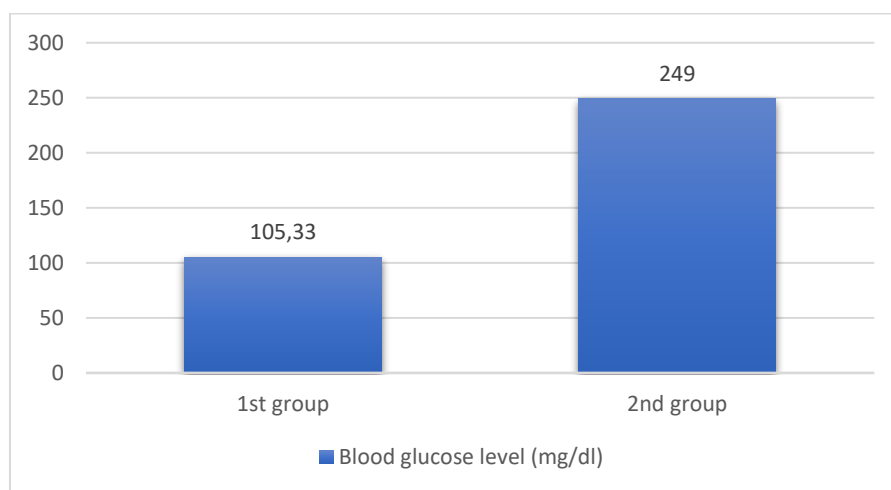


Figure 1. Average blood glucose levels in group 1 and group 2

Note : Group 1: the group that was not given treatment

Group 2: alloxan-induced group

The picture above shows that the average glucose level in group 1 is still within normal limits, while the average glucose level obtained from group 2 is 249 mg/dl, where the average is more

than 200 mg/dl. These results indicate that alloxan induction in group 2 resulted in a hyperglycemic condition and was considered to have reached a

diabetes condition (Hidayaturrahmah et al., 2017).

The results of measuring LDL cholesterol level in each group consisting of group 1, group 2, and group 3 can be seen in Table 1.

LDL Cholesterol Level

Table 1. LDL cholesterol level in each group

No.	LDL cholesterol level in each group (mg/dl)		
	Group 1	Group 2	Group 3
1	8	12	7
2	7	11	9
3	9	12	14
4	4	13	11
5	7	13	12
6	6	10	6
7	6	11	10
8	9	12	7
9	9	11	10

Note:

group 1: the group that was not given treatment

group 2: alloxan-induced group

group 3: group that was induced by alloxan and given catfish oil extract

Descriptive statistical analysis, namely the mean (mean), standard deviation, minimum, and

maximum obtained from the LDL cholesterol level variable from each group can be seen in Table 2.

Table 2 Descriptive statistical analysis of variable LDL cholesterol degree

	Group (mg/dl)		
	Group 1	Group k 2	Group 3
Average	7,2222	11,6667	9,5556
Standard Deviation	1,71594	1,00000	2,60342
Minimum	4,00	10,00	6,00
Maximum	9,00	13,00	14,00

Note:

group 1: the group that was not given treatment

group 2: alloxan-induced group

group 3: group that was induced by alloxan and given catfish oil extract

The data were tested for normality and homogeneity, the results of the homogeneity test showed the significant results in group 1 obtained 0.255 ($p > 0.05$), group 2 obtained 0.364 ($p > 0.05$), and group 3 obtained 0.809 ($p > 0.05$). The results of the normality test in the three experimental animal groups showed that the data distribution was normal.

The results of the homogeneity test showed that the significance of the data obtained from the homogeneity test, namely the Levene test conducted in this study, was 0.056 ($p > 0.05$). The results obtained showed that the groups did not have different variants or homogeneous variants.

One-Way Anova Test Parametric Statistics Test Results

The results of the normality test and homogeneity test obtained data with homogeneous variants and normally distributed, so it was continued with the parametric statistical test, namely the One-Way Anova Test. The significance obtained from the One-Way Anova test is 0.001 ($p < 0.05$). These results indicate that there is a significant difference in the mean between the three groups (group 1, 2 and 3)

Least Significance Difference (LSD) Post-Hoc Test Results

The LSD Post-Hoc test was carried out in this study in order to determine the groups with specific differences in mean LDL cholesterol levels. The results of the post-hoc LSD test can be seen in Table 3.

Table 3. LSD post-hoc test results

Comparison Group	Group 1	Group 2	Group 3
Group 1	-	0,001	0,015
Group 2	0,001	-	0,026
Group 3	0,015	0,026	-

Note: group 1: the group that was not given treatment
 group 2: alloxan-induced group
 group 3: group that was induced by alloxan and given catfish oil extract

The results showed that the significance between group 2 and group 1 was 0.001 ($p < 0.05$), group 3 and group 1 was 0.015 ($p < 0.05$), also between group 3 and group 2 was 0.026 ($p < 0.05$). The results obtained showed that between group 2 and group 1, group 3 and group 1, and also between group 3 and group 2 there was a significant difference in mean LDL cholesterol levels.

DISCUSSION

The results of the One-Way Anova test showed differences between groups which proved that the catfish oil extract had an effect on LDL cholesterol levels in *Rattus norvegicus*. The post-doc test, namely LSD, was carried out in this study and showed that there was a significant difference between group 2 induced by alloxan at a dose of 150 mg/kg BW and group 1 not given 3, namely $p = 0.001$ ($p < 0.05$). These results prove that the induction of alloxan at a dose of 150 mg/kg BW, namely 26.078 mg/173.8525 gBW dissolved in 0.9% NaCl solution and injected intraperitoneally with a volume of 0.1 ml, can cause a statistically significant increase in LDL cholesterol degree. Previous research conducted by Husna et al (2019) is in accordance with the results of the post-hoc LSD test in this study, wherein this study an increase in LDL cholesterol levels in the experimental group of animals was induced by alloxan at a dose of 150 mg/kg BW compared to the group of animals. a trial that was not induced by alloxan at a dose of 150 mg/kg BW (Husna et al., 2019).

Alloxan has a form similar to glucose so that it is rapidly and selectively absorbed by pancreatic cells. The glucose transporter (GLUT2) carries alloxan from the plasma membrane to the cytosol. GSH (reduced glutathione), ascorbate, cysteine, and a protein-bound sulfhydryl group (-SH) cause a reduction process in pancreatic cells. In

glucokinase, which is the binding site for sugar, alloxan reacts with two sulfhydryl groups (-SH) thereby creating a disulfide bond and inactivating the enzyme (Handajani, 2021; Ighodaro et al., 2017; Rohilla & Ali, 2012).

The reduction process produces dialuric acid which is oxidized back to alloxan. A redox cycle is then formed which causes the formation of ROS and superoxide radicals, where superoxide radicals result in the release of ferric ions from ferritin so that the reduction process occurs and forms ferrous and ferric ions. Superoxide radicals also cause dismutase due to the presence of superoxide dismutase which produces hydrogen peroxide. The Fenton reaction causes the formation of hydroxyl radicals and also results in the death of pancreatic cells, causing a decrease in insulin production and an increase in blood glucose which ends in diabetes mellitus (Handajani, 2021; Rohilla & Ali, 2012; Walean et al., 2020).

Insulin deficiency causes activation of the hormone-sensitive lipase enzyme found in adipose cells, which normally inhibits this enzyme. In the absence of insulin, hormone-sensitive lipase enzymes can cause triglycerides to hydrolyze and release large amounts of fatty acids into the blood circulation and cause free fatty acid levels to become too much (Hall, 2016). LDL cholesterol, especially small dense LDL will then be formed due to excessive free fatty acid levels. The smaller and denser particle size of small dense LDL makes it easier for particles to enter the blood vessels and increase the possibility of thrombosis, it is also more easily oxidized so that it is atherogenic (Adam, 2014; Daniel, 2011).

Some of the excess free fatty acids can be used as a source of energy and partly for the formation of triglycerides in the liver. In the liver, triglycerides will be re-formed and become part of VLDL which is then exchanged for cholesterol

esters from LDL cholesterol, so that LDL has a lot of triglycerides but little cholesterol esters. The presence of hepatic lipase enzymes causes hydrolyzed triglycerides to form small dense LDL which is highly atherogenic because it is easily oxidized (Adam, 2014). Hypercholesterolemia in diabetes mellitus can also be caused by an increase in VLDL because insulin cannot inhibit lipoprotein lipase so that the release of VLDL from the liver is not inhibited, which will result in an increase in IDL and LDL production, as well as a reduced affinity for LDL receptors. This situation results in decreased LDL clearance and VLDL remnants and causes an increased LDL concentration (Hanum, 2013).

The results of statistical analysis using the post-hoc LSD test showed that there was a significant mean difference between group 2 induced by alloxan and group 3 induced by alloxan and given catfish oil extract, namely $p=0.026$ ($p<0.05$). The results obtained showed that the administration of catfish oil extract at a dose of 73 mg/kg BW, namely 12.9 mg/176,484 gBW dissolved in 0.5% CMC Na and administered by intragastric sonde with a volume of 1 ml, could reduce LDL cholesterol levels. Which is statistically significant.

The results of the post-hoc LSD test analysis obtained are in accordance with the research conducted by Sinulingga et al (2019), where the administration of 1.04 ml/20 gBB of processed Pindang Patin for 21 days caused a decrease in LDL cholesterol levels in mice. male (*Mus Musculus L.*) with a statistically significant high-fat diet (Sinulingga et al., 2019). The results of the post-hoc LSD test analysis are also supported by research conducted by Soltan (2012), where administration of 8% fish oil for 8 weeks can reduce LDL cholesterol levels in rats on a high-fructose diet statistically significant (Soltan, 2012).

The effect of reducing LDL cholesterol levels can occur due to the PUFA content, namely omega-3 contained in catfish, especially the content of DHA and EPA which have an atheroprotective function by reducing LDL synthesis. This effect is accomplished by inhibiting the SREBP-1 (sterol regulatory element-binding protein-1)-mediated pathway (Botham & Mayer, 2015; Pizzini et al., 2017). Omega-3 also has an effect on the mechanism of lipoprotein production in the liver, where omega-3 can inhibit VLDL synthesis so that it will reduce the production of LDL cholesterol (Sinulingga et al.,

2019). Not only has the potential to reduce LDL levels, omega-3s also have an effect on the LDL variant itself, namely reducing small dense LDL levels, the number of LDL particles, and increasing LDL particle size (Talebi et al., 2020). In addition, EPA and DHA can also slow down platelet aggregation and the atherogenic process (Ardi, 2019).

Another composition of catfish that has the potential to reduce LDL cholesterol levels is oleic acid which works by influencing its structural function on cell membranes that work in signal transduction and regulate functions, namely maintaining the function of LDL receptors by keeping the moisture from the membrane. The way oleic acid works can result in the entry of LDL cholesterol into liver cells in greater numbers, thereby reducing LDL cholesterol levels in blood circulation (Sinulingga et al., 2019).

CONCLUSION

The results obtained from research conducted by researchers, it can be concluded that the induction of alloxan at a dose of 150 mg/kgBW dissolved in 0.9% NaCl solution can increase the mean LDL cholesterol level of *Rattus norvegicus* which is statistically mean. Patin extract at a dose of 73 mg/kg BW dissolved in 0.5% CMC Na for 14 days, was able to reduce the mean LDL cholesterol level of *Rattus norvegicus*.

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