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# THE EFFECT OF KEREHAU LEAF EXTRACT (*CALLICARPA LONGIFOLIA* LAMK.) ON LIPID RATIOS AND AORTA HISTOPATHOLOGY OF MALE RATS OF WISTAR STRAIN

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**Keywords:**

Kerehau leaves, *Callicarpa longifolia* Lamk., Lipid ratios, Foam cells, Aorta, Histopathology

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**ABSTRACT:** Ethanol extract of kerehau leaves is known to have anti-inflammatory and antioxidant activities with the potential to prevent atherosclerosis. This study aims to determine the ability of kerehau ethanol extract in reducing the lipid levels, the lipid ratios, and the number of foam cells in the blood vessel walls. This study employed *in-vivo* and *ex-vivo* experiments on male rats of Wistar strain induced with high-fat feed and 25% fructose for 45 days. The rats were divided into 6 groups (n=3), namely negative control (0.5% CMC-Na), positive control (induction), simvastatin (0.9 mg/kg bw), and 3 groups of test extracts with a dose of 75, 150, and 300 mg/kg bw, respectively. Administration of CMC-Na, simvastatin, and kerehau ethanol extract was carried out orally along with the inducer. The parameters measured were the levels of triglycerides, TC, and serum HDL at before and after 45 days of treatment. Rat aortic arches were taken for histopathological examination at the end of the study. The results showed that kerehau ethanol extract at a dose of 75 mg/kg bw was able to reduce the TC levels (63.63 ± 11.23), increase the HDL levels (30.47 ± 2.78), reduce the lipid ratios {(Cardiac Risk Ratio) (2.09 ± 0.18) and Atherogenic Coefficient (1.09 ± 0.18)}, and reduce the number of foam cells (16 ± 1.41). It can be concluded that the kerehau ethanol extract at a dose of 75 mg/kgbw has the ability to lower the lipid levels (TC), the lipid ratios, and the number of foam cells.

**INTRODUCTION:** Atherosclerosis is a chronic arterial disease characterized by fats and other substances gradually built up, creating atheroma and plaque, around the artery walls. In the event of plaque rupture, local thrombosis occurs, leading to partial or total occlusion of the affected arteries. Atherosclerosis is a major cause of vascular disease worldwide. The main clinical manifestations of atherosclerosis include ischemic heart disease, ischemic stroke, and peripheral artery disease [1].

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Inflammation and oxidative stress have long been thought to be the key processes that encourage the initiation, development and rupture of atherosclerotic plaques. Oxidative modification of low-density lipoprotein (LDL) particles is hypothesized to be an important first step in the atherosclerotic process [2].

Statin therapy has been used as a foundation for the prevention of atherosclerosis and its complications for decades. However, although statin's ability to reduce LDL cholesterol has shown considerable success, cardiovascular disease remains the leading cause of death worldwide. Besides, statin therapy causes some side effects, ranging from mild myalgia, rhabdomyolysis, diabetes mellitus, to central nervous system complaints, preventing some patients to be unable to get optimal benefits

from the therapy. This finding suggests that other treatments aside from statins are still required [3].

Plant flavonoids with an anti-atherosclerotic activity have received substantial attention in research, and they have also shown to minimize the risk of atherosclerosis *in-vitro* and *in-vivo* in different animal models. Flavonoid compounds with their anti-inflammatory and anti-atherogenic properties have also decreased lipid levels. The development of flavonoid-based drugs is believed to have a significant effect on atherosclerosis and their related diseases [4].

One plant that contains flavonoids with anti-inflammatory and antioxidant activities is kerehau (*Callicarpa longifolia* Lamk.). A number of studies revealed that the ethanol extract of kerehau leaves acted as anti-inflammatory and wound healing [5] as well as a powerful antioxidant [6]. It is thus necessary to further investigate the activity of ethanol extract of kerehau leaves as anti-atherosclerosis. Therefore, this study was conducted to figure out the potential of ethanol extract of kerehau leaves as anti-atherosclerosis on Wistar rats induced with hyperlipidemia. This method was selected based on the fact that hyperlipidemia has been a widely recognized risk factor for atherosclerosis [3].

## MATERIALS AND METHODS:

**Plant Material Collection:** The plant parts used in this study were the leaves of kerehau (*Callicarpa longifolia* Lamk.) found in Muara Muntai of Kutai Kartanegara District in East Kalimantan, Indonesia. To ensure the correctness of the type of plant used, plant identification was carried out in the Laboratory of Plant Anatomy and Systematics, Faculty of Mathematics and Natural Sciences of Mulawarman University, Samarinda.

**Preparation of Kerehau Leaf Ethanol Extract:** As much as 3 kg of dried kerehau leaves (*Callicarpa longifolia* Lamk.) were extracted by the maceration method using 96% ethanol solvent (1:5) about three times, 24 h each. The liquid extract was then concentrated using a rotary evaporator at a temperature of 40-45 °C. The thick extract was suspended in 0.5% CMC-Na before use.

**Induction Making:** This study used induction of high-fat feed and 25% fructose drinking water in order to produce a hyperlipidemic rat model. The high-fat diet consists of a mixture of standard feed (pig pellets), butter (Orchid butter), and quail egg yolks, with a ratio of 80:15:5. The fructose used was High Fructose Syrup (HFS) 55%, which was diluted with distilled water to 25% concentration [7].

**Test Animals:** This study was conducted at the Veterinary Laboratory of Bandung School of Pharmacy, Bandung, Indonesia. The test animals used were 18 male rats of Wistar strain aged 2-3 months with weights of 150-200 g. The animals were adapted for 7 days, and they were given ad libitum access to food (standard feed) and water in a 12-hour light/12-hour dark cycle.

The procedure for the maintenance and use of the animals in this study was approved by the Research Ethics Commission of Padjajaran University in Bandung (Code of Ethics no. 72/UN6.KEP/EC/2018).

**Test Animal Treatment:** The test animals were divided into 6 groups randomly (n=3) as follows [7]:

**1) (-) Control:** Group with standard feeding (15 g/day of pig pellets) and 0.5% of CMC-Na.

**2) (+) Control:** Group with induction (15 g/day of high-fat feed and 25% fructose ad libitum) and 0.5% of CMC-Na.

**3) Simvastatin:** Group with induction and simvastatin suspension at 0.9 mg/kg bw.

**4) Kerehau Ethanol Extract 1:** Group with induction and suspension of ethanol extract of kerehau leaves at 75 mg/kg bw.

**5) Kerehau Ethanol Extract 2:** Group with induction and suspension of ethanol extract of kerehau leaves at 150 mg/kg bw.

**6) Kerehau Ethanol Extract 3:** Group with induction and suspension of ethanol extract of kerehau leaves at 300 mg/kg bw.

Induction was carried out for 45 days. On day 0 and day 46, we examined the levels of triglycerides (TG), total cholesterol (TC), and serum high-density lipoproteins (HDL) in the rats. Afterward, the aortic arches of two rats from each group were taken out.

The organs were stored in 10% Buffered Neutral Formalin (BNF) solution and made histological preparations with hematoxylin and eosin staining. The aortic histology preparations were observed and the number of foam cells in the tunica intima and tunica media per cross-section was calculated. The number of foam cells in all rats in each treatment group was summed and calculated on average. The levels of TG, TC, and HDL were used to calculate the lipid ratios, including Cardiac Risk Ratio, Atherogenic Index, and Atherogenic Coefficient with the following equations [8]:

Cardiac Risk Ratio = Total Cholestrol (mg/dL) / HDL (mg/dL)

Atherogenic Index = Log triglyceride (mg/dL) / HDL (mg/dL)

Antherogenic Coefficient = Total Cholesterol – HDL (mg/dL) / HDL (mg/dL)

**Data Analysis:** To test differences in lipid levels and lipid ratios, a one-way ANOVA test was administered in each treatment group. The results of the analysis are significant if $p < 0.05$ at a 95% confidence level.

## RESULTS AND DISCUSSION:

**Activity of Kerehau Leaf Ethanol Extract on Lipid Levels:** The activity of the ethanol extract of kerehau leaves on lipid levels was tested by measuring the lipid profiles of the rat serum samples before treatment (T0) and after 45 days of treatment (T46), which included TG, TC, and HDL levels **Table 1**.

**TABLE 1: AVERAGES OF LIPID LEVELS ON T0 AND T46 PERIODS**

| Group | Average level (mg/dL) ± SD | | | | | |
|---|---|---|---|---|---|---|
| | Triglyceride | | Total Cholesterol | | HDL | |
| (-) Control | 95.47±31.62 | 54.90±23.45* | 68.80±15.20 | 62.37±4.74* | 26.07±14.07 | 28.37±14.42* |
| (+) Control | 64.73±19.24 | 218.03±94.98 | 88.63±53.43 | 95.20±15.56# | 30.37±9.31 | 12.13±2.14# |
| Simvastatin | 86.53±7.19 | 145.73±36.25 | 82.30±21.65 | 62.10±2.00* | 27.20±2.78 | 36.53±13.93* |
| Ker. Eth. Extr.1 | 80.00±17.23 | 175.93±59.13 | 78.70±30.93 | 63.63±5.42* | 27.87±4.18 | 30.47±2.78* |
| Ker. Eth. Extr.2 | 56.43±11.49 | 176.07±58.31 | 94.23±70.22 | 63.17±11.23* | 31.53±17.81 | 30.07±1.00* |
| Ker. Eth. Extr.3 | 53.93±12.27 | 201.50±35.87 | 87.37±32.47 | 73.97±3.49* | 29.83±5.28 | 36.43±1.99* |

*Difference is significant when compared to the (+) control group ($p<0.05$)
#Difference is significant when compared to the Simvastatin group ($p<0.05$)

As seen in **Table 1**, the kerehau ethanol extract showed its activity against lipid profiles. The provision of fructose induction at 25% and high-fat diet aimed to obtain a hyperlipidemic rat model. The (+) control group, which was induced by 25% fructose and high-fat diet, increased the TG and TC levels while decreased the HDL levels. Based on the results of the LSD (Least Significant Different) test, there were significant differences ($p<0.05$) between the lipid levels in the (+) control and (-) control groups after the induction phase for 45 days. This finding indicates that the induction of 25% fructose and high-fat feeding could produce a hyperlipidemic rat model. Several studies have shown that the administration of fructose induction and high-fat diets could raise the levels of TG and TC while declining the levels of HDL.

A study conducted by Zhao *et al*., [9] using induction of 20% fructose drinking water for 28 days, found an increase in TG and TC levels and a significant decrease in HDL levels ($p<0.01$). In addition, Rahmawati *et al.*'s [7] study using induction of 27.5% fructose drinking water and high-fat diet (80% standard feed, 15% butter, and 5% quail egg yolk) revealed that the treatment could increase the lipid ratios (comparison between LDL and HDL levels) and foam cell number in the vascular walls of the rat aortic arch. These findings suggested that high-fructose drinking water can be used as induction of hyperlipidemia.

High-fat feed consisting of butter and quail egg yolks help enhance lipid levels. Butter contains saturated fatty acids, which can increase TC and LDL cholesterol [10], while quail egg yolks have a higher TC and LDL cholesterol compared to chicken egg yolks [11]. Cholesterol and egg yolk diets raise the risk of cardiovascular events; therefore, patients with the risk of vascular diseases should avoid such diets [12].

**Triglycerides (TG):** Hyper-triglycerides can be associated with two other lipid abnormalities, such as the formation of small dense LDL and HDL reduction [13]. The kerehau ethanol extract activity

on the average TG levels of the rats is shown in **Table 1**. The average TG levels in each group increased except for the (-) control group. The simvastatin group and kerehau ethanol extract groups at all three test doses did not show any significant differences ($p>0.05$) with the (+) control group. This shows that simvastatin and all three test doses of kerehau ethanol extract have not been able to reduce TG levels. In these test groups, however, a significant rise in the TG levels was associated with the fructose induction, as it showed a dominant impact.

Simvastatin is a drug that has been successfully tested for its activity in declining the levels of TC and LDL[14]; however, clinically, the TG levels often remain high in some CHD patients when cholesterol is highly controlled by statins [15]. This is because statins are HMG-CoA reductase inhibitors (enzymatic rate inhibitors that play a role in the synthesis of cholesterol in the liver), whose main benefit is to lower cholesterol rather than triglycerides [16].

**Total Cholesterol (TC):** The activity of ethanol extract of kerehau leaves on the average of TC of the rats are shown in **Table 1**. The average TC levels in each group decreased except for the (+) control group. The TC levels in three kerehau ethanol extract groups were lower than those in the (+) control group, and these differences were statistically significant ($p<0.05$). It can be interpreted that kerehau ethanol extract at the three test doses can reduce the levels of TC.

**High-Density Lipoprotein (HDL):** HDL plays an important role in re-transporting cholesterol from peripheral tissues to the liver, making it has an antiatherogenic property. In addition, HDL particles have antioxidant, anti-inflammatory, antithrombotic, and antiapoptotic properties that contribute to inhibiting atherosclerosis [17]. The kerehau ethanol extract activity on the average HDL levels of the rats is shown in **Table 1**.

The average HDL levels in the (+) control group declined and showed significant differences ($p<0.05$) when compared to the other groups. The average HDL levels in the simvastatin, kerehau ethanol extract 1, and kerehau ethanol extract 3 groups revealed an increase, while the kerehau ethanol extract 2 group showed a small decrease in HDL levels.

**TABLE 2: AVERAGES OF LIPID RATIOS ON T0 AND T46 PERIODS**

| Group | Average value ± SD | | | | | |
|---|---|---|---|---|---|---|
| | Cardiac risk ratio | | Atherogenic index | | Atherogenic Coefficient | |
| | T0 | T46 | T0 | T46 | T0 | T46 |
| (-) Control | 3.01±1.25 | 2.52±0.97* | 0.09±0.03 | 0.07±0.02* | 2.01±1.25 | 1.52±0.97* |
| (+) Control | 3.58±3.39 | 8.15±2.62# | 0.06±0.03 | 0.20±0.04* | 2.58±3.39 | 7.15±2.62# |
| Simvastatin | 3.10±1.05 | 1.87±0.69* | 0.07±0.01 | 0.07±0.03* | 2.10±1.05 | 0.87±0.69* |
| Ker. Eth. Extr.1 | 2.87±1.12 | 2.09±0.18* | 0.07±0.01 | 0.07±0.01* | 1.87±1.12 | 1.09±0.18* |
| Ker. Eth. Extr.2 | 2.95±1.14 | 2.09±0.30* | 0.07±0.04 | 0.07±0.01* | 1.95±1.14 | 1.09±0.30* |
| Ker. Eth. Extr.3 | 2.97±1.04 | 2.04±0.17* | 0.06±0.01 | 0.06±0.00* | 1.97±1.04 | 1.04±0.17* |

*Difference is significant when compared to the (+) control group ($p<0.05$)
#Difference is significant when compared to the Simvastatin group ($p<0.05$)

**Activity of Kerehau Leaf Ethanol Extract on Lipid Ratios:** Lipid ratios are the combination of lipid parameters that reflect the proportion of atherogenic lipoproteins (lipoproteins that raise cardiovascular risk) and antiatherogenic lipoproteins (lipoproteins that reduce cardiovascular risk). Some lipid ratios proposed as the indicators of cardiovascular risk include cardiac risk ratio (CRR), atherogenic index (AI), and atherogenic coefficient (AC) [8]. Compared to a single lipid parameter, the lipid ratios are considered a better predictor of cardiovascular disease [18]. The results of testing the activity of ethanol extract of kerehau leaves on the lipid ratios can be seen in **Table 2**.

**Cardiac Risk Ratio (CRR):** CRR is the ratio between TC and HDL (TC/HDL). CRR values of ≥3.5 are associated with a risk of cardiovascular disease [19]. **Table 2** depicts that the average CRR values in the simvastatin, kerehau ethanol extract 1, kerehau ethanol extract 2, and kerehau ethanol extract 3 groups were 1.87, 2.09, 2.09, and 2.04, respectively, indicating that there was no risk of

cardiovascular disease, whereas the (+) control group showed a risk for cardiovascular disease with an average CRR value of 8.15.

**Atherogenic index (AI):** AI is calculated as the ratio between the logarithm results of TG and HDL {(Log TG)/HDL} levels. AI is a useful parameter for the diagnosis and prognosis of cardiovascular disease with a high predictive value [20]. AI has been used by some practitioners as a significant predictor of atherosclerosis, and it has been suggested that AI values of -0.3 to 0.1 are associated with low cardiovascular risk, 0.1 to 0.24 with moderate cardiovascular risk, and above 0.24 with high cardiovascular risk [21]. The results of the study showed that the AI averages in the simvastatin, kerehau ethanol extract 1, and kerehau ethanol extract 2 groups were all 0.07, while only the kerehau ethanol extract 3 group was 0.06. These results were classified as low cardiovascular risk, whereas the (+) control group had a moderate cardiovascular risk with an average AI value of 0.20.

**Atherogenic Coefficient (AC):** AC is the ratio between non-HDL and HDL {(TC-HDL)/HDL cholesterol. The recommended AC value is <3.0[22], and in this study, the average AC values in the simvastatin, kerehau ethanol extract 1, kerehau ethanol extract 2, and kerehau ethanol extract 3 groups were 0.87, 1.09, 1.09, and 1.04, respectively, indicating that there was no risk of cardiovascular disease, whereas the (+) control group had a risk of cardiovascular disease with an average AC value of 7.15.

**Activity of Kerehau Leaf Ethanol Extract on Aortic Histopathology:** Testing the activity of ethanol extract of kerehau leaves on aortic histopathology was conducted by examining and calculating the number of foam cells in the aortic tunica intima and tunica media using a microscope at 400x magnification (ocular 10x, objective 40x). The test results can be seen in **Table 3**. This study found that the induction of 25% fructose and high-fat feed, comprising a combination of standard feed, butter, and quail egg yolks with a ratio of 80:15:5, were able to enhance the number of foam cells in the rat aortic tunica intima and tunica media. This result was in line with previous studies that reported that the administration of fructose and high-fat diets with similar composition could grow the number of foam cells in the tunica intima and tunica media of the rat aorta [7]. Microscopic images in each group can be seen in **Fig. 1**.

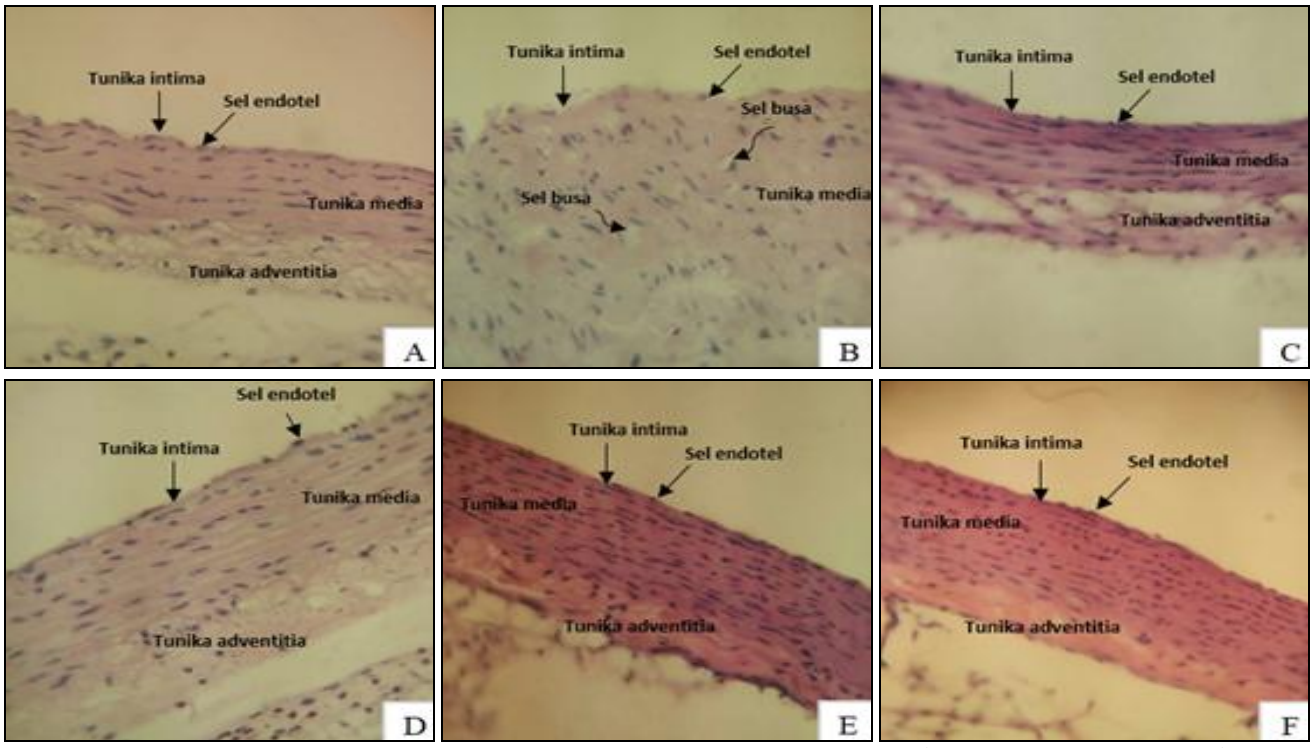


**FIG. 1: MICROSCOPIC IMAGES OF CROSS-SECTIONS OF AORTA (400X MAGNIFICATION)**
A = (-) Control; B = (+) Control; C = Simvastatin; D = Ker. Eth. Extr. 1; E = Ker. Eth. Extr. 2; F = Ker. Eth. Extr. 3

As shown in **Fig. 1**, the microscopic images of the cross-sectional areas of the rat aorta in the (-) control group illustrate normal endothelial cells with parallel positions on the tunica intima, smooth muscle cells horizontally oriented on the tunica media, and normal collagen and connective tissue appearance at the tunica adventitia. On the other hand, those of the (+) control group outline disorientation of smooth muscle cells and foam cell appearance in the tunica media. The foam cell formation was caused by the macrophages that penetrated the endothelial layer and then accumulated in the tunica intima and tunica media of the arteries.

Under normal conditions, endothelial cells function to maintain vascular homeostasis by preserving a balanced release between vasoconstriction and vasodilation factors as well as prothrombotic and antithrombotic substances that inhibit monocyte adhesion to the endothelial layer which prevents inflammation [22].

**TABLE 3: RESULTS OF FOAM CELLS CALCULATION**

| Group | Average number of foam cells (n=2) |
|---|---|
| (-) Control | 8.5±2.12 |
| (+) Control | 66.5±4.95 |
| Simvastatin | 15.0±0.00 |
| Ker. Eth. Extr.1 | 16.0±1.41 |
| Ker. Eth. Extr.2 | 39.0±15.56 |
| Ker. Eth. Extr.3 | 22.0±2.83 |

In conditions such as hyperlipidemia, hypertension, smoking, aging, and diabetes, endothelial lining can experience dysfunction in which there is an increase in permeability in the endothelial layer leading LDL and other circulating cells such as monocytes/macrophages and T-lymphocytes to enter the endothelial layer. The accumulated LDL can then undergo an oxidation process that produces an oxidized form of LDL. Oxidized LDL shows various damaging effects on vascular cells, including a rise in inflammatory cytokines, chemotactic factors, fibrinolytic regulators, and procoagulant activity. Oxidized LDL also directly elevates oxidative stress in blood vessels. The oxidative stress accelerates the process of atherosclerosis and thrombosis in blood vessels [23].

The microscopic features of the cross-sections of the rat aorta in the kerehau ethanol extract groups at all three test doses provide similar images as in the (-) control group. This suggests that kerehau ethanol extract helps improve aortic histopathological features when compared to the (+) control group. The group administered with kerehau ethanol extract at a dose of 75 mg/kg bw yielded the number of foam cells closest to the group of simvastatin at 0.9 mg/kg bw.

Research on the isolation of flavonoid compounds in kerehau leaves carried out by Pasaribu *et al.* [24]

**CONCLUSION:** The ethanol extract of kerehau leaves (*Callicarpa longifolia* Lamk.) has shown its potentials in improving atherosclerosis by reducing the lipid ratios and improving the histopathological features of aorta in hyperlipidemia induced rats.

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