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Hepatoprotective Effect of Parijoto Fruit Extract (*Medinilla speciosa* Blume) on Male Mice Fed with High-Fat Diet

Inasa Nabila[1*], Maria Selvester Thadeus[2], Ryan Herardi[3]
[1]Undergraduate Study Program of Medical Science, Universitas Pembangunan Nasional Veteran Jakarta, Jakarta 12450, Indonesia
[2]Anatomical Pathology Department of Medical Faculty, Universitas Pembangunan Nasional Veteran Jakarta, Jakarta 12450, Indonesia
[3]Internal Medicine Department of Medical Faculty, Universitas Pembangunan Nasional Veteran Jakarta, Jakarta 12450, Indonesia

ABSTRACT

This study aims to determine the effectiveness of parijoto fruit extract (*Medinilla speciosa* Blume) in improving the condition of fatty liver in male balb/c mice (*Mus musculus* L.) fed with a high-fat diet. Thirty male balb/c mice weighing 20 to 30 g were randomly divided into six groups, i.e: 1) Standard feed and Carboxymethyl Cellulose Sodium or CMC-Na 0.5% (K1); 2) High-fat diet and CMC-Na 0.5% (K2); 3) High-fat diet and simvastatin 0.026 mg/day (K3); 4) High-fat diet and parijoto fruit extract 5.6 mg/20 g BW (P1); 5) High-fat diet and parijoto fruit extract 8.4 mg/20 g BW (P2); 6) High-fat diet and parijoto fruit extract 11.2 mg/20 g BW (P3). Standard feed, high-fat diet, simvastatin, and parijoto fruit extract were administered for 56 days. On the 57th day, the total of 30 mice were terminated and the livers were then removed for H&E staining histopathological slides. Data on the degree of fatty liver on histopathological slides were collected and analyzed using the Kruskal-Wallis test and followed by the Mann-Whitney test. The histopathological analysis showed Medinilla speciosa Blume extract at a dose of 5.6 mg/20 g BW in group P1 prevent the steatosis degree compared to high-fat feed mice in group K2 ($p>0.05$). Parijoto fruit extract could act as the potential treatment for fatty liver.

Keywords: fatty liver, histopathological, high-fat diet, medinilla speciosa blume, mice

INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) is defined as a liver condition with a fat content exceeding 5% of the total normal liver weight in people who rarely or do not drink alcohol (Kneeman *et al.* 2012). The spectrum of fatty liver disease ranges from simple hepatic steatosis to cirrhosis. Although not all cases of hepatic steatosis will progress, early intervention is important because these patients are at risk of developing liver cirrhosis and associated complications such as hepatocellular carcinoma (Huang *et al.* 2020).

NAFLD is the most common cause of liver disease worldwide. Globally, the prevalence of NAFLD has continued to increase over the last three decades, increasing from 391.2 million in 1990 to 882.1 million in 2017 (Ge *et al.* 2020). In Indonesia, the prevalence of NAFLD reaches around 30% of the total adult population. The incidence of NAFLD is believed to be influenced by high-calorie diets, particularly those heavy in cholesterol, saturated and trans fatty acids, and other fats (Salehi-Sahlabadi *et al.* 2021). NAFLD is most commonly found in hyperlipidemic patients with a prevalence of 90%, followed by 80–90% in obese adults and 30–50% in diabetic patients (Milić & Štimac 2012). The high prevalence of NAFLD due to hyperlipidemia indicates that effective therapy is needed in this disease to prevent disease progression leading to cirrhosis and liver failure (Kneeman *et al.* 2012). Statins are a class of drugs that are often used to treat hyperlipidemia. This drug can lower Low-Density Lipoprotein (LDL) cholesterol, triglycerides, and increase High-Density Lipoprotein (HDL) through the mechanism of HMG-CoA Reductase inhibition. However, long term ingestion of statins causes adverse health effects such as liver injury and muscle toxicity. Other side effects include myopathy,

[*]**Corresponding Author:** tel: +6287780215787, email: inasanabila@upnvj.ac.id
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rhabdomyolysis, and acute renal failure. Thus, attention is now directed to natural products of plant origin (Salvamani *et al.* 2014).

So far, only a few studies have been conducted on the effect of parijoto fruit on the treatment of NAFLD. Research results from Elfrida (2015) showed that parijoto fruit extract at a dose of 0.9 mg/200 g BW/day had a significant effect on fatty liver tissue of the rats. Apart from the small number of studies, this study also modified the duration of research time and selected more suitable test animals. Balb/c strain mice were used as test animals because a high-fat diet in balb/c mice was able to induce hyperlipidemia more effectively than mice and C57BL/J6 strain mice, which are atherogenic strains of mice that have been genetically modified (Madariaga *et al.* 2015), so it is hoped that a clear fatty liver can be formed to be assessed. As a result, the purpose of this study was to assess the effect of parijoto fruit extract on improving histopathological features of fatty liver in male mice balb/c strain. Parijoto is a typical plant of the slopes of Mount Muria in Kudus Regency, Central Java which is used by local people as traditional medicine to treat various diseases (Wibowo *et al.* 2012). The benefits of parijoto are believed to be uterine fertilizers, diarrhea medicine, thrush, anti-inflammatory, anticancer, antibacterial, and lowering lipid profiles (Hanum *et al.* 2017). Based on research conducted by Wachidah (2013), the fruit of the parijoto plant contains tannins, flavonoids, and saponins which have the potential as lipid profile-lowering drugs because they have antioxidant and antihyperlipidemic activity. Study of 70% ethanol extract of parijoto fruit (*Medinilla speciosa* Blume) on the liver histopathology of male white rats induced by hyperlipidemia that has been done previously gave effective results at a dose of 90 mg/200 g BW in liver histopathology improvement carried out for 42 days (Elfrida 2015). The researchers expected that this study could show the potentials of parijoto fruit extract in improving histopathological feature of fatty liver with modified doses.

METHODS

Design, location, and time

This is true-experimental research, using a randomized post-test only control group design. The material origin located in the slopes of the Muria Mountains in Kudus, Central Java, Indonesia. Maintenance and treatment on experimental animal was conducted at the Pharmacology and Therapeutic Laboratory, Faculty of Medicine, University of Padjadjaran, Bandung, West Java, Indonesia. The research was carried out from November 2021 to June 2022. This study had obtained approval by The Research Ethics Committee of Medicine Faculty, Universitas Pembangunan Nasional Veteran Jakarta, Jakarta, Indonesia (2/1/2022/KEPK).

Materials and tools

Medinilla speciosa Blume for the treatment were extracted with 70% ethanol as solvent. Modeling of fatty liver was done by inducing high-fat diet for 56 days. Mice liver tissue stained with hematoxylin and eosin, were observed under a light microscope from the entire field of view at 40 times magnification to determine area and 400 times magnification to confirm the fat accumulation.

The tools used in this study were rotary evaporator, water bath, O'Hauss scale, gastric probe for oral administration, disposable syringe, surgical instruments, alcohol swabs, and blanks.

Procedures

Parijoto fruit extraction. A sample of 3,000 g of parijoto fruit with a purplish pink color was separated from foreign matter and twigs that were carried away, then washed, drained, and dried at a temperature of 70˚C. Parijoto fruit was mashed and then weighed as much as 800 g for maceration. Parijoto fruit extract (*Medinilla speciosa* Blume) was prepared by maceration method using 70% ethanol solvent for 24 hours then filtered using filter paper. The extract was evaporated in a rotatory evaporator at 70˚C to separate 70% ethanol solvent from the extract (Kurniawati 2015; Legawati *et al.* 2020) The remaining filtrate was followed by evaporation using a waterbath at a temperature of 70˚C. The weight of parijoto fruit extract obtained was 12 g and the yield was 1.5% of the total extracted sample.

Phytochemical screening. Phytochemical screening was carried out to ensure the presence of active secondary metabolites that have biological activity from parijoto fruit simplicia used in this study. Tests were qualitatively carried out on saponin, tannin, flavonoid, alkaloid, steroid, terpenoid by by color reaction or precipitation.

Parijoto fruit extract suspension preparation. Parijoto fruit extract suspension was made every week. Parijoto fruit extracts were weighed at 0.294 g (280 mg/kg BW), 0.441 g (420 mg/kg BW), and 0.588 g (560 mg/kg BW) respectively. Doses were modified from prior research on parijoto fruit on liver rats with a dose modification close to 90 mg/200 g BW. Each extract was dissolved in 0.5% CMC-Na gradually until a suspension was formed and the volume was made up to 35 ml (Kurniawati 2015; Legawati *et al.* 2020).The extract was administered orally to mice using oral gavage, 1 ml each mice, expressed as mg/20 g of body weight for 56 days.

Simvastatin suspension preparation. The dose of simvastatin in mice was 0.026 mg/20 g BW then dissolved in a 0.5% CMC-Na (Elfrida 2015). The suspension was administered orally to mice using oral gavage, expressed as mg/20 g BW for 56 days along with high-fat feed induction period.

High-fat feed induction. The high fat feed given to mice was prepared by mixing and stirring until evenly distributed 8,000 g of standard pellets, 2,500 g of flour, 750 g of palm oil. Sixteen duck egg yolks are mixed with 1,000 g of goat fat until soft and runny while adding hot water as needed. The two mixtures then mixed together until smooth (Kodariah & Wahid 2020).

Animals study. Total number of samples in this study was thirty male balb/c mice (*Mus musculus* L.). The mice were 8 weeks old and weighed 20–30 g. The mice were acclimiatized for seven days according to standard animal housing conditions (with the temperatur was kept at 25±2˚C and maintained with 12 hour light-dark cycles), fed standard CP511 pellet and water ad libitum.

Experimental procedure. The randomization was done where the mice were divided into 6 groups (n=5); each group was given different treatments and treated for a period of 56 days: standard feed and CMC-Na 0.5% for group I (K1), high-fat diet and CMC-Na 0.5% for group II (K2), high-fat diet and simvastatin 0.026 mg/day for group III (K3), high-fat diet and parijoto fruit extract for group IV–VI (P1–P3). The dose of parijoto fruit extract for P1–P3 was 5.6, 8.4 and 11.2 mg/20 g BW, respectively. The doses in this study were base on modification of previous studies by Kurniawati (2015) and Elfrida (2015). The diet was administered ad libitum and the treatment was administered using oral gavage for 56 days. The animal handling during treatment was the same as during the acclimatization phase.

Microscopic study. At day 57, part of liver tissue was drawn from the terminated mice, fixed in 10% formalin, embedded in paraffin, and finally cut into 5 mm sections. The slides were stained with hematoxylin-eosin. The histopathological fatty changes of the liver were observed using Olympus CX23 Microscope from the entire field of view at 40 times magnification to determine area and 400 times magnification to confirm the fat accumulation.

The percentage of distribution of fat deposits was assessed semi-quantitatively using the Brunt method histological criteria that degreed from 0 to 3, as follows: 1) Degree 1: Fatty liver was found in 33% hepatocytes; 2) Degree 2: Fatty liver was found in 34%–66% hepatocytes; 3) Degree 3: Fatty liver was found in >66% hepatocytes (Brunt 2016; Aufazhafarin *et al.* 2021). Fat deposits distribution was firstly by estimating the liver lobes area which contained fat accumulation in hepatocytes (40 times), confirmating the steatosis (400 times), and then categorized into three degree.

Data analysis

All data obtained were presented as mean. Analysis was done with Kruskall-Wallis statistical test, followed by post-hoc Mann Whitney test with a significant value of $p<0.05$.

RESULTS AND DISCUSSION

Phytochemical screening was carried out to ensure the content of active compounds contained in the extract of parijoto fruit used in this study. The results of the phytochemical tests carried out as shown in Table 1 show that extract of the parijoto fruit used contained compounds in the form of saponin, tannin, flavonoid, alkaloid, and steroid.

The degree of steatosis

The results of this study based on the degree of steatosis indicate that there is impact of treatment of parijoto fruit extract in preventing fatty liver.

The degrees based on the microscopic observations represent the percentage of the fatty

Table 1. The secondary metabolites of parijoto fruit

Secondary metabolite	Conclusion	Explanation
Saponin	c	Foam is formed for more than one second
Tannin	+/Galat	Formed in black
Flavonoid	+	A clear red layer forms on top of the amyl alcohol layer
Alkaloid	+	Formation of orange color
Steroid	+	Formed in green and red
Terpenoid	-	No color formed

liver found in hepatocytes (Table 2). Based on the degree of steatosis of the liver of mice (Table 2), the highest percentage of steatosis occurred in the negative control group (K2). Eighty percent of mice in group K2 had a severe degree of steatosis (>66%) and as many as 20% had a moderate degree of steatosis (34–66%).

Second, the administration of simvastatin 0.026 mg/20 g BW in mice or a dose of 10 mg/70 g BW (K3) was able to reduce the incidence of steatosis by more than 66%, as much as 60% is in accordance with the baseline (≤33%) and 20% at a moderate degree.

Third, the administration of parijoto fruit extract at a dose of 5.6 mg/20 g BW/day (P1) can prevent the degree of steatosis to not exceed 66%, and only as much as 60% is in accordance with the baseline, which is a value that is a normal benchmark range, found in the normal group without a high-fat diet (K1).

Fouth, administration of parijoto fruit extract with increasing doses of 8.4 and 11.2 mg/20 g BB (P2 and P3) did not show the prevention in the degree of steatosis. Sixty percent of mice in each group P2 and P3 had a severe degree of steatosis (>66%) and as many as 40% had a moderate degree of steatosis (34–66%).

Statistical analysis based on degree of steatosis from histopathological observation

Data on the degree of steatosis were analyzed using the Kruskal Wallis test. It shows a significant difference between the six treatment

Table 2. The effect of parijoto fruit extract on the degrees of steatosis

Group	Degree of steatosis			n
	Degree 1	Degree 2	Degree 3	
K1	4	1	0	5
K2	0	1	4	5
K3	3	1	1	5
P1	3	0	2	5
P2	0	2	3	5
P3	0	2	3	5

K1:Standard feed and carboxymethyl cellulose sodium or CMC-Na 0.5%
K2:High-fat diet and CMC-Na 0.5%
K3:High-fat diet and simvastatin 0.026 mg/day
P1:High-fat diet and parijoto fruit extract 5.6 mg/20 g BW
P2:High-fat diet and parijoto fruit extract 8.4 mg/20 g BW
P3:High-fat diet and parijoto fruit extract 8.4 mg/20 g BW

Table 3. The results of the difference in the significance of steatosis between groups

Groups	K1	K2	K3	P1	P2	P3
K1	x	0.007*	0.439	0.366	0.011*	0.011*
K2	0.007*	x	0.041*	0.120	0.513	0.513
K3	0.439	0.041*	x	0.811	0.077	0.077
P1	0.366	0.120	0.811	x	0.212	0.212
P2	0.011*	0.513	0.077	0.212	x	1
P3	0.011*	0.513	0.077	0.212	1	x

*Significant difference ($p<0.05$) as analysed by Mann-Whitney test
K1:Standard feed and carboxymethyl cellulose sodium or CMC-Na 0.5%
K2:High-fat diet and CMC-Na 0.5%
K3:High-fat diet and simvastatin 0.026 mg/day
P1:High-fat diet and parijoto fruit extract 5.6 mg/20 g BW
P2:High-fat diet and parijoto fruit extract 8.4 mg/20 g BW
P3:High-fat diet and parijoto fruit extract 8.4 mg/20 g BW

groups with a result of 0.021 ($p<0.05$). Then, followed by Mann-Whitney Test (Table 3).

The Mann-Whitney result showed that the negative control group (K2) fed a high-fat diet for 56 days had a significant difference with the normal control group (K0) with p=0.007. These results indicate that the administration of HFD in eight weeks succeeded in forming fatty liver in the presence of steatotic cells in hepatocytes.

The K3 group that was given simvastatin had a significant difference with the K2 group that was fed a diet high in fat and cholesterol (p=0.041). These results showed that the administration of simvastatin at a dose of 0.026 mg/20 g BW showed the prevention in the degree of steatosis which was better than the treatment groups P1, P2, and P3.

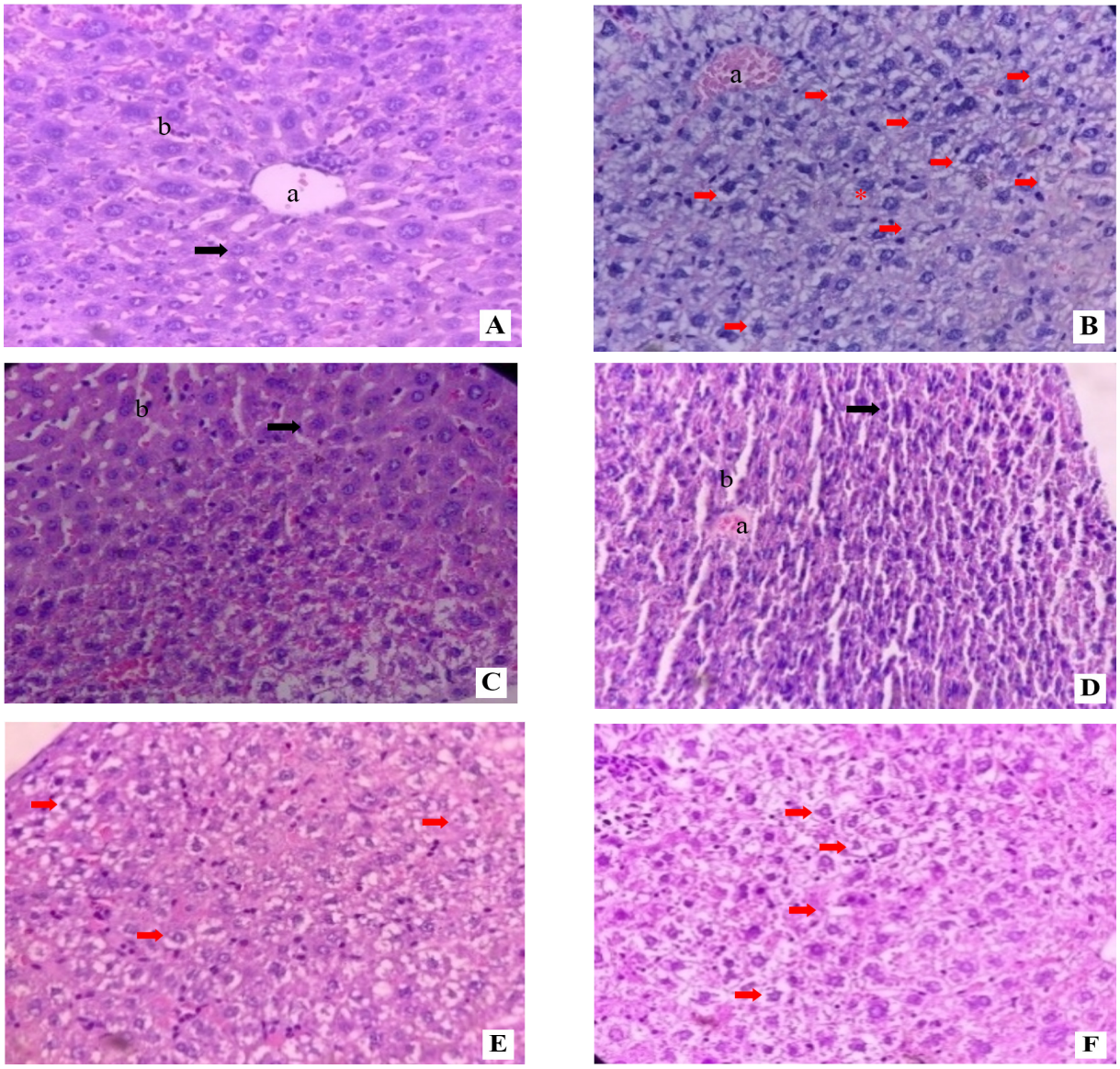
Based on the Mann-Whitney statistical result, Group P1 did not find a significant difference with K2 (p=0.120). These results showed that the administration of P1 parijoto fruit extract was not statistically significant. Although it was not statistically significant, but the histopathological picture in this study showed the administration of P1 parijoto fruit extract was able to improve the histopathological picture of fatty liver (Figure 1 (D)). This prevention is thought to be related to the proven active compound content of saponins, tannins, and flavonoids in the parijoto fruit extract in the phytochemical analysis that was also carried out in this study.

The results of Mann-Whitney statistical test, Group P2 and P3 did not find significant differences with all groups K2 (p=0.513, 0.513). In addition, Group K1 given standard feed had a significant difference with groups P2 and P3 (p=0.011 and 0.011). These results showed that the doses of parijoto fruit extract at 8.4 and 11.2 mg/20 g BW (P2 and P3) for 8 weeks were not optimal in preventing fatty liver.

Histopathology observation

The effects of parijoto fruit extract on liver histopathology of non-alcoholic fatty liver induced mice are offered in Figure 1. The histopathological results of HE staining are shown in Figure 1. Images were obtained at 400 times magnification. Group K1 is presented in Figure 1 (A). Liver tissue shows normal morphological structures in lobular architecture. The cell boundaries in the sinusoids are clear and regular. Hepatocytes are evenly distributed and tightly packed to form a plate arranged radially around the central vein, hepatocytes within normal limits, the cell nucleus in the middle, and there are no fat vacuoles in the cytoplasm.

In the K2 group, it was shown that high-fat diet can cause fatty liver with the presence of



(A) K1: Standard feed
(B) K1: High-fat diet
(C) K3: High-fat diet + Simvastatin
(D) P1: High-fat diet + Parijoto fruit extract (5.6 mg/20 g BB)
(E) P2: High-fat diet + Parijoto fruit extract (8.4 mg/20 g BB)
(F) P3: High-fat diet + Parijoto fruit extract (11.2 mg/20 g BB)
a:central vein; b:sinusoids; ➡:Normal hepatocytes; ➡:Steatosis

Figure 1. The effect of parijoto fruit extract on histopathological fatty changes of H&E staining in the liver of mice

steatotic cells in hepatocytes (Zhang *et al.* 2021). A greater amount of steatosis was seen in group K2 (Figure 1 (B)) compared to control groups K1 and K3 (Figure 1 (A and C)). In group K2, there was a change in the structure of the liver cells which became irregular, the cell boundaries were not clear, the arrangement of hepatocytes was wide and the sinusoids became irregular. Liver cells showed marked fat degeneration with clear fat vacuoles with cell nuclei pushed to the

periphery. Administration of high-fat diet will cause FFA in the liver to undergo β-oxidation in the mitochondria of hepatocytes or accumulate in the form of triglycerides. The accumulation of fat in the form of triglycerides in the liver can trigger an increase in liver lipotoxicity from an increase in free fatty acids, free cholesterol and other lipid metabolites. As a result, mitochondrial dysfunction can occur, causing a decrease in the results of burning fatty acids into energy from the β -oxidation process of triglycerides (Buzzetti *et al.* 2016). Triglycerides from the liver that are not transported on time can damage the lipid metabolism pathway that causes fatty liver (Fan *et al.* 2017).

The prevention of the fatty liver histopathological appearance occurred in group P1 which is presented in Figure 1 (D). In the P1 group, the liver parenchyma began to approach the normal hepatocyte picture, the parenchymal cell boundaries were clearer and the lipid degeneration was reduced with smaller vacuoles when compared to the K1 group, although the degree of steatosis in the P1 group was still higher than in the K3 and K1 groups. The improvement in the P1 group is thought to be related to the proven active compound content of saponins, tannins, and flavonoids in the extract of parijoto fruit in the phytochemical analysis which was also carried out in this study. Thus, parijoto fruit extract has the potential to improve the histopathology of fatty liver.

The mechanism of saponin content in improving the appearance of steatosis is by inhibiting the absorption of cholesterol and triglycerides and the reabsorption of bile acids in the intestine. Saponin can inhibit this absorption through inhibiting pancreatic lipase activity (Marrelli *et al.* 2016) and due to its heavy molecular weight, it is able to displace cholesterol from the food-mixed micelles and form cholesterol deposits that are difficult to pass through the intestinal mucus barrier, thereby causing a decrease in serum cholesterol concentrations (Luo *et al.* 2020). Tannin have the ability to increase the fecal excretion of cholesterol and bile acids through inhibition in the intestine, and also inhibit the occurrence of fatty liver through the activation of Adenosine Monophospate Activated Protein Kinase (AMPK) which is involved in the regulation of lipid metabolism. Activated AMPK inhibits the activity of enzymes and factors that regulate lipogenic processes in triglyceride synthesis. AMPK activation is also known to increase the expression of genes involved in fatty acid oxidation resulting in an increase in β-oxidation (Zou *et al.* 2014). Flavonoid act by reducing cholesterol formation in the liver through inhibition of HMG-CoA reductase (Zeka *et al.* 2017). Flavonoid also increase the activity of Lecithin Acyl Transferase (LCAT) which can reduce free cholesterol levels in the blood, increase the release of cholesterol from macrophages and increase the expression of ATP-binding Cassette (ABC) A1 and apolipoprotein A1 which is the basic material for the formation of HDL (Puspasari *et al.* 2016). The content of alkaloid also has antioxidant activity through the donation of hydrogen ions to free radicals and can act as pancreatic lipase inhibitors that can increase fat secretion in the faeces by reducing the breakdown of triglycerides into free fatty acids and glycerol (Artha *et al.* 2017).

Figures of hepatocytes with steatosis in the histopathology of groups P2 and P3 are presented in Figures 1 (E and F). In the histopathological picture of the group treated with parijoto fruit extract groups P2 and P3, fatty liver did not appear to have improved, macro and microvesicular steatosis was found where hepatocytes filled with fat granules with enlarged sizes. Cell boundaries and sinusoids appear indistinct and irregular. Hepatocytes with micro and macrovesicular steatosis were seen in groups P2 and P3 (Figure 1 (E and F)). In macrovesicular steatosis, there are lipid droplets in the hepatocyte cytoplasm that push the nucleus to the cell periphery. In microvesicular steatosis, there are small lipid droplets in the hepatocyte cytoplasm and the nucleus remains in the center of the cell (Takahashi & Fukusato 2014). These results showed that the doses of parijoto fruit extract at 8.4 and 11.2 mg/20 g BW (P2 and P3) for 8 weeks did not show any improvement in the histopathological picture of fatty liver compared to 5.6 mg/20 g BW (P1). The lack of improvement in the histopathology of fatty liver of mice in the treatment group of parijoto fruit extract doses 2 and 3 may be a condition that often occurs when testing new drug candidates which is a dose optimization phenomenon, where the dose will provide a maximum pharmacological response at a certain dose with minimum likelihood of undesirable symptoms. Thus, these

results indicate that the doses of 8.4 and 11.2 mg/20 g BW are not optimal in improving the histopathology of fatty liver.

Improvement of the histopathological picture of fatty liver occurred in the K3 group which is presented in Figure 1 (C). The degree of steatosis in the K3 group given simvastatin showed a better histopathological improvement than the P1, P2, and P3 treatment groups. The liver parenchyma began to approach the normal hepatocyte picture, was more clearly demarcated, and reduced lipid degeneration compared to the K1 group. This improvement in the degree of steatosis is due to simvastatin, a pharmacological agent that is commonly used in antihyperlipidemic therapy that works by inhibiting the HMG-CoA reductase enzyme during the cholesterol synthesis process (Indonesian Society for Endocrinology (PERKENI) 2019). The degree of steatosis in the K3 group given simvastatin at a dose of 0.026 mg/20 g BW showed better histopathological improvement compared to the P1, P2, and P3 treatment groups. Thus, simvastatin is able to improve the histopathological picture of fatty liver, and its effectiveness is still better than the administration of parijoto fruit extract therapy.

The current standard for the management of non-alcoholic fatty liver disease in humans, which has been evaluated histologically and has been included in the American Association for the Study of Liver Diseases is given Vitamin E (Perumpail *et al.* 2018). Vitamin E (rrr a-tocopherol) given at a daily dose of 800 IU/day can improve the improvement of liver steatosis in adults, especially non-diabetics. With the findings of this study, it is hoped that parijoto fruit extract can be used as a supplementation modality or can be used daily, especially by the local community which can be given as a non-alcoholic fatty liver disease prevention therapy in humans.

CONCLUSION

The administration of parijoto fruit extract 5.6 mg/20 g BB/day was able to improve the degree of steatosis which was more than 66%, but only 60% was in accordance with the baseline. Increasing the dose of parijoto fruit extract (*Medinilla speciosa* Blume) at doses of 8.4 and 11.2 mg/20 g BW did not increase the response to improvement in the degree of steatosis in the histopathological picture of fatty liver of male mice (*Mus musculus* L) balb/c strain. It can be concluded that parijoto fruit extract dose of 5.6 mg/20 g BW/day could act as the potential treatment for fatty liver. Thus, further research is needed on toxicity tests for knowing the side effects that can be caused by parijoto fruit extract before it can be implemented in human.

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DECLARATION OF INTERESTS

The authors have no conflict of interest.

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