Physalis angulata Linn. As a Potential Liver Antifibrotic Agent In Rats

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ABSTRACT

Background: *No drug with a liver antifibrotic effect for treating non-alcoholic fatty liver disease (NAFLD) has been approved. Physalis angulata Linn., better known to Indonesians as ciplukan, can treat various metabolic and inflammatory diseases naturally. This study aims to determine the effect of ethyl acetate fraction of P. angulata in the NAFLD rat model by examining alanine aminotransferase (ALT), cholesterol levels, and liver histopathological features, which are methods to evaluate the course of the disease and the potential antifibrotic effect.*

Method: *This research is an in vivo study on male Wistar rats conducted at the Animal Laboratory, Faculty of Medicine, Universitas Padjadjaran, from September to November 2020. Rats were grouped randomly into seven groups of 5 each. The NAFLD models were created by giving a diet containing 20% margarine for four weeks.*

The intervention groups were given vitamin E, ethyl acetate fraction of P. angulata, and both combinations. The statistical analysis examined differences in each group based on their histopathological features, ALT, and cholesterol levels.

***Results*:** *Histopathological results in the group given P. angulata at a dose of 0.32 mg resembled normal liver, and the ALT level was similar to vitamin E. The administration of P. angulata at a dose of 0.16 mg improved cholesterol levels.*

Conclusions: *P. angulata ethyl acetate fraction at a dose of 0.32 mg improved the histopathological and serum ALT levels in the NAFLD rat model, which could be the basis for the mechanism of P. angulata's antifibrotic ability in NAFLD conditions.*

Keyword: *antifibrotic, ciplukan, fibrosis, NAFLD, Physalis angulata*

ABSTRAK

Latar belakang: *Sampai saat ini belum ada obat dengan efek anti fibrosis sebagai bagian penatalaksanaan non-alcoholic fatty liver disease (NAFLD). Physalis angulata Linn., biasa dikenal di Indonesia sebagai ciplukan, memiliki berbagai efek pada penyakit metabolik dan berbagai inflamasi. Penelitian ini bertujuan untuk melihat efek fraksi etil asetat dari P. angulata pada tikus model NAFLD dengan menilai kadar alanin aminotransferase (ALT), kolesterol, dan tampilan histopatologi hati, guna mengevaluasi perkembangan alami dari penyakit serta efek anti fibrosisnya.*

Metode: *Penelitian dilakukan secara in vivo pada tikus Wistar jantan yang bertempat di Laboratorium Hewan, Fakultas Kedokteran Universitas Padjadjaran pada bulan September sampai November 2020. Tikus dikelompokkan secara acak ke dalam tujuh grup yang masing-masing beranggotakan lima tikus. Model tikus NAFLD dibuat dengan memberikan pakan mengandung margarin 20% selama empat minggu. Kelompok yang mendapatkan intervensi diberikan vitamin E, fraksi etil asetat P. angulata, atau kombinasi keduanya. Selanjutnya diperiksa uji beda antar grup berdasarkan gambaran histopatologi, kadar ALT, dan kolesterol.*

Hasil: *Hasil histopatologi pada kelompok yang diberikan P. angulata dengan dosis 0,32 mg menghasilkan gambaran hati normal, kadar ALT juga sejalan dengan kelompok yang diberikan Vitamin E. Pemberian P. angulata dengan dosis 0,16 mg memperbaiki kadar kolesterol.*

Kesimpulan: *P. angulata dengan dosis 0,32 mg memperbaiki gambaran histopatologi hati serta kadar ALT pada tikus model NAFLD, hal ini dapat menjadi dasar potensial mekanisme anti fibrosis P. angulata pada kondisi NAFLD.*

Kata kunci: *anti fibrosis, ciplukan, fibrosis, NAFLD, Physalis angulata.*

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the world.[1] In 2019, the highest prevalence of NAFLD in Asia is occupied by Indonesia, with 51.04%.[2] The mortality rate from NAFLD is still high and is generally caused by the progression of the disease, leading to cirrhosis and terminal liver disorders. Oxidative stress is an essential factor in the development of NAFLD.[3] Increased serum levels of free fatty acids (FFA) and cholesterol, insulin resistance, adipocyte proliferation, and gut microbiome dysfunction are all possible outcomes of obesity caused by dietary habits and environmental factors. Activity in the gut microbiome raises FFA levels and triglyceride. Lipopolysaccharide (LPS), produced by intestinal microbes, causes stress and inflammation in the endoplasmic reticulum (ER). Free cholesterol and other lipid metabolites cause oxidative stress, mitochondrial dysfunction, and ER stress, which in turn cause hepatic inflammation and fibrogenesis.[4] NAFLD will lead to more serious liver diseases such as nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma.[1] Previous research has shown that consuming high-fat foods causes liver steatosis and liver damage, which are part of NAFLD. This method can be a good model for the initial stage of NAFLD.[5–7] Consuming 20% margarine showed extensive liver necroinflammation, the features of NAFLD. It increased aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, and lactate dehydrogenase (LDH) levels compared to the control group in rats.[8]

However, to date, no drugs are approved for treating NASH based on the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).[9] The FDA and EMA are supporting drugs that have two effects: improve NASH without exacerbating liver fibrosis or reduce liver fibrosis by one class without worsening NASH.[10] Vitamin E is an antioxidant that affects NASH and is the therapy with the highest anti-steatohepatitis effect. The European Association for the Study of the Liver and the Italian Association for the Study of the Liver were still waiting for further research because previous studies regarding the use of vitamin E showed inconsistent results and were only sometimes in line.[9,11] In the pioglitazone *versus* vitamin E *versus* placebo for the treatment of nondiabetic patients with nonalcoholic steatohepatitis (PIVENS) clinical trial, vitamin E significantly improved steatohepatitis without significantly affecting fibrosis.[1,12] Vitamin E and pioglitazone only reduced liver cell damage and inflammation without improving fibrosis.[13] Most antifibrotic drugs today are costly; there is a need to develop a potential natural substance to produce an efficient effort in the fight against fibrosis. *Physalis angulata* Linn. (Figure 1), better known to Indonesian as *ciplukan* is a group of plants commonly used for antidiabetic, antiviral, immunomodulatory, anti-inflammatory, antioxidant, analgesic, and anti-tumor.[14] A secosteroid known as physalin has a structure that is comparable to glucocorticoids and is one of the chemical compounds of *P. angulata* that has an effect that reduces inflammation.[15] Significant results occurred in improving liver fibrosis induced by carbon tetrachloride (CCl_4) by administration of *P. angulata* herb.[16] In some research, the safety of *P. angulata* herb in humans has been demonstrated.[17–19] Compared to the standard extract method, the fractionation method would result in more concentrated isolates.[20]

The histopathology of NAFLD is the most reliable way to evaluate its condition. Cholesterol levels are yet another indicator of fatty liver. One of the serum indicators of liver damage is the level of ALT.[21,22] Research using PAL herb is one of the newest in this subspecies, particularly concerning liver fibrosis. This study aimed to determine the effect of ethyl acetate fraction of PAL in the NAFLD rat model by examining ALT levels, cholesterol levels, and liver histopathological features, which are methods to evaluate the course of the disease and the potential antifibrotic effect.

METHOD

Plant Material

All parts of *P. angulata,* except the roots, were collected from December 2019 to February 2020 from a few regions in West Java, Indonesia. The collected herbs were processed at the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran.

Ethyl Acetate Fraction of *P. angulata* Preparation

Initially, *P. angulata* herbs underwent an extraction process. The extraction method was the cold maceration technique using a solvent and stirred several times at room temperature. All parts of *P. angulata* herbs (except the roots) were soaked with 50% ethanol for 3 × 24 hours. A rotational vacuum evaporator was used to concentrate the filtrate. Then, thick freeze-dried *P. angulata* was extricated into a dry extract. A parcel of the thick ethanol extract was weakened in hot water and fractionated utilizing the liquid–liquid extraction strategy with ethyl acetic acid derivation as the dissolvable. Each extractant was then evaporated using a rotary vacuum vaporizer, while the raffinate was evaporated using a freeze dryer. From the fractionation process, ethyl acetate fraction and water fraction were obtained. In this study, we used the ethyl acetate fraction. Although the fractional method is more expensive than extraction, it will yield more concentrated isolates than those produced by the extraction method.

NAFLD Model

The NAFLD induction process was done by providing a high-fat diet containing trans fatty acids with 20% margarine for four weeks.[8]

Animals

Based on Mead's formula, the estimated minimum number of subjects with seven treatment groups is between 17 and 27 rats. In total, 28 rats should be used, and each group will consist of four. Therefore, the authors used five rats in each group to anticipate death or other uncontrolled events.[23] Male adult Wistar rats, 12 weeks old and 150–200 g body weights, were purchased from the Division of Animal Laboratory of PT. Biofarma, Parongpong, West Java, Indonesia. Rats were acclimatized for 14 days before the experiment and received food and drink *ad libitum*. They were

caged in 30 x 25 x 15 cm containers with wood shavings. There was a place to eat and drink, always filled and cleaned regularly. Each container contained five rats that were classified according to the treatment group. The room was well-ventilated because it had sufficient air circulation and was not stuffy. The lighting principle of a 12-hour dark and 12-hour light cycle in the room. The standard room and cage conditions allowed the experimental animals to move freely and express their natural behavior. However, the biological needs of the experimental animals were limited because all of the experimental animals were male to minimize confounding factors in the study.

After undergoing the treatment phase for eight weeks (four weeks induction of NAFLD condition and four weeks administration of additional interventions), the rats entered the termination phase. At the end of the experiment, all rats were terminated, and surgery was performed to remove the liver after blood samples were obtained by puncturing the heart with a 23G needle while being anesthetized with ketamine and xylazine at doses of 100 mg/kg and 5 mg/kg.[24] The guidelines for the welfare of laboratory animals by the Research Ethical Committee of Universitas Padjajaran refer to American Veterinary Medical Association (AVMA) guidelines for the Euthanasia of Animals: 2020 Edition and National Research Council of the National Academies, Guide for the Care and Use of Laboratory Animals (Eight Edition), 2011, approved this research with approval number 1009/UN6.KEP/EC/2020.

Treatments

Rats were grouped randomly into seven groups of five each, consisting of the normal group that did not receive any intervention. The negative control group was only induced with 20% margarine. After being induced with 20% margarine, the other five groups also received treatment: the group that received standard NAFLD therapy with vitamin E (positive control group), the group that received ethyl acetate fraction of *P. angulata* 0.16 mg (PAL-1), and the group that received ethyl acetate fraction of *P. angulata* 0.32 mg (PAL-2). In addition, there was also a group that received a combination of vitamin E and PAL-1, as well as vitamin E and PAL-2.

All interventions were administered orally every day for four weeks. The dose of *P. angulata* fraction given was based on randomized controlled trial (RCT) studies on humans using *P. angulata* capsules. Capsule formulated with a composition of *P. angulata* extract and filler in a ratio of 1:1. The *P. angulata* extract capsules used in that study weighed 250 mg, so the total *P. angulata* extract was 125 mg/capsule. Because the dose given in the previous RCT study was three times one capsule each, the amount of extract was 375 mg/day/subject.[18] To determine the dose in rats, the dose of extract in humans was multiplied by the Laurence and Bacharach coefficient for rats, which was 0.0026, so that the extract dose in rats was 0.975 mg and the yield of the fraction was 0.165, then the dose of fraction per rat is 0.16 mg. In this study, two stages of fractions were given, namely 0.16 mg (*P. angulata*-1), and the dose doubled to 0.32 mg (*P. angulata*-2). The *P. angulata* fractions were dissolved in 0.5% carboxymethyl cellulose and administered orally.

The standard therapy for NAFLD is vitamin E at a dose of 800 mg/day in humans.[25] To determine the dose in rats, the dose in humans was multiplied by the Laurence and Bacharach coefficient for rats, which was 0.018. Therefore, the dose in rats was 14.4 mg/rat/day.

Sample Preparation

The serum followed centrifugation for 3 minutes at 15,000 rpm at 4 °C. Liver organs were taken as soon as possible after the rats were sacrificed. Then, the liver was weighed, washed using physiological NaCl solution, and fixed using formaldehyde solution to make histological preparations with hematoxylin and eosin staining. Later, the slides were examined under a light microscope.

Biochemical Assays

This study focuses on serum ALT because ALT is more specific for liver function. The authors did not use AST level to ensure that the findings accurately reflect liver injury because AST distribution is more common in the heart than in the liver.[21,22] ALT examination of rat serum was performed to assess liver function. ALT activities on serum samples were measured using an ALT LOT kit (Glory diagnostic 16887, Linear Chemicals, SLU, Spain). The steps for the analysis were carried out using the kit protocol. Cholesterol examination of rat serum was examined as part of the pathogenesis of NAFLD. Cholesterol on serum samples was measured using a cholesterol kit (Glory diagnostic, LOT 16777, Linear Chemical, SLU, Spain).

Histopathological Examination

According to Kleiner et al., for clinical histopathologic investigations, it is recommended to classify the histologic changes and NAFLD

activity based on biopsy into the following classes: no significant evidence of fatty liver disease (not NAFLD/0), steatosis (1), steatohepatitis (2), or cryptogenic cirrhosis/fibrosis (3). Not NAFLD is defined as deficient steatosis for a diagnosis of steatosis (< 5%) without other changes (ballooning or fibrosis) that would propose steatohepatitis. Steatosis definition is no particular changes were found to propose a frame of steatohepatitis.[25] This category may incorporate spotty lobular inflammation and/or mild degrees of fibrosis with insignificant findings. Ballooning injury is a characteristic of steatohepatitis. The ballooning injury could be a cellular alter in which the cells get extended, and the cytoplasm sporadically clumped with clear, non-vesiculated zones. It is said to be cryptogenic fibrosis/cirrhosis if the presence of fibrosis with any degree of portal inflammation or minor to no steatosis and ballooning.

Statistical Analysis

In the histopathological investigations data, analysis was carried out to compare each treatment group with the negative control group using Fisher's exact test. The data obtained in the ALT serum data section were not normally distributed, and the variance was not homogeneous. Therefore, a Kruskal–Wallis analysis was carried out first, regardless of whether the results were significant or not, followed by a bivariate analysis with Mann–Whitney in each treatment group compared to the negative control group to find out whether there were at least distinct differences between the negative control group when compared to just one other group or the differences did not appear before due to inadequate dosage. For data analysis, cholesterol was tested using the one-way analysis of variance (ANOVA) test because the data were normally distributed and the variance was homogeneous. Because the one-way ANOVA analysis showed statistically significant results, it was continued with post-hoc analysis with the least significant difference (LSD). For all statistical analysis results in this study, $p < 0.05$ is a significant value. All statistical analyses used SPSS software version 20 (IBM Corp., USA).

RESULTS

Histopathological Changes

Our induction method is related to histopathological outcomes in this population. Based on Table 1, Fisher's exact test results defined a significant value in the normal group compared to the negative control group ($p = 0.036$). A significant value was found in the 0.32 mg of *P. angulata* herbs group compared to the negative control group ($p = 0.024$), which means the 0.32 mg of *P. angulata* herbs was the only treatment that improved the histopathological outcome in this population.

Table 1. Histopathological clinical investigations analysis

Variables	Histopathological degree, median (min–max)	*p-value*
Normal	0 (0.00–0.00)	**0.036***
20% margarine (negative control)	2 (1.00–3.00)	
20% margarine (negative control)	2 (1.00–3.00)	1.000
20% margarine + vitamin E	2 (1.00–2.00)	
20% margarine (negative control)	2 (1.00–3.00)	1.000
20% margarine + vitamin E + PAL-1	1.5 (1.00–2.00)	
20% margarine (negative control)	2 (1.00–3.00)	0.286
20% margarine + vitamin E + PAL-2	1 (0.00–3.00)	
20% margarine (negative control)	2 (1.00–3.00)	0.524
20% margarine + PAL-1	1 (1.00–2.00)	
20%margarine (negative control)	2 (1.00–3.00)	**0.024***
20% margarine + PAL-2	0 (0.00–1.00)	

p-value is obtained from the Fisher's exact test result; 20% margarine is the NAFLD induction method; Vitamin E is the standard treatment of NAFLD; PAL-1, 0.16 mg of *P. angulata* ethyl acetate fraction; PAL-2, 0.32 mg of *P. angulata* ethyl acetate fraction. **significant difference*

Figure 1. *Physalis angulata* Linn.

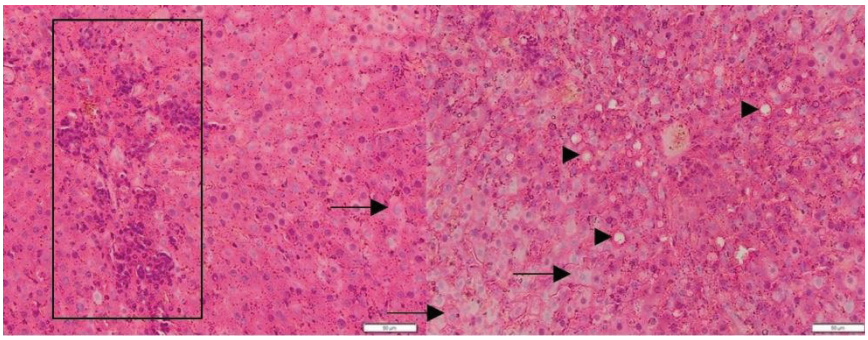

Figure 2. Histological picture of the 20% margarine-only group

In this group, who only received NAFLD induction without treatment, histological signs of NAFLD were found in the form of the intracellular vacuole (arrowheads), hepatocellular ballooning (arrow), and portal inflammation (rectangular) (magnification ×100).

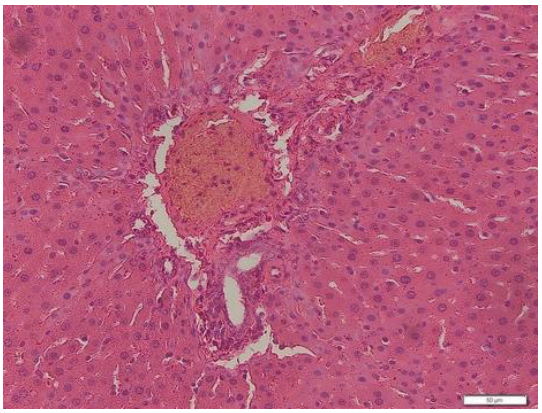

Figure 3. Histological picture of the group treated with 0.32 mg of *P. angulata* herbs

After being induced with 20% margarine, this group was treated with 0.32 mg of *P. angulata* herbs. Histological results of the liver were normal, without any signs of inflammation. (magnification ×100).

The histopathological result showed that in the 20% margarine group (Figure 2), there was steatosis or fat accumulation in the form of intracellular vacuoles, hepatocellular ballooning, and portal or intralobular inflammation, which are the conditions found in NAFLD. Histopathological results in the group given 20% margarine and 0.32 mg of *P. angulata* herbs (Figure 3) showed a hepatocellular picture resembling normal liver cells without steatosis, hepatocellular ballooning, and inflammation.

Serum Marker (ALT)

The results of the Kruskal–Wallis analysis showed the *p*-value of the ALT level > 0.05, which means there was no statistically significant difference between the ALT levels in those groups. The bivariate analysis results with Mann–Whitney can be seen in Figure 4. It is known that the rat group that was given 20% margarine and 0.32 mg of *P. angulata* herbs showed significantly lower serum ALT levels than the negative control group, with $p = 0.029$. The significant difference in ALT serum levels resembled the group of rats given standard vitamin E therapy with $p = 0.032$, which means that 0.32 mg of *P. angulata* herbs had similar effectiveness to vitamin E in this study.

Cholesterol Serum

The result of the one-way ANOVA test in the cholesterol group obtained information on the *p*-value of the cholesterol variable < 0.05; thus, it can be explained that there is a statistically significant mean difference between the cholesterol variable in the normal, negative control, and treatment groups. Through LSD analysis (Figure 5), a significant difference was found between the normal and negative control groups ($p < 0.05$). In addition, the administration of 0.16 mg of *P. angulata* herbs also improved the cholesterol serum levels compared to the negative control group ($p < 0.05$).

DISCUSSION

Histopathological results of the liver in rats receiving 20% margarine showed severe cytoplasmic vacuolation, hepatocyte hypertrophy, and hepatocyte ballooning, which showed extensive areas of necroinflammation. The histologic and pathogenic features of NAFLD are very clear in this group.[8] The statistical test result also showed that our induction method affected the histopathological outcome (Table 1).

In Figure 2, intracellular vacuole, hepatocellular ballooning, and portal inflammation were found. Hepatocellular ballooning is a crucial histological finding in the diagnosis of nonalcoholic steatohepatitis (NASH).[26] In the initial classification system of NAFLD, ballooning was the main distinguishing feature of NASH, indicating a greater risk of disease

progression.[26,27] Hepatocellular ballooning has also been shown to correlate with fibrosis.[28] Portal inflammation was found in 87% of NAFLD patients.[29] Liver biopsy presentation with more than mild portal inflammation was associated significantly with the amount of steatosis, the presence of ballooning, and advanced fibrosis.[30] It was also strongly correlated with the incidence of fibrosis, with a correlation coefficient of 0.76.[29] Those histopathological features were not present in the 0.32 mg of P. angulata herbs group (Figure 3), which might mean that the P. angulata ethyl acetate fraction at a dose of 0.32 mg could prevent liver deterioration in NAFLD patients from a histological point of view and is supported by the administration of 0.32 mg of P. angulata herbs is associated with histopathological improvement statistically (Table 1).

However, at the P. angulata dose of 0.16 mg, there was no significant association with the histopathological outcome of the population (Table 1). This could indicate the possibility that the P. angulata ethyl acetate fraction would show good results only in adequate doses. The group that received vitamin E with or without the P. angulata ethyl acetate fraction also showed no significant results, and this could be due to possible drug interaction between vitamin E and P. angulata fraction, which made the insignificant effect or the dose of vitamin E in this study was not sufficient to overcome the 20% margarine that we used.

In a study by Dhibi et al., consumption of 20% margarine caused an increase in AST, ALT, alkaline phosphatase, and LDH levels compared to the control group. In addition, there was an increase in triglyceride levels and a higher inflammatory response in the plasma of rats fed 20% margarine.[8] Our study showed no statistically significant difference in ALT serum levels between the normal and negative control groups. Figure 4 showed a possibility that the potential of 0.32 mg of P. angulata herbs was similar to the current NAFLD standard therapy (vitamin E) in reducing serum ALT levels or preventing their increment.

Elevated AST and ALT are markers of hepatocellular injury. ALT is a more specific marker of liver injury than AST.[21,22] Hepatocellular damage is found in NAFLD conditions. Excessive fat accumulation in the liver will cause hepatocellular lipotoxicity through oxidative stress, including in the ER and mitochondria, which can cause hepatocyte apoptosis. Prolonged ER stress can produce reactive oxygen species and activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), or c-Jun N-terminal kinase, which causes an inflammatory process.[11,31] Physalin E has an anti-inflammatory effect by inhibiting NF-κB activation.[32] Hindrance of NF-κB in Kupffer cells results in diminished liver fibrosis, but the underlying mechanisms remain elusive. Whereas the part of NF-κB enactment in hepatocytes and Kupffer cells that leads to liver fibrosis is as it were not entirely caught on, there was growing evidence that NF-κB acts as a critical arbiter of fibrosis in hepatic stellate cells and hepatic myofibroblasts (hMF). Stellate cells are involved in the deposition of extracellular matrix components. These cells transform into hMF that will form scars.[33]

P. angulata herb contains active compounds, including saponins, tannins, flavonoids, polyphenols, secosteroids (physalin, withangulatin), and alkaloids.[34] One chemical compound that has an anti-inflammatory effect is a secosteroid (physalin). The structure of physalin is 13,14-seco-16, 24-ring ergosterol has a functional group with the highest level of oxidation group. It has a similar structure to glucocorticoids, so it has an anti-inflammatory effect with fewer side effects than glucocorticoids.[15] Physalin B, F, and G can inhibit nitric oxide (NO) production from macrophages stimulated by tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS), and interferon-gamma (IFN-γ). Physalin does not work through the glucocorticoid receptor, as evidenced by the addition of the glucocorticoid receptor antagonist RU-486, which does not affect the effect of physalin.[32]

Various studies have proved that P. angulata has an antioxidant effect through its natural phenol content. Phenol components such as flavonoids, tannins, phenylpropane, and other phenolic components have immunomodulatory activity through the complement system or intracellular biochemical reactions.[34] Permatasari et al. found a relationship between P. angulata leaf methanol extract and malondialdehyde levels as a marker of oxidative stress with a significant negative correlation.[30] Oxidative stress is set to be an essential factor in the development of NAFLD.[11] This supported the results of our research, which makes P. angulata comparable to vitamin E in terms of antioxidizing effects.

In the pathogenesis of NAFLD, lipid peroxidation occurs in cell membranes and is a process of oxidative degradation of polyunsaturated fatty acids (PUFAs). This process causes functional and structural membrane damage.[35] Phospholipids contained in the membrane containing trans fatty acids will attract cholesterol.[36] This is in line with our study, where the consumption of 20% margarine containing trans fatty acids causes

an increase in serum cholesterol, as in Figure 5. Free cholesterol will accumulate in the lysosomes of Kupffer cells, triggering an inflammatory response.[37,38] The accumulation of free cholesterol in hepatic stellate cells causes an increase in toll-like receptor 4 (TLR-4) expression and sensitivity of stellate cells to transforming growth factor beta (TGF-β), which plays a role in fibrosis.[39,40] Therefore, lowering cholesterol levels in patients with NAFLD may play a role in overcoming inflammation and fibrosis in the liver. This effect can be matched with the treatment group results with 0.16 mg of P. angulata herbs in Figure 5. The ethanol extract of P. angulata herb incorporates a substantial antioxidant impact due to its flavonoid substance. Phenol subordinates from P. angulata are crucial as substances that can avoid oxidative push in chronic inflammatory diseases.[41,42] Antioxidant activity in all parts of the plant was highest in fruit and stem extracts using the standards of superoxide, NO, hydrogen peroxide, and hydroxyl radical.[42] Kusumaningtyas et al. found that the antioxidant activity of P. angulata was influenced by the total phenol contained in it.[18]

Another advantage of our research method is the ability to produce ethyl acetate fraction products from *P. angulata* herbs. The fractionation process produces fractions that contain more concentrated compounds. The fraction is obtained from further extract processing so that the active substance is more isolated.[20] All of the above evidence can form the basis of *P. angulata*'s potential as an antifibrotic agent. Everything has a cause-and-effect correlation between the herbs, pathogenesis, and even the biomolecular stage. In addition, we succeeded in establishing a NAFLD model histopathologically and based on blood serum examination. The induction method we used aligns with the pathogenesis and pathophysiology of NAFLD, making this study more focused. This research is still a preliminary study, and further research should be carried out to assess liver fibrosis conditions directly, including by conducting histological analysis using other stains, such as Masson's trichrome stain, and utilizing other more advanced comparison liver fibrosis parameters. Considering the results of our research, which are pretty unique regarding vitamin E and the *P. angulata* fraction, it is necessary to conduct further research on the relationship between the two. The quest for the optimal dose of *P. angulata* fraction still needs to be done.

CONCLUSION

In conclusion, *P. angulata* ethyl acetate fraction at a dose of 0.32 mg improved the histopathological and serum ALT levels in the NAFLD rat model with 20% margarine, which could be the basis for the mechanism of *P. angulata*'s antifibrotic ability in NAFLD conditions.

CONFLICT OF INTERESTS

The authors affirm no conflict of interest in this study.

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