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Hepatoprotective effect of the ethyl acetate fraction of Indonesian *Artocarpus* species against CCl_4 induced liver injury in rat

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ABSTRACT: The liver—the center of metabolism and detoxification—is especially susceptible to damage from toxins and must be protected. This study aimed to evaluate the hepatoprotective effects of the ethyl acetate fraction from three Indonesian Artocarpus species (*Artocarpus altilis*, *A. champeden*, and *A. heterophyllus*) in rats with acute liver injury induced by carbon tetrachloride (CCl_4). The hepatoprotective effect was assessed through biochemical parameters (AST, ALT, and ALP) and histological analysis of the liver of the test group and compared to the normal control group (Na CMC 0.5%) and the positive control group (silymarin 100 mg/kg). There was no significant difference between the AST, ALT, and ALP levels in the 500 mg dose group, the control and the silymarin group ($p > 0.05$). Histological analysis of the liver tissue showed a microscopic profile similar to that of the control and silymarin groups. This study showed that the ethyl acetate fraction of *Artocarpus altilis*, *A. heterophyllus*, and *A. champeden* has synergistic potential as a hepatoprotector against liver injury caused by CCl_4. Consequently, the leaves of these species can be used as complementary medicine in treating acute liver damage.

KEYWORDS: Ethyl acetate fraction; *Artocarpus altilis*; *Artocarpus heterophyllus*; *Artocarpus champeden*; acute liver damage.

1. INTRODUCTION

The liver is the main organ in which metabolism and detoxification occur. This organ involves many biochemical pathways in its working mechanism, such as oxidation, reduction, hydrolysis, and conjugation [1,2], making it especially susceptible to injury that may result from chronic exposure to drugs such as paracetamol, biological agents such as viruses, or chemicals such as carbon tetrachloride (CCl_4) [2]. Such injuries result in changes in the structure and function of the liver, cirrhosis, fibrosis, and even liver cancer if left untreated[3]. Drugs are one of the leading causes of liver damage[4]. In Indonesia, there has been an increase of around 15–20% in cases of liver disorders in the last five years [5].

Liver disease treatment is quite expensive, so many people prefer natural medicine to prevent and treat this disease[3]. In addition, synthetic drugs currently used are inadequate and have adverse effects. Therefore, many people turn to natural medicines, which are believed to be more efficient, economical, and safe [3].

The *Artocarpus* genus is a plant from tropical and subtropical Asia well-known for its diverse secondary metabolites and promising pharmacological potential. Among the various pharmacological properties of *Artocarpus* species are their antioxidant and anti-inflammatory [6], antiplatelet [7], antityrosinase [8], anticancer [9,10], antidiabetic [11], gastroprotective [12], and diuretic [13] properties. *A. altilis* (Breadfruit), *A. champeden* (Cempedak), and *A. heterophyllus* (Jackfruit) are the three best-known and easily collected species of *Artocarpus* in Indonesia. These three species are known as rich sources of polyphenolic compounds and are used as traditional medicines to treat various diseases, such as hypertension, kidney disorders, rheumatism, and liver damage. This study aims to evaluate the hepatoprotective potential of ethyl acetate extracts of *A. altilis*, *A. champeden*, and *A. heterophyllus* leaves in CCl_4-induced liver injury in the rat.

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2. RESULTS

2.1 Effect of Ethyl Acetate Fraction on Biochemical Parameters

The results of the liver damage parameter analyses are shown in Figure 1. Data on AST, ALT, and ALP levels were normally distributed and homogeneous ($p > 0.05$). AST levels of all dose groups were significantly different from the normal control ($p < 0.05$) and not significantly different from the silymarin group ($p > 0.05$). The results of the ALT examination showed that the ethyl acetate fraction at all dose ranges differed considerably from the CCl_4 group ($p < 0.05$), except for the 500-dose group, which showed ALT levels equivalent to those of the control and silymarin groups ($p > 0.05$). For ALP, the CCl_4 group showed very high levels of ALP compared to the control and silymarin groups ($p < 0.05$). At doses of 125 and 250, the ethyl acetate fraction reduced ALP levels, but not on par with the control group ($p < 0.05$) or silymarin ($p < 0.05$). At a dose of 500 mg, the test group showed ALP levels equivalent to the silymarin-positive control group ($p > 0.05$).

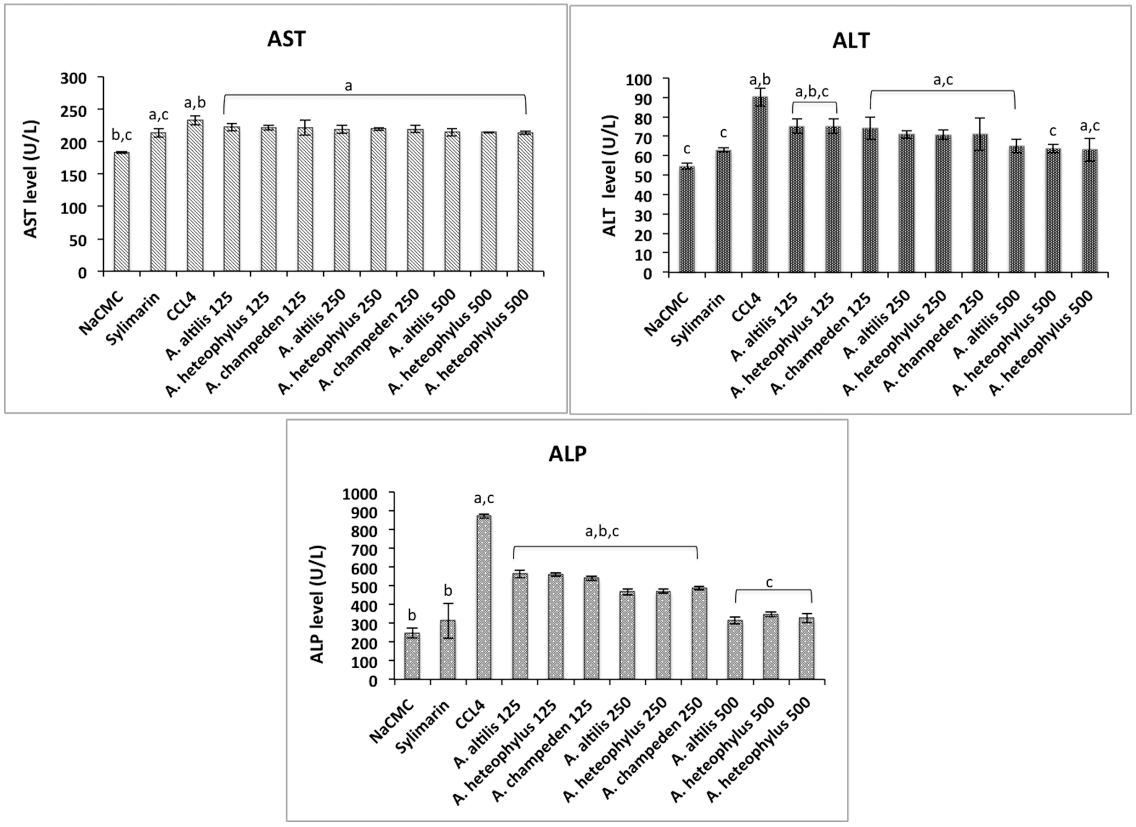

Figure 1. Effect of extract on AST, ALT and ALP levels in the rat liver damaged: Value expressed as mean ± SD, (n = 5). Statistical analysis (P < 0.05) by parametric ANOVA followed by Tukey HSD test. [a]p<0.05: significantly different from normal control, [b]p<0.05 significantly different from positive control, [c]p<0.05 significantly different from negative control.

2.2 Effect of the Extract on Macroscopic and Microscopic Characteristics of the Liver

The macroscopic observations of the liver are shown in Table 1. The morphology of the liver in the test group appeared to resemble that of the control group and the silymarin group, namely brownish-red in color and a smooth surface. The CCl_4 group had an abnormal appearance characterized by a mottled surface and a pale red color. The liver index of the test group was within the normal range, while that of the diseased group was abnormal.

Table 1. The effect of ethyl acetat fraction of Artocarpus on macroscopic profile of the rat liver.

Groups	Color	Texture	Liver index
NaCMC	Brownish-red	Fine	0.029
Sylimarin	Brownish-red	Fine	0.028
CCL4	Pale-red	Grainy	0.033
A. altilis 125 + CCL_4	Brownish-red	Fine	0.029
A. heteophylus 125 + CCL_4	Brownish-red	Fine	0.028
A. champeden 125 + CCL_4	Brownish-red	Fine	0.030
A. altilis 250 + CCL_4	Brownish-red	Fine	0.030
A. heteophylus 250 + CCL_4	Brownish-red	Fine	0.028
A. champeden 250 + CCL_4	Brownish-red	Fine	0.029
A. altilis 500 + CCL_4	Brownish-red	Fine	0.027
A. heteophylus 500 + CCL_4	Brownish-red	Fine	0.028
A. champeden 500 + CCL_4	Brownish-red	Fine	0.030

Histopathological observation was determined based on grade scoring [14]. A score of 0 represents a normal liver—with no changes in histological structure. Score 1 means light damage, ranging from 1–25%. Score 2 is for moderate damage ranging from 26–50%, and a score of 3 represents heavy damage ranging from 51–75%. Finally, severe damage ranges from 75–100%. The results of quantifying the protective effect of the ethyl acetate fraction on liver damage based on histological observations are shown in Table 2.

Table 2. The effect of fraction ofethyl acetate of Artocarpus on liver injury of rats

Groups	Injury of the score [14]			
	Inflammation	Steatosis	Cholestasis	Necrosis
Normal	0	0	0	0
CCl4	2	2	2	3
Sylimarin	1	0	0	0
A. altilis 125 + CCL_4	2	1	1	1
A. heteophylus 125 + CCL_4	1	2	1	2
A. champeden 125 + CCL_4	2	2	2	1
A. altilis 250 + CCL_4	1	1	0	1
A. heteophylus 250 + CCL_4	1	2	1	1
A. champeden 250 + CCL_4	1	1	1	1
A. altilis 500 + CCL_4	1	0	0	0
A. heteophylus 500 + CCL_4	1	1	0	0
A. champeden 500 + CCL_4	1	0	1	0

A value 0-3 indicate liver damage scoring referring to [14]: score 0 = no visible cell damage; score 1 = light damage; score 2 = moderate damage and score 3 = severe damage.

Microscopic observation provided a histopathological analysis of the liver (Figure 2). In the control group (Figure 2A), there were no changes in the histological structure of the liver tissue, and the cells appeared to be arranged radially with the central vein; hepatocyte cells were round or oval, did not exhibit inflammation, degeneration, steatosis, cholestasis, or necrosis, and with no dilation of the sinusoids. The silymarin group (Figure 2B) showed a profile similar to that of the control group, although mild inflammation was found. The CCl_4 group (Figure 2C) revealed severe liver damage, as indicated by moderate inflammation,

steatosis, cholestasis, and severe necrosis (Table 2). In addition, there was congestion in the central vein, which is indicated by the presence of a red mass and sinusoidal grooves that are not visible. When compared to the CCl_4 group, the ethyl acetate fraction at a dose of 125 mg (Figure 2D, 2G, and 2J) showed a decrease in damage, as there was no congestion in the central vein and sinusoidal grooves, which began to be visible but not clearly. Liver damage appeared to decrease in the group that received 250 mg ethyl acetate fraction treatment. The 500 mg dose group (Figure 2F, 2I, and 2L) showed the best results—inflammation and steatosis were mild, and no necrotic cells were found. In addition, cholestasis and congestion were not found, and sinusoidal grooves were clearly visible and arranged radially in the central vein.

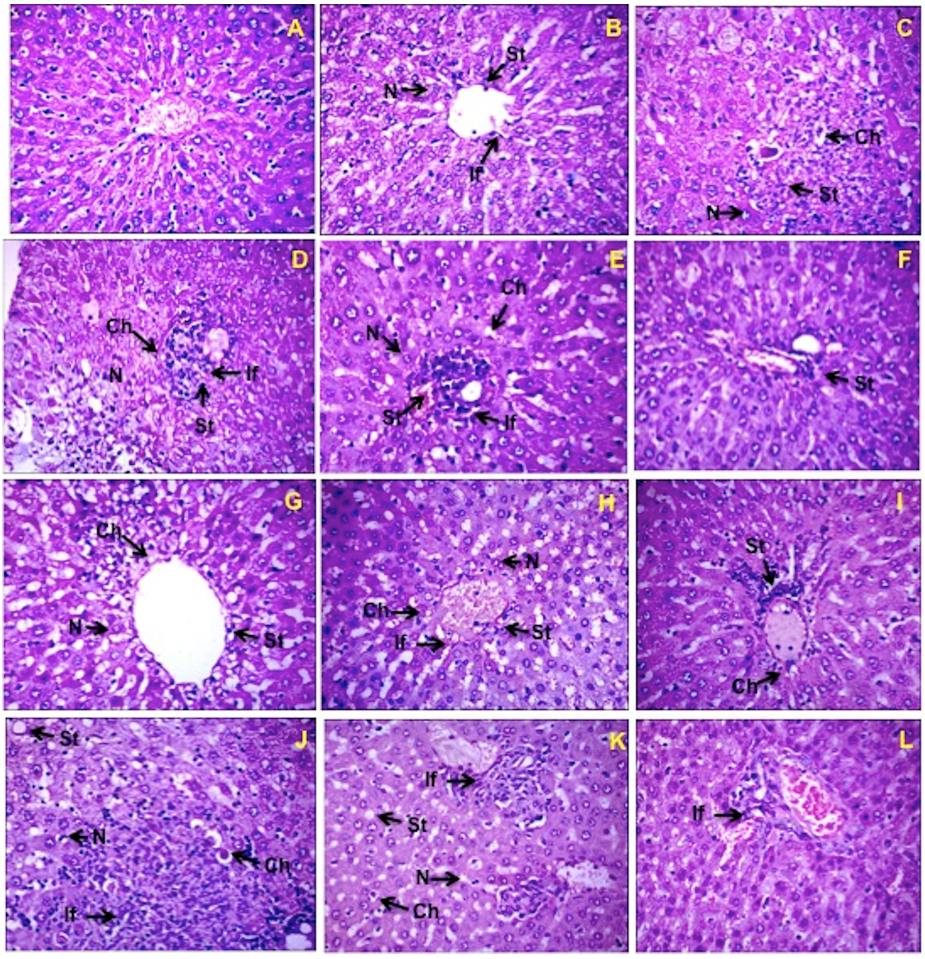

Figure 2. Histopathological analysis of rat liver (40×10 magnification): Normal control group (A), Silymarin group (B), CCl4 group (C), Groups *A. altilis* 125–500 mg (D–F), Groups *A. heterophyllus* 125–500 mg (G–I), Groups *A. champeden* 125–500 mg (J–L); Inflammation (If), Necrosis (N), Steatosis (St), Cholestasis (Ch).

3. DISCUSSION

The liver is the most vital organ for metabolizing exogenous and endogenous substances, including various xenobiotics. In addition, it is involved in several biochemical processes, notably nutrition, energy, and reproduction. Any disturbance originating from the liver can affect physiological and biochemical functions [2,4]. CCl_4 is a common toxicant used to evaluate the hepatoprotective properties of a substance because the model can define metabolic and morphological changes that occur in liver injury with high reproducibility. Carbon tetrachloride (CCl_4) is metabolized by the microsomal cytochrome P450 enzyme into a trichloromethyl metabolite (–CCl3), which can react with oxygen to form trichloromethyl peroxyl radical (CCl3O2•) [1,2]. These radicals are highly reactive, so they can covalently bind to proteins and lipids or remove hydrogen atoms from unsaturated fatty acids resulting in lipid peroxidation, inhibiting antioxidant enzymes, and causing nucleic acid mutations. The results are oxidative stress, inflammation, apoptosis, and necrosis [15,16].

The AST, ALT, and ALP are liver necrosis and damage biomarkers. The concentration of this enzyme is high in the liver and is released into the blood when the liver is damaged. Clinically, an increase in serum ALT, AST, and ALP indicate liver disease. ALT is more specific than AST, and an increase in ALP indicates cholestasis [17]. The trichloromethyl peroxyl radical ($CCl3O2•$) formed due to the toxic agent CCl_4 causes an increase in the production of this biomarker enzyme [2,18]. This study verifies that CCl_4 causes liver injury characterized by increased serum AST, ALT, and ALP levels. The increased activity of AST, ALT, and ALP may be due to hepatocyte cellular necrosis, which causes an increase in cell permeability [19]. However, the levels of this enzyme decreased significantly in animals that received treatment with the ethyl acetate fraction of *A. altilis*, *A. heterophyllus*, and *A. champeden*, demonstrating that the test substance can protect the liver from injury due to the CCl_4 hepatotoxin. The data of this study were consistence with previous studies, which found that *Moringa oleifera* [20] and *Polygonum orientale* [14] extracts caused a significant reduction in ALT, AST, and ALP levels in CCl4-induced animal models, indicating protection against liver damage.

AST is a sensitive indicator of mitochondrial problems, particularly in the centrilobular area of the liver. The test results showed that the AST levels in the test group significantly differed from those in the normal control group. The AST is a transaminase enzyme found not only in the liver but also in other organs such as the brain, kidneys, heart, and skeletal muscles. As a result, AST levels remain high in the blood circulation, making AST a non-specific parameter for liver damage [2,21]. Concerning ALT, levels dropped in line with increasing doses of the test substance. At a dose of 500 mg, the test group exhibited almost the same ALT levels as the group that received silymarin ($p > 0.05$), indicating that ethyl acetate fraction prevents CCl4-induced hepatotoxicity. ALT is the most common transaminase enzyme found in the liver. ALT is very accurate in monitoring hepatocellular status, and increased ALT is rarely caused by nonliver disorders, so ALT is a specific parameter of liver damage [16]. Alkaline phosphatase (ALP) is a marker enzyme for assessing the integrity of the plasma membrane and endoplasmic reticulum of tissues. ALP is involved in the hydrolysis of phosphate monoesters. Elevated ALP level often indicates cholestasis caused by liver injury [16,19]. The test results also showed that CCl_4 caused an increase in ALP levels three times greater than the levels of the normal group. It indicates severe liver damage. An increase in ALP levels to 3–10 times normal after administering CCl_4 indicates cholestasis and intra- and extra-biliary obstruction [22]. Administering ethyl acetate fraction significantly reduced ALP levels, and at a dose of 500 mg, the ALP levels did not differ from silymarin controls ($p > 0.05$). The results of this study indicate that the ethyl acetate fractions of *Artocarpus altilis*, *A. champeden*, and *A. heterophyllus* are hepatoprotective effects. Flavonoids contained in the ethyl acetate fraction of all three species are thought to suppress the release of AST, ALT, and ALP by inhibiting cytochrome P450, which catalyzes trichloromethyl biotransformation [23]. The significant decrease in AST, ALT, and ALP levels with increasing doses suggests that the primary mechanism of hepatoprotection may be through a reduction of lipid peroxidation.

The macroscopic of the test group liver's (Table 1) demonstrated that administration of the ethyl acetate fraction of *Artocarpus altilis*, *A. champeden*, and *A. heterophyllus* could prevent hepatomegaly as well as silymarin. A normal liver is red-brown, has a smooth surface, and has a rubbery consistency. The liver index of the test group was within the normal range, namely 2–3%—the same as the normal control group and the silymarin group. Silymarin is able to prevent hepatomegaly by inhibiting the production of leukotrienes, the 5-lipoxygenase cycle, and free radicals in liver Kupffer cells [24]. Liver weight in the CCl_4 group was more than 3% of body weight (Table 1) because the liver experienced steatosis and cholestasis, causing an accumulation of fat and bile in the liver cells, which increased liver weight.

Typical morphological features of acute liver injury include structural organization disorders and acute inflammatory reactions in the periportal area due to dystrophic and necrotic lesions in the lobular hepatocyte center [15]. Microphotographs of the normal control group's livers showed regular and evenly spaced hepatocyte cells, and no signs of damage such as inflammation, necrosis, steatosis, or cholestasis were observed (Figure 2A). The silymarin group experienced mild inflammation but not significantly, compared to the normal control (Figure 2B and Table 2). In contrast, the CCl_4 group showed severe inflammation and necrosis and moderate grades of steatosis and cholestasis (Figure 2C and Table 2). Necrosis is characterized by changes in the cell nucleus and the widening of the sinusoids. Dilation of the sinusoids occurs because the CCl_4, which was present in the liver cells, entered the sinusoids through the subendothelial gap, causing swelling and damage to cell organelles [22]. In the test group, the degree of liver injury decreased with increasing doses. At a dose of 500 mg, the photomicrograph was not significantly different from the silymarin and normal groups; no inflammation, steatosis, or necrosis was found. It indicates that the three *Artocarpus* fractions produced liver protective effects. Based on the liver damage score (Table 2), the histopathological profile in this study follows previous reports where animals injured with CCl_4 showed severe damage as indicated by necrosis, inflammation, and severe cholestasis[25]. The decrease in the damage score in the

animals treated with the extract showed an improved structure of hepatic cells, ensuring the protective effect of the extract

The CCl_4 metabolism leads to the accumulation of reactive oxygen species, especially O_2 and H_2O_2. The severity of oxidative injury caused by these species depends on the outcome of the interaction between the oxidant and the body's protective system. In the body, there is a complex defense system consisting of antioxidant enzymes such as SOD, CAT, and glutathione peroxidase and several non-enzymatic antioxidants, including GSH, vitamins, and ubiquinone, which can help protect against oxidative damage [16]. Silymarin works as a hepatoprotector by stabilizing free radicals and affecting intracellular glutathione, thereby increasing the number of natural antioxidants in the body [26]. The hepatoprotective effect shown by the test group is believed to be related to the antioxidant and anti-inflammatory activity of flavonoid compounds. *A. altilis* extract is known to be able to increase the activity of the antioxidant enzyme superoxide dismutase (SOD) and reduce H_2O_2 level [6]. *Artocarpus altilis* extract has also been shown to reduce MDA and increase catalase [27]. As well as *A. heterophyllus*, and *A. champeden* have been reported to have antioxidant and anti-inflammatory activity [6,28–30]. Antioxidants play a role in protecting cells from oxidative damage from the cellular level to the organ level [31]. Beside H_2O_2, Nitrogen oxide (NO) is a reactive oxygen species known as a mediator of acute and chronic inflammation. Flavonoid and chromone compounds from *A. heterophyllus* and *A. altilis* have been shown to inhibit NO production [6,32,33]. In the previous studies, we found that *A. altilis* extract suppressed pro-inflammatory cytokines (IL-1β, IL-6) and stimulated the expression of anti-inflammatory cytokines (IL-10) [34]. Several previous hepatoprotector effect studies also reported that one of the protective mechanisms of the extract was inhibiting the production of pro-inflammatory cytokines [14,23]. Therefore, it can be argued that the hepatoprotective effect demonstrated in this study resulted from its ability to decrease pro-inflammatory cytokine levels.

Besides inhibiting NO production, another mechanism that can provide a hepatoprotective effect is inhibiting the NF-κB signaling pathway, the TGF-β pathway, and the MAPK pathway [3]. Previous studies have determined that the compound Moracin-C from *A. heterophyllus* interfered with the mitogen-activated protein kinase pathways (MAPKs) and nuclear factor-κB (NF-κB) pathways primarily by reducing the nuclear translocation of the NF-κB p65 subunit. The compound Moracin C has also been shown to inhibit proinflammatory cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α-) [35]. The results of this research can explain the mechanism of liver protection shown by leaf extracts from *Artocarpus altilis*, *A. heterophyllus*, and *A. champeden*.

4. CONCLUSIONS

Based on the analysis of biochemical and histopathological parameters, it can be concluded that the three *Artocarpus* species examined have a hepatoprotective effect. This study has proven that the ethyl acetate fraction of *Artocarpus altilis*, *A. heterophyllus*, and *A. champeden* can potentially be developed as medicinal plants to treat liver damage.

5. MATERIALS AND METHODS

5.1 Plant Material and Chemicals

Plant materials (leaves) of *A. altilis*, *A. champeden*, and *A. heterophyllus* were obtained from the Ogan Ilir area, South Sumatra, Indonesia. Plant authentications are recorded at the Purwodadi Botanic Garden, Indonesian Institute of Science, No. B-390/IPH.6/KS.02/XI/2020. The chemicals used include 96% ethanol (PT Brataco), n-hexane (PT Brataco), ethyl acetate (PT Brataco), silymarin (Hubei Nokete Pharmaceutical Co. Ltd., Singapore), CCl_4 (Sigma Aldrich), Carboxymethylcellulose Sodium (Na-CMC), olive oil, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), kit reagents, and hematoxylin-eosin (HE) staining.

5.2 Experimental Animals

The experimental animals to be used in this study were male wistar rats aged 7-8 weeks, body weight 190–210 g. Before the treatment, the animals were habituated to room conditions with a temperature of 22°C and 55.5% humidity and a 12 hour light-dark cycle. During the adaptation period, the rats were given standard food and water ad libitum. After the adaptation period, the experimental animals were randomly divided into 12 groups (5 per group). The normal control group was given 0.5% Na-CMC, the negative group received CCl_4, and the positive group received silymarin (100 mg/kg in 1% CMC). Groups 4–12 were test groups that received the ethyl acetate fraction of *A. altilis*, *A. heterophyllus*, and *A. champeden* leaves orally, each at three

dose levels (125, 250, and 500 mg/kg). The treatment of animals was approval by the Health Ethics Committee, Palembang Ministry of Health Health Polytechnic No. 528/534/537/KEPK/Adm2/XII/2020.

5.3 Preparation of Ethylacetate Fraction

Artocarpus altilis, *A. champeden*, and *A. heterophyllus* leaves (2 kg) were washed with running water and then dried in an oven at 40°C. Up to 500 g of dried leaves were then grind into a coarse powder. The powder was macerated in 2 L of 96% ethanol for 48 h. The extraction process was completed by remaceration twice for 48 h. The filtrate was filtered through filter paper and concentrated using a rotary evaporator at 60°C. The viscous extract obtained was fractionated sequentially in a separatory funnel with n-hexane and ethyl acetate as solvents. Each fraction was concentrated using a rotavapor (Biobase®) to produce a thick extract.

5.3 Hepatoprotective Effect Test

This research method follows Ahmed Rakib [2]. The test groups were orally administered the ethyl acetate fraction of *A. altilis*, *A. heterophyllus*, and *A. champeden* leaves for 7 consecutive days. On the eighth day, all groups (except the normal control group) received an intraperitoneal injection of CCl_4 (1 mL/kg 10% solution in olive oil) one hour after the last daily treatment. On the 9th day, blood was taken from the eye retroorbitalis, and the animals were sacrificed by inhalation of petroleum ether. Blood was centrifuged at 15,000 rpm for 10 min, and the serum was analyzed for AST, ALT, and ALP levels. The sacrificed rats were then dissected from the pubis to the thorax. Liver tissue was examined macroscopically and microscopically [2].

5.4 Macroscopic and Microscopic Observations of the Liver

The liver was extracted and washed with 0.9% sodium chloride, then weighed and observed macroscopically for color, weight, and surface texture [23]. The liver index was calculated by dividing the mean liver weight by the average body weight of the rats. The liver organs were immersed in phosphate buffer solution and then fixed with formalin buffer for 18 h. The sample was dehydrated in an alcohol series from 70% to 100% concentration, followed by clearing using xylol, embedding in paraffin 56–58°C, and then dissection. The preparations were stained with hematoxylin eosin (HE) and observed under a microscope [15].

5.5 Statistical analysis

The data is presented as mean ± standard deviation of the mean (n=5). Data analysis was performed using SPSS 16.0 for the windows program. The normality of the data distribution was analyzed using Shapiro-Wilk. The data, which were normally distributed, were then tested with one-way ANOVA and continued with the Post hoc test to determine the differences between groups. The differences were considered significant at $p < 0.05$.

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