Jurnal Kedokteran Hewan March 2024, 18(1):16-22
P-ISSN : 1978-225X; E-ISSN : 2502-5600 DOI: https://doi.org/10.21157/j.ked.hewan.v18i1.32339

EFFECT OF CIPLUKAN STEMS AND LEAVES EXTRACT ADMINISTRATION (*Physalis angulata* L.) ON THE DEGREE OF hepatic lobes INFLAMMATION IN DYSLIPIDEMIA INDUCED WISTAR RATS

Nazala Safira[1], Katon Pamungkas[1], Rizki Fajar Utami[2], Dwi Nur Ahsani[3], and Miranti Dewi Pramaningtyas[4]*
[1]Faculty of Medicine, Indonesian Islamic University, Yogyakarta, Indonesia
[2]Department of Biochemistry, Indonesian Islamic University, Yogyakarta, Indonesia
[3]Department of Histology, Indonesian Islamic University, Yogyakarta, Indonesia
[4]Department of Physiology, Indonesian Islamic University, Yogyakarta, Indonesia
*Corresponding author: miranti.dewi@uii.ac.id

ABSTRACT

The aim of this study was to determine the effect of stems and leaves ciplukan extract on the degree of liver lobe inflammation in dyslipidemia-induced wistar rats. This research was an experimental study with a post-test only design. A total of 24 Wistar rats were allotted into 4 groups with 6 rats per group. Hepatic organs were then histologically stained with Hematoxylin-Eosin stain. Inflammation reading was done by looking for inflammatory foci in the preparation. The result was then analyzed using Kruskal-Wallis test. The results showed that groups I and II had one severe inflammation. Group III had two severe degrees of inflammation. Group IV had no severe inflammation. The analysis revealed that there was no significant difference in the degree of inflammation between groups (P= 0.964). Administration of ciplukan stem and leaf extract did not affect the degree of liver lobe inflammation in Wistar rats induced by dyslipidemia.

Key words: inflammation, dyslipidemia, liver, NAFLD, *Physalis angulata*

ABSTRAK

Tujuan penelitian ini adalah mengetahui pengaruh pemberian batang dan daun ciplukan terhadap derajat inflamasi lobus hepar pada tikus wistar yang diinduksi dislipidemia. Penelitian ini merupakan penelitian eksperimental dengan desain post-test only. Subjek penelitian diambil dari 24 tikus wistar yang terdiri dari 4 kelompok yaitu kelompok I, II, III, dan IV. Organ hepar kemudian dibuat preparat histologi dengan pewarnaan Hematoksilin-Eosin. Pembacaan inflamasi dilakukan dengan mencari fokus inflamasi pada preparat. Hasil tersebut kemudian dianalisis menggunakan uji Kruskal-Wallis. Hasil penelitian menunjukkan grup I dan II memiliki satu inflamasi derajat berat. Grup III memiliki dua inflamasi derajat berat. Grup IV tidak memiliki inflamasi derajat berat. Hasil tersebut menunjukkan tidak ada perbedaan derajat inflamasi yang signifikan antar kelompok (P= 0.964). Pemberian ekstrak batang dan daun ciplukan tidak memengaruhi derajat inflamasi lobus hepar pada tikus wistar yang diinduksi dislipidemia.

Kata kunci: inflamasi, dislipidemia, liver, NAFLD, Physalis angulata

INTRODUCTION

According to 2018 RISKESDAS data, 21.2% of the population aged ≥ 15 years had total cholesterol in the range of 200-239mg/dL, while 24.3% had HDL less than 40mg/dL. Apart from that, 24.4% had Low Density Lipoprotein (LDL) above 100 mg/dL and 27.9% had triglyceride levels above 150 mg/dL (Badan Litbangkes RI, 2018). These data show that the Indonesian population is at considerable risk of experiencing dyslipidemia. Dyslipidemia is a collective disorder of lipoproteins so that blood fat levels, including total cholesterol, LDL, High Density Lipoprotein (HDL), or triglycerides are abnormal. The condition of dyslipidemia is influenced by several risk factors (Jamesin *et al*. 2018).

Risk factors that play a large role are consumption of foods high in fat, especially LDL and a sedentary lifestyle. In conditions of dyslipidemia, there is a decrease in HDL levels which function as a natural antioxidant. The decrease in HDL results in endothelial dysfunction in blood vessels resulting in increased permeability. Increased permeability in blood vessels causes LDL to enter the endothelium. A lot of LDL in blood vessels can increase the risk of forming atheroma plaque which can cause atherosclerosis.

Consuming excessive amounts of high-fat foods over a long period of time without balanced physical activity will cause the accumulation of fat stores in the form of triglycerides in the liver and several other tissues. Due to slight changes in the liver, more free fatty acids and triglyceride deposits in peripheral tissues cause peripheral lipid transporters, namely HDL, to experience an imbalance. This imbalance can result in lipotoxicity. Lipotoxicity will suppress the peroxisome proliferator-activated receptor (PPRα) gene and stimulate endoplasmic reticulum stress and the release of reactive oxygen species (ROS) from mitochondria.

Suppressing PPRα will reduce lipid oxidation and prevent the release of very-low-density lipoprotein (VLDL) from the liver. Meanwhile, endoplasmic reticulum stress stimulates the release of pro-inflammatory cytokines such as Interleukin-1β (IL-1β), interleukin-18 (IL-18), and Tumor Necrosis Factor-α (TNF-α). The release of pro-inflammatory cytokines will cause the release of the transcription factor NF-κB and activate pro-inflammatory Kupffer M1 cells.

Activation of M1 Kupffer cells will cause Non-alcoholic Fatty Liver Disease (NAFLD) and inflammation of the liver. Apart from that, consuming high-fat foods over a long period of time will also result in an imbalance in the gut microbiota and increased fat uptake into the liver. As a result, NAFLD and liver inflammation will increase. This shows that high lipid levels cause damage and are associated with increased levels of malondialdehyde (MDA) which functions as a bio-marker for oxidative stress due to inflammation (Ardhani *et al*. 2017; Jamesin *et al*. 2018; Marra and Lotersztajn, 2013; Nozaki *et al*. 2015; Onwe *et al*. 2015; Peng *et al*. 2020).

NAFLD conditions can progressively lead to liver fibrosis and end in liver cirrhosis. Several efforts can be made to improve lipid levels in the blood, one of which is pharmacological therapy (Jamesin *et al*. 2018; Kumar *et al*. 2020). The pharmacological therapy that is often taken is using statin chemical drugs, one of which is simvastatin. However, chemical drugs have various side effects on the liver and kidneys (Jose 2016). With these effects, alternative medicines are needed that have fewer side effects, one of which is herbal medicine (Sumayyah and Salsabila 2017). The herbal medicine that can be used is ciplukan (*Physalis angulata* L.).

Ciplukan fruit contains flavonoids such as quercetin and kaempferol (Rengifo-Salgado and Vargas-Arana, 2013). Quercetin consumed for 8 weeks will reduce blood triglyceride levels (Huang *et al*. 2020). In addition, quercetin also plays a role in reducing NF-κB transcription factors and hepatic mRNA in the production of pro-inflammatory interleukins (Yi *et al*. 2021). Kaempferol suppresses stimulation of the NF-κB transcription factor so that the cytokine TNF-α is reduced. This reduction can be seen on the 14th day (Alam *et al*. 2020). Kaempferol has an effect on stimulating PPRα in the liver. With PPRα stimulation, lipid oxidation and VLDL production will increase (Xiang *et al*. 2021).

Ciplukan stems and leaves contain several components such as physalins and myricetin type flavonoids. Myricetin can correct intestinal microbial imbalance and improve intestinal barrier function so that hepatic triglyceride accumulation can be reduced (Sun *et al*. 2021). Myricetin also regulates ROS and decreases the transcription factor NF-κB and has the effect of reducing IL-1β and TNF-α levels (Wang *et al*. 2019). Physalins B, D, F, G have anti-inflammatory effects by reducing IL-1β and TNF-α levels through inhibiting the transcription factor NF-κB. In addition, physalins E has an effect in regulating lipopolysaccharides produced by intestinal microbial imbalance so that the NF-κB transcription factor and pro-inflammatory cytokines decrease (Meira *et al*. 2022). Although there have been many studies showings the potential of ciplukan leaves and stems. However, there has been no research that has looked at the effect of ciplukan stem and leaf extract on the degree of inflammation of the liver lobes, so this research needs to be carried out.

MATERIALS AND METHODS

Research Design

This research was experimental with the research design used being post-test only to determine whether or not there was an effect of leaf and stem extract of ciplukan on the degree of lobar inflammation in the liver of Wistar rats induced by dyslipidemia. This research used the liver of male Wistar rats (*Rattus norvegicus*), 2-3 months old, and weighing 150-280 g. The research group consisted of 4 groups with a minimum of 5 experimental animals per group. In total there were 24 Wistar rats. The first group (I) was a negative control (6 rats) which was given with a standard diet, egg yolk 2 mL/200 g BW, and propylthiouracil (PTU) 0.01%. Group II was a positive control (7 rats) and given a standard diet, egg yolk 2 mL/200 g BW, PTU 0.01%, and simvastatin 0.18 mg/200 kg BW. Group III was treated with a standard diet, egg yolk 2 mL/200 g BW, PTU 0.01%, and ciplukan extract 400 mg/Kg BW (5 rats). Meanwhile, group IV was treated with a standard diet, egg yolk 2 mL/200 g BW, PTU 0.01%, and ciplukan extract 800 mg/Kg BW (6 rats). The administration of egg yolk, PTU, simvastatin, and extract was carried out using gastric lavage All groups met the minimum number of samples per group which was calculated using the Frank and Wallen formula with a minimum of 5 rats per group. The intervention was carried out for the entire group for 6 weeks. The choice of 400 mg and 800 mg ciplukan extract doses refers to research conducted by Afriyeni and Surya in 2019. This research has received ethical approval from the Ethics Commission of the Faculty of Medicine, Gadjah Mada University based on the Certificate No. KE/ FK/0223/EC/2022.

Blood Lipid Profile Examination

Blood for lipid profile examination was taken from retro orbital blood and then put into a tube and centrifuged at a speed of 3000 rpm for 20 minutes. The serum that settles at the top was analyzed using the precipitation method to see the levels of LDL. In addition, blood cholesterol and triglycerides were analyzed using the enzymatic photometric test method. High-density lipoprotein levels were examined using the Burstein serum method.

Research Preparations

Wistar rats that had been intervened for 6 weeks were terminated and the largest hepatic lobus were taken and fixed in 10% formaldehyde for 24 hours. After that, the liver was dehydrated by placing in alcohol with a concentration of 70% to 100%.

Clearing was conducted to remove alcohol from the tissue. Clearing using xylol solution. After dehydration, the tissue was placed in xylol I for 1 hour and continued into xylol II for ½-1 hour. Immersion is carried out by removing the liquid during the cleaning stage and replacing it with paraffin. The process of this stage is to soak the preparation in a paraffin solution that has been melted at a temperature of 56° C. Soaking was carried

out three times using paraffin I, II, and III. Each soaking was carried out for 2 hours, 1 hour and 2 hours alternately. Next step was casting and cutting. The casting stage was carried out so that the stock could be cut. Initially the liquid paraffin was placed in a plastic mold or metal plate. Then the tissue was inserted into the mold using heated tweezers. The block cutting process begins by cutting the liver organ in large lobes horizontally with a length of 1.5 cm and a width of 0.5 cm. These organs were divided into equal lengths. After that, cutting was carried out using a microtome with a cutting thickness of around 5-7 micrometers. Then, the resulting cut was attached to a glass object that has been coated first using albumin adhesive (Jusuf, 2009).

Hematoxylin Eosin Staining

Staining was carried out using Mayer hematoxylin-eosin staining. Initially, the samples were hydrated with 100% alcohol for 2 minutes and this was done twice. Then continued with hydration using 95%, 90%, 80%, 70% alcohol for 2 minutes each and closed by immersion in distilled water for 3 minutes. After that, incubation was carried out in Mayers hematoxylin solution for 15 minutes and washed using running water for 15-20 minutes. The final stage is to observe in a microscope whether the solution was still too blue and wash it again under running water until the blue color is sufficient if it was still too blue (Jusuf, 2009).

Liver Histology Observation

Observation of liver histology was carried out with a magnification of 100x and 400x. Readings use Region of Interest (ROI) in 5 fields of view which was considered representative or hotspot approach. These results were then included in the Brunt criteria to determine the inflammation category (Brunt *et al*. 2011; Nagarkar *et al*. 2016).

MalondialdehydeLevels Test

Measurement of malondialdehyde (MDA) levels was carried out using spectrophotometry based on the purple color change due to the formation reaction of thiobarbituric acid-MDA complex. Blood samples were taken from venous blood as much as 4 ml after 6 weeks of intervention. The blood was then put into a tube and centrifuged at a speed of 3000 rpm for 20 minutes at a temperature of 4 degrees Celsius. A separate serum was then performed to check MDA levels using the hunter method.

Data Analysis

The data analysis process was carried out using One-Way ANOVA and Kruskal-wallis statistical test.

RESULTS AND DISCUSSION

The amount of liver has met the minimum sample required. Apart from rat livers, blood was also taken to analyze the lipid profile. The rats lipid profile consisted of average total cholesterol, triglycerides, LDL, and HDL (Table 1).

Dyslipidemia induction was carried out using administration of 2 mL /200 g BW egg yolk and 0.01% PTU for 6 weeks. Rats were considered dyslipidemic when the LDL and triglycerides levels increased above the normal range and HDL decrease below the normal range. The normal LDL limit for rat was 25.45-59.98 mg/dL. Meanwhile, triglycerides were in the range of 72-130 mg/dL and normal HDL was 36.36-54.55 mg/dL (Ihedioha *et al*. 2013). In negative control group, LDL was 76.63 mg/dL, triglycerides 135.72 mg/dL, and HDL 27.7 mg/dL. This data indicated that the control group already experienced dyslipidemia. The increase in LDL levels is in accordance with research conducted by Retnaninggalih *et al*. (2014) which used chicken egg yolk and PTU 2 mg/Kg BW/day for 2 weeks. Research conducted by Ranti *et al*. (2021) showed that giving egg yolk and 0.01% PTU for 7 days could cause an increase in total cholesterol, LDL and a decrease in HDL.

In previous research (Afriyeni and Surya 2019), it was known that the stems and fruit of the ciplukan plant reduced cholesterol, triglyceride and LDL levels in rats. In this research, it appeared that the total cholesterol of the stem extract decreased significantly at doses of 200, 400 and 800 mg/kg BW. Apart from that, ciplukan at a dose of 400 mg/kg BW also significantly reduced blood triglyceride and LDL levels. This effect occurs due to antioxidant activity in the form of quercetin and kaempferol which were included in the flavonoids in ciplukan stems.

Research conducted by Baghdadi (2014) showed that quercetin had the effect of reducing total cholesterol, triglyceride and LDL levels but did not increase HDL in rat induced by hypercholesterolemia. In research conducted by Moon *et al*. (2012) it was found that quercetin greatly increased the expression of the LDL receptor (LDLR) gene by increasing the clearance of circulating LDL levels. Quercetin and kaempferol may have a significant synergistic effect in reducing LDL levels by increasing LDL clearance by hepatocytes. Quercetin in the form of glycosides or quercetin 3-glucoside (Q3G) increases the expression of the LDL receptor and reduces the secretion of Proprotein convertase subtilisin/kexin type 9 (PCSK9) resulting in faster clearance of LDL (Yusof *et al*. 2017; Adorni *et al*. 2020).

Table 1. Mean (±Standard Deviation) lipid profiles of rats

Group	Total cholesterol	Triglycerides	LDL	HDL
I (K-)	188.84±0.229 mg/dl	135.71±0.181 mg/dl	76.36±0.11 mg/dL	27.70±0.038 mg/dL
II (K+)	99.65±0.120 mg/dl	77.37±0.103 mg/dl	33.42±0.048 mg/dL	69.64±0.094 mg/dL
III (KA)	144.96±0.175 mg/dl	113.56±0.152 mg/dl	52.18±0.075 mg/dL	44.26±0.06 mg/dL
IV (KB)	117.08±0.142 mg/dl	87.14±0.116 mg/dl	61.03±0.083 mg/dL	44.87±0.065 mg/dL

LDL= Low-Density Lipoprotein, HDL= High-Density Lipoprotein, K-= Given yellow egg and PTU, K+= Given yellow egg, PTU, and simvastatin, KA= Given yellow eggs, PTU, and 400 mg ciplukan extract, KB= Given yellow eggs, PTU, and 800 mg ciplukan extract

The MDA levels of experimental rats was presented in Table 2. Data from Table 2. indicated that there was an effect of giving ciplukan extract on serum MDA levels between groups. In conditions of dyslipidemia, it could cause an increase in ROS levels which can influence the emergence of an oxidative stress response. ROS compounds could react to various biomolecules such as lipids, lipoproteins, carbohydrates, proteins, nucleic acids and connective tissue macromolecules. Free radical compounds could damage lipids, especially lipids in low-density lipoprotein (K-LDL) cholesterol.

LDL-cholesterol was very easily oxidized compared to other lipoproteins because its composition consists mostly of polyunsaturated fatty acids or PUFA (Werdhasari 2014). The continuous process of free radicals in lipid tissue would cause a lipid peroxidation process which produces final products including MDA. The amount of MDA could be used as an indicator of damage caused by free radicals or an indicator of the activity of the lipid peroxidation process. It means that when the number of K-LDL levels decreases, the process of lipid peroxidation will decrease so that the final result of lipid peroxidation, namely MDA, will also experience a decrease in levels. Based on previous research (Binmowyna *et al*. 2020), it is known that there was a significant increase in serum MDA levels in rats induced by dyslipidemia within an induction period of 60 days.

The bioactive contents of ciplukan such as alkaloids, flavonoids, saponins, steroids, tripemoids, phenolics could reduce dyslipidemia conditions. The contents of ciplukan could reduce free fatty acids and triglycerides in the body so that oxidative stress and mitochondrial stress could be reduced. The lower levels of triglycerides and total cholesterol when compared to the control group could be a sign that the

Table 2 . Measurement of Serum monoaldehyde (MDA) Levels of experimental animals

Group	Mean ± Standard Deviation	ANOVA ^{sig}
I (K-)	9.77 ± 0.40 nmol/g	0,000 *
II (K+)	1.44 ± 0.21 nmol /g	
III (KA)	4.84 ± 0.19 nmol /g	
IV (KB)	3.78 ± 0.22 nmol /g	

K-= Given yellow egg and PTU, K+= Given yellow egg, PTU, and simvastatin, KA= Given yellow eggs, PTU, and extract ciplukan 400 mg, KB= Given yellow eggs , PTU, and extract ciplukan 800 mg

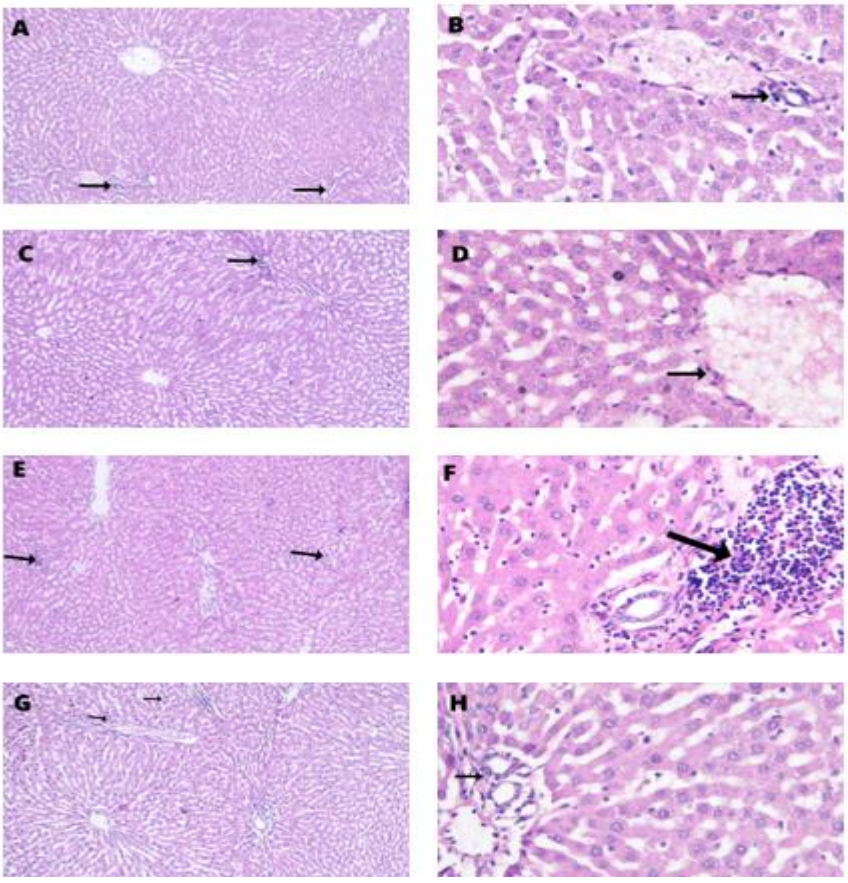

Figure 1. Liver histopathology image with Haematoxylin-Eosin (HE) staining. Group I liver magnification 100x (A) and 400x (B), Group II liver magnification 100x (C) and 400x (D), Group III liver magnification 100x (E) and 400x (F), and Group IV liver magnification 100x (G) and 400x (H).

blood lipid profile was reduced after being given ciplukan extract. It could be seen that the lipid profile in the 800 mg dose group was lower than the 400 mg dose. A reduced lipid profile would cause reduced oxidative stress so that the resulting MDA yields are less (Ardhani *et al*. 2017; Jamesin *et al*. 2018; Marra and Lotersztajn 2013; Nozaki *et al*. 2015; Onwe *et al*. 2015; Peng *et al*. 2020).

The liver organ that has been terminated is then prepared and observed (Figure 1). Observation of the preparations was carried out by looking for inflammatory focus in five fields of view in the region of interest (ROI) or hotspot approach. The magnification used starts from weak magnification (100x) and strong magnification (400x). The inflammatory focus obtained was then included in the Brunt criteria (2011). These criteria consist of 4 degrees. Normal grade (0) did not indicate the presence of an inflammatory focus. Mild grade (1) indicated less than 2 inflammatory foci. Moderate degree (2) indicated 2-4 focus of inflammation and severe degree (3) indicated more than 4 inflammatory foci. The results were presented in Table 3.

Groups I, II, III, and IV had a median degree of moderate inflammation with the lowest degree being mild inflammation. All groups had severe liver disease except in group IV. Group IV did not have severe inflammation. The most severe degree of inflammation was only moderate. The inflammatory focus data in these criteriawas then processed using statistical software. The result showed that there was no effect from administering ciplukan extract on the degree of inflammation among group treatments.

Induction of dyslipidemia in Wistar rats increases the degree of inflammation of the non-alcoholic fatty liver disease activity score (NAS). It was seen in group I (K-) which was only given egg yolk induction and PTU without the intervention of ciplukan extract. This was in accordance with research conducted by Zălar *et al*. (2021) using 75% standard diet, 9% lard, 5.5% powdered egg yolk, 7.5% sugar, 2.5% cholesterol, 0.3 % sodium cholate, and 0.2% PTU for 4 weeks. However, research conducted by Zălar *et al*. (2021) showed an increase in the degree of inflammation starting from mild to moderate. Meanwhile, the results of this study showed an increase in the degree of inflammation from mild to severe.

The increased degree of inflammation is caused by high lipid profile levels. A high lipid profile causes lipotoxicity and continues with suppression of the PPRα gene, endoplasmic reticulum stress, and the release of ROS from mitochondria. This is in line with Gou *et al*. (2016) which stated that giving high-fat foods as an inducer resulted in disruption of the PPRα gene. Disruption of the PPRα gene causes a decrease in lipid oxidation and prevents the release of VLDL from the liver. Meanwhile, endoplasmic reticulum stress stimulates interleukin-1β (IL-1β), interleukin-18 (IL-18), and Tumor Necrosis Factor-α (TNF-α) which results in the transcription factor NF-κB and M1 Kupffer cells being active. Kupffer M1 cells will secrete pro-inflammatory cytokines and C-C chemokine receptor 2 (CCR2). These chemokines cause monocytes to migrate towards hepatocyte cells (Jamesin *et al*. 2018; Marra and Lotersztajn 2013; Nozaki *et al*. 2015).

The results of histopathological observations showed that administration of ciplukan stem and leaf extract did not reduce the degree of inflammation significantly. In the research group, it appeared that the degree of inflammation in group 4 (KB) with a dose of 800 mg was lower compared to group 1 (K+) as a control with standard therapy, although the results were not significant. The results of the degree of inflammation in this study are different from Zhang *et al*. (2021) which stated that administration of one of the substances in the Physalis species can reduce the degree of inflammation by inhibiting the NF-κB transcription factor and pro-inflammatory cytokines, namely interleukin-1β. (IL-1β), interleukin-6 (IL-6), and Tumor Necrosis Factor-α (TNF-α). This inhibition causes inflammatory activity to decrease. Apart from that, another study conducted by Fitri *et al*. (2017) showed that ciplukan extract at a dose of 2 mL/kg BW reduced serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels in male rat Swiss strain.

A non-significant reduction in the degree of inflammation can be influenced by administering PTU. PTU administered increases the risk of hepatotoxicity and increases the degree of liver inflammation (Karamikhah *et al*. 2015). In addition, there are still few studies regarding the effect of extract extracts on the degree of liver lobe inflammation, so there is a lack of reference for dosage and duration of intervention. Pre-analytic factors of liver fixation also influence the results of preparing preparations.

PTU administration also affects liver inflammation. It could be seen from the results that the degree of inflammation in each group ranges from mild to severe. This was because the side effects of PTU increase the risk of hepatotoxicity. Previous research conducted by Karamikhah *et al*. (2015) found that PTU at a dose of 100 mg/kg could cause lipid peroxidase, resulting in ROS and oxidative stress in the liver. This is in accordance with the results of this study which showed an increase in inflammatory activity in all induction groups, both K-, K+, A, and B

Table 3. Analysis of the degree of inflammation of rats liver

Criteria	Group	N	Median (min-max)	p value
Inflammation	I (K-)	6	2 (1-3)	0.964
	II (K+)	7	2 (1-3)	
	III (KA)	5	2 (1-3)	
	IV (KB)	6	2 (1-2)	

groups with 0.01% PTU administration. In addition, Sun *et al*. (2021) stated that administration of PTU can cause microbial imbalance in the intestine, increase lipopolysaccharides, and destroy the intestinal barrier. This causes an increase in hepatic inflammatory activity along with the digestive tract.

The limitations of this study were the low dose of intervention and the small number of studies regarding the effect of ciplukan extract on liver histopathological changes, especially increasing the degree of inflammation, so the effect of the intervention was less than optimal. In this study, no analysis was carried out on the digestive system, especially the intestines, to see the specific effects of liver inflammation via the digestive tract. Apart from that, pre-analytic factors of liver fixation also influence the results of preparation. Therefore, further studies are needed using other intervention doses, analysis of the digestive system, other variables related to liver inflammation, and pre-analytic factors related to appropriate fixation so that the preparation results could be observed properly.

CONCLUSION

Based on the results of statistical tests, it could be concluded that there is no effect of ciplukan stem and leaf extract (*Physalis angulata* L) on the degree of inflammation of the liver lobes of Wistar rats induced by dyslipidemia.

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