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# Histopathology of Rats Intestinal Treated with High-Fat Diet and Neem Leaf Extract

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***ABSTRACT***

*The high-fat diet can increase free radicals, which can damage the intestinal histological structure. The ethanolic neem leaf extract contains antioxidants that have the potential to help improve the intestinal histological structure. This study aims to examine the effectiveness of ethanolic neem (A. indica* A. Juss*) leaf extract on the histopathological structure of the small intestine in rats (R. norvegicus* L.*) hyperlipidemia. This study used 24 rats divided into 6 groups: normal control, positive control, high-fat diet + simvastatin, the test groups dose I, II, and III (ethanolic neem leaf extract 75 mg/200 gBW, 100 mg/200 gBW, and 125 mg/200 gBW). The results of the analysis using ANOVA test on the height of villi, mucous thickness, and number of goblet cells in the small intestine part duodenum, jejunum, and ileum showed no significant difference (p>0.05). The results of the morphology of epithelial cells in treatments P0, P2, and P5 showed normal epithelial cells, whereas treatments P1, P3, and P4 showed erosion epithelial cells. Based on the result, the ethanolic neem (A. indica* A. Juss*) leaf extract can suppress the damage effects of epithelial cells and the histological structure of the small intestine in rats (R. norvegicus* L.*) hyperlipidemia.*



## INTRODUCTION

The era of globalization has led to changes in people's lifestyles and food consumption. Changes in food nutrition consumption patterns are marked by increased fast-food consumption with common ingredients of high fat, salt, and low fiber that can affect both the lipid profile and subsequently the health of the Indonesian people (Sartika, 2011). Consumption of a high-fat diet is the main cause of increasing total cholesterol levels in blood. These dietary changes lead to various diseases, one of them is high cholesterol

(hypercholesterolemia) (Dalimartha, 2001).

Hypercholesterolemia can cause an increase in free radicals and a decrease in the antioxidant mechanism associated with oxidative stress (Duarte *et al*., 2010). Wresdiyati *et al.* (2011) stated the hypercholesterolemia state causes damage to the cell membranes that form free radicals and therefore disrupts organ functions. The increase in free radicals can cause tissue damages in the small intestine and may interfere with the absorption of food nutrients. The damages that occurred give an image of desquamation of the small intestinal villi, which leads to intestinal malabsorption and maldigestion (Ananto *et al*., 2017).

The prevention of oxidative stress can be done by maintaining the homeostasis of the prooxidant mechanism (free radicals) with antioxidants. Antioxidant consumption derived from natural ingredients can help to maintain the homeostasis of the prooxidant mechanism with antioxidants. One of the natural ingredients containing antioxidants is neem leaves (*Azadirachta indica* A. Juss.).

Neem plants are empirically used as medicine for reducing cholesterol as they contain alkaloids, flavonoids and terpenoids (Arief *et al*., 2012). Biswas *et al*. (2002) stated that neem leaves can be used to reduce total cholesterol in the blood, LDL- and VLDL-cholesterol, triglycerides, and total lipids in serum. The effect of reducing cholesterol levels occurs because the flavonoid contained in the ethanol extract of neem leaves is able to reduce blood cholesterol (Kumar et al., 2010).

Flavonoid is a group of polyphenol compound known to have traits such as capturing free radicals, hydrolytic and oxidative enzyme inhibitors as well as working as anti-inflammatory (Pourmorad *et al*., 2006). Balaji & Cheralathan (2015) stated that neem leaves extract has a role as antioxidant compound that can delay or slow down the speed of oxidation.

Based on the facts above, this study was performed to obtain evidence and information regarding the histopathology of rats (*Rattus norvegicus* L.) intestinal tenue induced by high-fat diet that exposed with ethanolic neem (*Azadirachta indica A.* Juss) leaf extract in terms of epithelial cell morphology,

height of vili, mucous layer thickness, and the number of goblet cells in duodenum, jejunum, and ileum.

## RESEARCH METHODS

### Tools

The tools used are rat cages, water bottles, food containers, oven, digital scale, analytical scale, cannula tip injection syringes, measuring cup, cholesterol test kit, dissecting set, rotary microtome, embedding cassette, tissue processor, base mold, dropping pipette, object glass, cover glass, microscope, and photomicrograph.

### Animal

The materials used are 24 white male rats (*Rattus norvegicus* L.) with 2 months of age and ± 200g of body weight, ethanolic neem leaf extract, simvastatin, duck egg yolks, cooking oil with 9 times frying, food pellets, and water.

### Making High-Fat Diet

High-fat diet consists of a mixture of commercial food with reused cooking oil and duck egg yolk. The reused cooking oil used in this study was obtained from one liter of cooking oil used to fry 450g white tofu for ± 10 minutes at a temperature of 150-165$^{o}$C with deep fat frying techniques (Muhartono *et al*., 2018) for nine (9) times frying (Hanung *et al*., 2019).

### Making of Ethanolic Neem Leaf Extract (*Azadirachta indica* A. Juss)

Neem leaf were dried in an oven at 45-50$^{o}$C. Dried leaf was crushed mechanically by hand to form a grainy powder. Neem leaf extraction was done by maceration method using 70% ethanol and evaporated to a powdered extract.

### Research Design

The animals test used were acclimated. Acclimation was carried out for ± 1 week. During the acclimation period, the animals test were given standard feed and drinking water on an ad libitum basis. Animals test will be weighed to determine the initial body weight after the acclimation period. This study used completely randomized design along with 6 treatments and 4 replicates, which was: negative control (P0) was given commercial food, positive control (P1) was given high-fat diet and duck egg yolk orally 2,5 ml/200gBW, P2 was given high-fat diet and duck egg yolk orally 2,5 ml/200gBW + 8 mg/200gBW

simvastatin in 1 ml of distilled water, P3 was given high-fat diet and duck egg yolk orally 2,5 ml/200gBW + 75 mg/200gBW ethanolic *A. Indica* leaf extract in 1 ml of distilled water, P4 was given high-fat diet and duck egg yolk orally 2,5 ml/200gBW + 100 mg/200gBW ethanolic *A. Indica* leaf extract in 1 ml of distilled water, and P5 was given high-fat diet and duck egg yolk orally 2,5 ml/200gBW + 125 mg/200gBW ethanolic *A. Indica* leaf extract in 1 ml of distilled water. High-fat diet was given every morning for 45 days as much as ± 30g, and 75 ml of water every day. Duck egg yolk was given once every two days in the morning, while simvastatin and ethanolic neem leaf extract was given orally every afternoon for 44 days with a whipped tip injection syringe.

**Making of Microscope Slide**

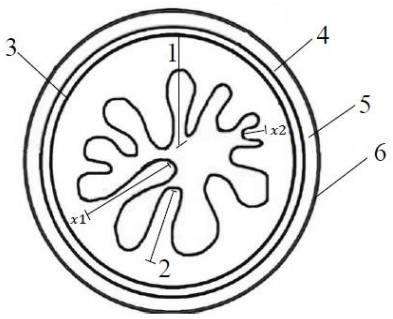
The histopathology microscope slide of the small intestine was made by using paraffin method. The steps taken after the fixation process were to make thin cuts of tissue ± 4 mm thick with orientation appropriate to the organ trimming. The blade used for trimming was the No. 22-24 scalpel blade. The number of tissue pieces that can be contained in the embedding cassette ranges from 1-5 pieces according to the size of the organ. Tissue dehydration was performed after trimming using a tissue processor, it is intended to remove the water contained in the tissue by using a dehydrated liquid, a high concentration alcohol. The dehydrated samples will go through a clearing process, by cleaning the tissue using a clearing agent, xylol. The cleaning agent was then replaced by paraffin by inserting liquid paraffin solution so that the paraffin was penetrated through the tissue. This process is called impregnation (Isdadiyanto, 2019).

Then the tissue was embedded in the embedding cassette called a block. The tissue in the blocks that had been cooled was trimmed into 4 μ thick slices with a rotary microtome. The slices were attached to a glass object previously smeared with mayer's egg albumin and dropped in distilled water and then left to dry at room temperature. The next step after the microanatomy microscope slide dry was staining the slide. The staining method used was the Hematoxylin Ehrlich-Eosin staining method, then mounting it by dripping enough of it and

covering it with a cover glass (Isdadiyanto, 2019).

**Microscope Slide Observation and Data Analysis**

The observed variables were the height of vili, mucous layer thickness, number of goblet cells, and epithelial cell morphology. Observations of epithelial cell morphology, height of vili, mucous layer thickness, number of goblet cells were carried out in four fields of view. The mucous layer thickness and the height of villi were determined based on the longest and shortest measurements, then averaged. Observation of the epithelial cell morphology in the form of descriptions. Each intestinal incision was observed and measured as shown in Figure 1.




Explanation:
1. Mucous Layer
2. Villi
3. Muscularis mucous
4. Submucous
5. Muscularis externa
6. Serosa
$x1$. Longest Size
$x2$. Shortest Size

**Figure 1.** Schematic of Measuring Height of Villi and Mucous Layer Thickness

Measurement of mucous layer thickness and the height of villi were carried out as follows:

$$\bar{x} = \frac{x1 + x2}{2}$$

The data obtained were analyzed by Analysis of Variance (ANOVA) at the level of 95% ($\alpha = 0.05$). Data analysis using SPSS 25 for Windows.

## RESULT AND DISCUSSION

The result of the study on vili height, mucous layer thickness, and the number of goblet cells of the small intestine in rats (*R. norvegicus* L.) hyperlipidemia that exposed with ethanolic neem (*Azadirachta indica* A. Juss) leaf extract can be seen in Table 1, 2, and3.

**Table 1**. The analysis result of the average of vili height, mucous layer thickness, and the number of goblet cells of the small intestine of the duodenum in rats hyperlipidemia that exposed with ethanolic neem leaf extract.

| Treatment | Research Variable (Duodenum) | | |
|---|---|---|---|
| | Vili Height (µm) $\bar{X}$±SD | Mucous Thickness (µm) $\bar{X}$±SD | The Number of Goblet Cells $\bar{X}$±SD |
| P0 | 467.69 ± 45.04 | 614.50 ± 44.59 | 107.00 ± 41.28 |
| P1 | 427.00 ± 35.66 | 539.30 ± 35.59 | 133.50 ± 32.27 |
| P2 | 484.76 ± 28.45 | 620.14 ± 62.81 | 170.50 ± 26.19 |
| P3 | 454.29 ± 36.38 | 596.16 ± 38.30 | 157.50 ± 51.04 |
| P4 | 450.79 ± 145.30 | 598.36 ± 183.29 | 160.75 ± 30.54 |
| P5 | 528.90 ± 47.85 | 674.90 ± 67.76 | 128.50 ± 8.73 |

Description: Data are presented in the form of significance level of 95%. The analysis showed mean $\bar{X}$ ± standard deviation (SD) which has that the results were not significantly different been tested with the ANOVA test with a ($p > 0.05$).

**Table 2**. The analysis result of the average of vili height, mucous layer thickness, and the number of goblet cells of the small intestine of the jejunum in rats hyperlipidemia that exposed with ethanolic neemleaf extract.

| Treatment | Research Variable (Jejunum) | | |
|---|---|---|---|
| | Vili Height (µm) $\bar{X}$±SD | Mucous Thickness (µm) $\bar{X}$±SD | The Number of Goblet Cells $\bar{X}$±SD |
| P0 | 327.42 ± 130.87 | 470.62 ± 83.54 | 133.00 ± 36.91 |
| P1 | 426.20 ± 84.97 | 539.01 ± 71.96 | 130.25 ± 13.25 |
| P2 | 311.04 ± 66.82 | 435.53 ± 71.74 | 107.25 ± 21.33 |
| P3 | 428.00 ± 11.53 | 550.23 ± 13.62 | 130.75 ± 13.94 |
| P4 | 388.98 ± 129.97 | 514.56 ± 157.67 | 158.25 ± 44.92 |
| P5 | 360.88 ± 168.52 | 464.34 ± 187.62 | 126.50 ± 32.21 |

Description: Data are presented in the form of significance level of 95%. The analysis showed mean $\bar{X}$ ± standard deviation (SD) which has that the results were not significantly different been tested with the ANOVA test with a ($p > 0.05$).

**Table 3**. The analysis result of the average of vili height, mucous layer thickness, and the number of goblet cells of the small intestine of the ileum in rats hyperlipidemia that exposed with ethanolic neem leaf extract.

| Treatment | Research Variable (Ileum) | | |
|---|---|---|---|
| | Vili Height (µm) $\bar{X}$±SD | Mucous Thickness (µm) $\bar{X}$±SD | The Number of Goblet Cells $\bar{X}$±SD |
| P0 | 315.98 ± 40.45 | 450.84 ± 74.49 | 126.75 ± 70.56 |
| P1 | 298.80 ± 52.00 | 397.17 ± 59.65 | 198.75 ± 68.74 |
| P2 | 304.25 ± 52.30 | 404.44 ± 60.16 | 139.00 ± 14.85 |
| P3 | 206.27 ± 24.14 | 290.39 ± 33.80 | 118.50 ± 73.89 |
| P4 | 282.26 ± 112.05 | 409.04 ± 147.12 | 163.00 ± 48.55 |
| P5 | 267.92 ± 33.49 | 365.60 ± 49.97 | 112.50 ± 23.23 |

Description: Data are presented in the form of significance level of 95%. The analysis showed mean $\bar{X}$ ± standard deviation (SD) which has that the results were not significantly different been tested with the ANOVA test with a ($p > 0.05$).

Statistical analysis shows that the average height of vili, mucous layer thickness, and the number of goblet cells in the duodenum, jejunum, and ileum were not significantly different ($p > 0.05$) between treatment groups. This can be seen from the average size of vili height, mucous layer thickness, and the number of goblet cells between treatment groups (Tables 1, 2, and 3) which are relatively the same. The treatment did not affect the vili height, mucous layer thickness, and the number of goblet cells proved that the distribution of neem leaf extract at different doses for 44 days did not affect the vili structure, mucous layer, and the number of goblet cells so it can protect and doesn't cause damage against the vili, mucous layer, and goblet cells in the duodenum, jejunum, and ileum. Based on the research of Ghatule *et al*. (2012) showed that neem leaf extract given at an oral dose of 5 g/kg did not show acute toxicity such as increased motor activity, saliva secretion, intestinal spasms, coma and death after being observed for 1 week. Research by Apriyanto (2002), stated that giving ethanol extract of neem leaves at a dose of 6247.7 mg/kg bw gave abnormalities in the intestine in the form of erosion of the epithelium and epithelial mucosa and an increase in the number of goblet cells.

Research conducted by Soares *et al*. (2015) stated that high-fat feeding will lead to food retention in the small

intestine due to reduced motility. This retention of food in the small intestine can increase the length of the vili. The composition of the types of fat in the high-fat diet in this study contained high linoleic acid, oleic acid and palmitic acid. The high-fat diet in this study also contained high cholesterol, which suggests that the high-fat content in this treatment are responsible for the difference in the height of the vili.

Linoleic acid, oleic acid, and palmitic acid are non-saturated fatty acids which undergo a deep-frying process so they contain saturated fatty acids. The deep-frying process, besides causing the formation of long-chain saturated fatty acids, also causes thermal polymerization and oxidation reactions that form trans-fatty acids. Trans-fat is believed to be a source of oxidative stress (Martin *et al*., 2007). Trans-fatty acids can increase LDL cholesterol levels, but at the same time reduce HDL cholesterol levels (Tuminah, 2009). The results showed that giving ethanolic neem leaf extract could increase HDL levels, reduce LDL and TG cholesterol levels in the blood serum of white rats (Isdadiyanto *et al*., 2020).

Treuting *et al.* (2012) stated that diet is one of the factors that affects the microanatomy structure of the mucous layer. This study indicates that the largest number of goblet cells found in the ileum treatment of P1. Research by *Soares et al*. (2015) stated that giving a high-fat diet leads to an increase in the number of goblet cells and inflammatory activity as part of the adaptation process.

The epithelial cells of the small intestine of the duodenum, jejunum, and ileum of each treatment group are shown in figures 2, 3, and 4

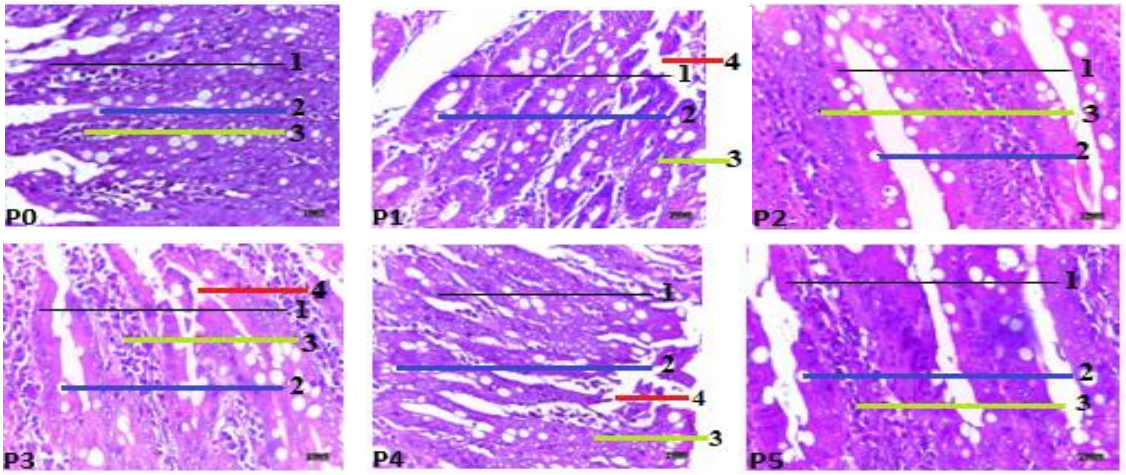



**Figure 2.** Duodenum structure of rats hyperlipidemia that exposed with ethanolic neem (*Azadirachta indica* A. Juss) leaf extract. Magnification 400x.

Description: Black arrows: 1. Epithelial cells, Blue arrows: 2. Goblet cells, Green arrows: 3. Lacteal, Red arrows: 4. Erosion of epithelial cells.

The results of observations of the morphology of small intestine epithelial cells in duodenum in treatment P0, P2, and P5 show that normal epithelial cell are marked with structures that did not change, while treatments P1, P3, and P4 show that the occurrence of epithelial cell erosion was marked by the loss of epithelial cells (Figure 2. P1, P3, and P4 No.4).

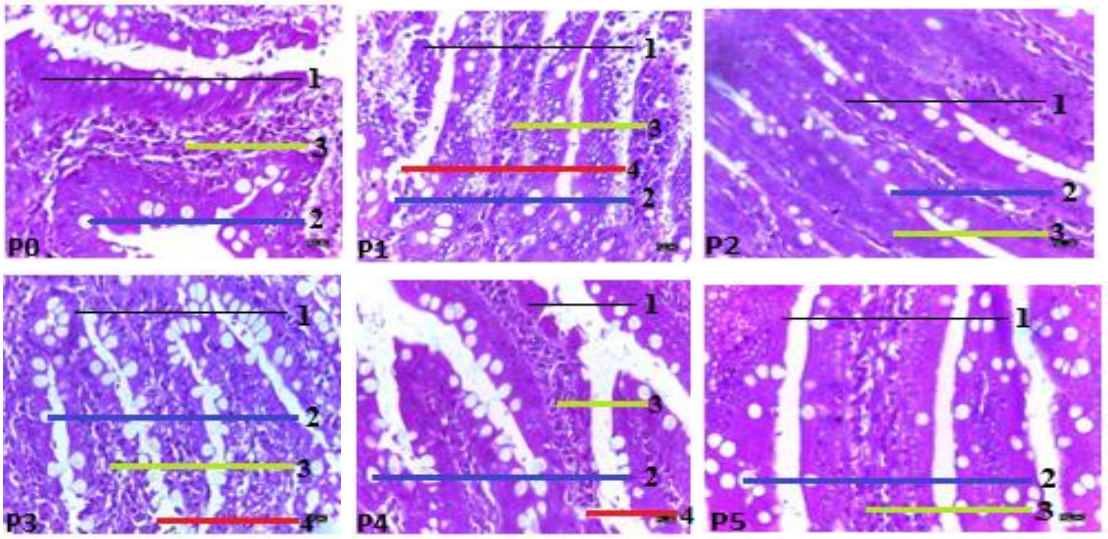



**Figure 3.** Jejunum structure of rats hyperlipidemia that exposed with ethanolic neem (*Azadirachta indica* A. Juss) leaf extract. Magnification 400x.

Description: Black arrows: 1. Epithelial cells, Blue arrows: 2. Goblet cells, Green arrows: 3. Lacteal, Red arrows: 4. Erosion of epithelial cells.

The result of the observation of the morphology of small intestine epithelial cells in jejunum in treatment P0, P2, and P5 show that normal epithelial cells are marked with structures that did not change, while P1, P3, and P4 show that the occurrence of epithelial cell erosion was marked by the loss of epithelial cells. (Figure 3. P1, P3, and P4 No.4).

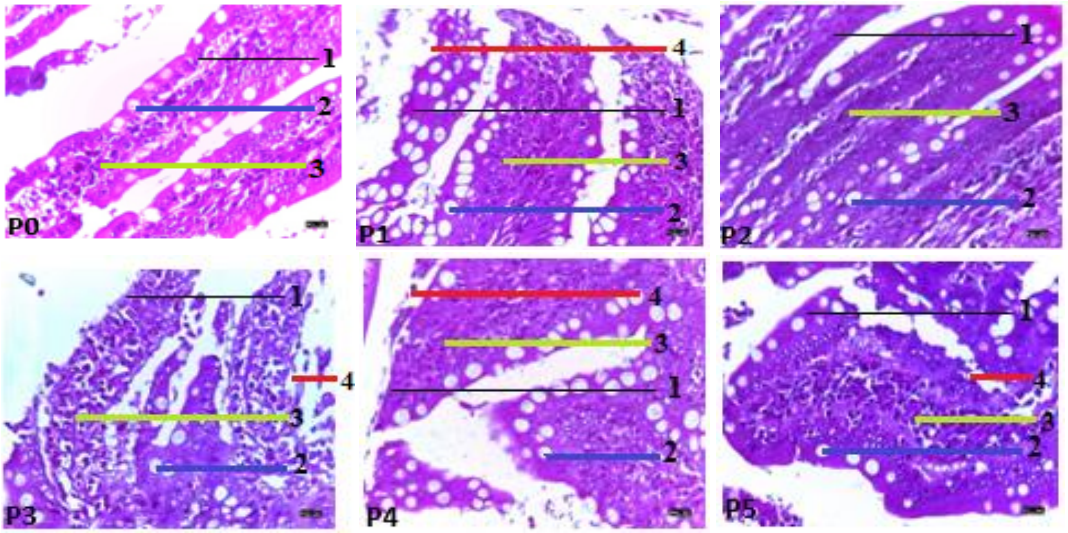



**Figure 4.** Ileum structure of rats hyperlipidemia that exposed with ethanolic neem (*Azadirachta indica* A. Juss) leaf extract. Magnification 400x.

Description: Black arrows: 1. Epithelial cells, Blue arrows: 2. Goblet cells, Green arrows: 3. Lacteal, Red arrows: 4. Erosion of epithelial cells.

The result of observation of the morphology of small intestine epithelial cells in the ileum in treatment P0 and P2 show that normal epithelial cells are marked with structures that did not change, while P1, P3, P4, and P5 show that the occurrence of epithelial cell erosion was marked by the loss of epithelial cells (Figure 4. P1, P3, P4, and P5 No. 4).

High-fat diet can cause histological damage to the intestinal tenue by the presence of the missing

intestinal epithelium in a particular focus, which also called epithelial erosion (Horii & Kobayashi, 2002). Research conducted by Ananto *et al*. (2017) claims that rats inducted by peroxide lipids can cause changes in epithelial cells. Free radical increases can cause epithelial cell damage and it can interfere with the process of nutrient absorption. Lipid peroxidation is the process of converting unsaturated fatty acid into free radicals through hydrogen absorption (Murray *et al*., 2003). Epithelial cell erosion is one of the most damaging of the intestines, where the intestines lose parts of the epithelial cells in the mucosal lining of the small intestine. Epithelial erosion of the intestines can interfere with nutrient absorption (Sulastri *et al*., 2018).

The duodenum and jejunum epithelial cells in treatment P0, P2, and P5 didn't experience structural changes, so nutrients in food can be absorbed by the intestinal tenue. This shows that P5 treatment with 125 mg/200gBW of neem leaf extract is able to withstand the damage to epithelial cells. The ileum epithelial cells in treatment P0 and P2 also experience no structural change. The absence of impaired absorption of nutrients can be seen from the weight of the body in the normal range. This is in accordance with the opinion of Wolfensohn & Lloyd (2003) that the body weight of normal rats ranges from 150-600g. The result of the analysis of body weight at the end of the treatment shows no significant effect ($p>0.05$) between treatment groups. Body weight in the normal range indicates that the ethanolic neem leaf extract does not affect the metabolism of the test animals. Epithelial cells located on the surface of the intestinal tenue serve as absorptive cells in nutrients absorption, so that when there's a disturbance in the epithelial cells, there will be impaired absorption of nutrient (Campbell *et al*., 2019).

Epithelial cells duodenum, jejunum, and ileum in treatment P1, P3, and P4 experienced erosion. Erosion of epithelial cells is thought to be the result of feeding a large amount of high-fat diet, which interferes with the absorption process and the ethanol extract of neem leaf at a dose of 75 mg/200gBW and a dose of 100

mg/200gBW which has not been able to inhibit the disruption of the absorption process. Epithelial cells in treatment P1 experienced more erosion than P3 and P4. Epithelial cells in treatment P4 experienced less erosion compared to treatment P3. This shows that the distribution of 100 mg/200gBW of neem leaf extract is more capable of reducing epithelial cell damage than the 75 mg/200gBW neem leaf extract. The distribution of 100 mg/200gBW of neem leaf extract was able to reduce epithelial cell erosion as shown in Figure P4 where the number of epithelial cells that experienced erosion was relatively small compared to other groups that experienced erosion. Erosion of ileum epithelial cells in the P5 treatment is thought to have occurred due to damage to the ileum during the preparation of the microscope slide. This is indicated by histological preparation or microscope slide in the P5 treatment of the damaged ileum.

Better structure of epithelial cell is thought to be because the flavonoid compounds contained in neem leaf are able to bind and reduce the number of ROS that causes epithelial cell damage. The mechanism of flavonoids against free radicals is by suppressing the formation of ROS, binding ROS, and increasing regulation or protection of antioxidant defenses. Flavonoids bind ROS by donating hydrogen atoms and electrons to hydroxyl, peroxyl, and peroxynitrite radicals (Kumar & Pandey, 2013). Flavonoids suppress the formation of ROS by inhibiting the activity of the enzyme superoxide dismutase, as an antioxidant, the enzyme is able to protect cells in the body from attack by free radicals. The distribution of neem leaf extract in this study is believed to be able to suppress epithelial cell erosion. Treatment P5 with 125 mg/200gBW of neem leaf extract was able to withstand epithelial cell erosion, P4 treatment with 100 mg/200gBW of neem leaf extract experienced relatively small amount of epithelial erosion compared to P3 treatment with 75 mg/200gBW of neem leaf extract. This proves that the higher of concentration neem leaf extract make the better the effect on epithelial cells in the intestine tenue.

Food materials that enter the body will undergo absorption, metabolism, distribution and excretion processes. Wiratmoko & Rafie (2014) stated that the small intestine has a special epithelium which has a large surface area. The structure of the villi in the mucosa can optimize absorption of food. Damage to the small intestine occurs when there is a disruption of the balance between defensive factors that maintain mucous integrity and aggressive factors that damage mucous defenses. This is a result of increased aggressive factors or decreased defensive factors. High-fat feeding can be an aggressive factor, causing side effects to interfere with food absorption. Giving ethanolic neem leaf extract at different doses can be a defensive factor that can inhibit the disruption of food absorption.

Neem leaf has various active compounds that can be used in traditional medicine, including alkaloid, flavonoids, terpenoids, azadirachtin, nimbine, nimbidine, meliantriol, salanin and tannins (Kumar *et al*., 2010). Biswas *et al*. (2002) stated that neem leaf can be used to reduce total cholesterol in the blood, LDL- and VLDL-cholesterol, triglycerides, and total lipids in serum. Compounds that affect the formation of cholesterol are quercetin flavonoids. Quercetin as a compound can reduce cholesterol levels by reducing the absorption of cholesterol and bile acids in the small intestine, thereby increasing the excretion of cholesterol through feces (Yasmin *et al*., 2010).

Hyperlipidemia can cause an increase in free radicals and a decrease in the antioxidant mechanism associated with oxidative stress (Duarte *et al*., 2010). The increase in free radicals can cause increased tissue damage that occurs in the small intestine so that it can interfere with the absorption of food nutrients. The damage that occurs gives an image of desquamation of the small intestinal vili, which leads to intestinal malabsorption and maldigestion (Ananto *et al*., 2017). Quercetin flavonoid compounds act as antioxidants and can prevent LDL oxidation by binding to free radicals and transitioning metal ions to

inhibit lipid peroxidation. The distribution of neem leaf extract in this study is believed to have a component that is able to withstand the effects of hyperlipidemia so that it does not cause intestinal tenue dysfunction that can be seen from the histological structure of the various treatment groups that are relatively the same. This mechanism also causes suppression of erosion of the tenue intestinal epithelial cells.

## CONCLUSION

Based on the research results, it can be concluded that the exposure of ethanol extract of neem leaves (*Azadirachta indica* A. Juss) at the highest dose of 125 mg/ 200gBB is able to suppress the effect of damage caused by hyperlipidemia of epithelial cells and the structure of white rats (*Rattus norvegicus* L.) small intestine.

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