Combined *Carica papaya* and *Alstonia scholaris* Leaf Water Extracts Prevent Hypercholesterolemia and Lipid Peroxidation in Cholesterol Fed Rats

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Abstract. Papaya (*Carica papaya*) and pulai (*Alstonia scholaris*) are recognized for their antihypercholesterolemic and antioxidant effects with minimal side effects. An equal combination of their leaf extracts demonstrated a synergistic effect as an HMG-CoA inhibitor, comparable to that of lovastatin *in vitro*. This study evaluated cholesterol levels and lipid peroxidation inhibition in a hypercholesterolemic animal model induced by a high-fat, high-cholesterol diet and propylthiouracil treatment. Sprague- Dawley rats were divided into three groups: Normal, Hypercholesterolemia, and Extract groups, which were subjected to treatment protocols over six weeks. The combination extracts (200:200 mg/kg BW) were administered and blood samples were analyzed for cholesterol and malondialdehyde levels. The results showed that hypercholesterolemia induction increased blood cholesterol to 173.72±5.6 mg/dL and MDA level to 19,44±4,59 nmol/mL. The extract treatment effectively decreased blood cholesterol levels by 27% and prevented lipid peroxidation, as shown by a 52% reduction in MDA levels ($p<0.05$).

1 Introduction

In Indonesia, the mortality rate of coronary heart disease (CHD) is as high as 13.1%, and the disability rate due to stroke is 20.2% [1]. Atherosclerosis plays a significant role in the pathogenesis of both CHD and stroke, and hypercholesterolemia has been identified as a major risk factor [2]. In addition to hypercholesterolemia, free radicals play a role in the development of cardiovascular diseases. An imbalance between radical oxygen species (ROS) and antioxidants in the body, often called oxidative stress, can increase atherogenic progression through many mechanisms that correlate with ROS [3]. The body has a natural antioxidant system that reduces excess ROS. Nevertheless, this system is incomplete without exogenous antioxidants such as polyphenols, which play an important role in the antioxidant mechanisms of living organisms to prevent oxidative stress [3].

Statins, which are effective in lowering blood cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), are associated with an increased risk of side effects during long-term use. It has long been believed that natural ingredients have minimal side effects. Previous research has shown that papaya (*Carica papaya*) has antihypercholesterolemic and antioxidant activities. Animal studies have shown that papaya leaf ethanol extract inhibits HMGCR in the liver of Wistar rats and significantly reduces serum total cholesterol [2]. Nirmala *et al.* (2023) showed that the same extract reduced blood malondialdehyde (MDA) levels in rats subjected to high-intensity work [4]. Another plant, pulai (*Alstonia scholaris*), also exhibits anti-hypercholesterolemic and antioxidant activities. The indole alkaloid fraction reduced LDL-c and total triglyceride levels in rats fed a high-fat diet [5]. Meanwhile, methanol extract of pulai leaves exhibits

strong antioxidant activity *in vitro* [6].

Research shows that the combination of papaya leaf water extract (*Carica papaya* L.) and pulai leaf (*Alstonia scholaris* (L.) R.Br.) 1:1 produces a synergistic effect on HMGCR inhibition, comparable to that of the commercial drug lovastatin *in vitro* [7]. However, the effects of this combination on blood cholesterol and antioxidant levels in animal models have not been reported. This study aimed to measure the effect of administering a 1:1 combination of extracts on serum cholesterol levels and lipid peroxidation inhibition activity in hypercholesterolemic model rats induced by PTU and a high-fat high-cholesterol diet.

2 Materials and method

2.1 Materials

The experimental animals used were male Sprague-Dawley rats aged 8–10 weeks, with a total of 25 rats. The average initial weight of the rats was 153.45±8.22 g. Fresh papaya (*Carica papaya* L.) leaves and pulai (*Alstonia scholaris* (L.) R.Br.) leaves were obtained from Dramaga Research Forest, Forest Research and Development Center, Bogor, Indonesia. The materials used for extraction were distilled water and filter paper with pore sizes of 20–25 μm. The materials used for hypercholesterolemia induction were Rat Bio standard rat feed, vegetable oil, chicken eggs, lamb fat, and propylthiouracil (PTU). The reagents used for analysis were CHOD-PAP enzyme KIT (Dumo Lab, Austria), 1,1,3,3-tetramethoxypropane (TMP), trichloroacetic acid (TCA), and thiobarbituric acid (TBA).

2.2 Sample preparation and extraction

Both fresh leaf samples were cleaned, cut into small pieces, dried in an oven at 50 °C, ground, and sieved through a 50-mesh powder. The water content of the obtained powder was measured using the oven-drying method. Extraction was performed separately for each sample using the decoction method described by Sulistiyani *et al.* (2024) [7]. The decoction method was performed by heating the powder in distilled water at a ratio of 1:20 w/v in boiling water until the volume of the solution was reduced by half. The filtrate was filtered, concentrated, and powdered using a freeze dryer, and the yield of each extract was calculated using the following equation:

$$\%\text{yield} = (\text{extract weight (g)}/\text{powder weight (g)}) \times 100\% \quad (1)$$

2.3 Experimental animal setup

Rats were divided into three groups: normal control (n=5), hypercholesterolemic control (n=15), and extract-treated control (n=5). The rats were adapted for one week and given standard feed and water *ad libitum*. Every day, the rats were fed 20 g/day and treated, as shown in Table 1. The doses used were for the combination extract 200:200 mg/kg bb and PTU 0.5 mg/kg bb given orally [2,8]. The body weight and feed consumption of the rats were measured daily. Hypercholesterolemia induction and extract treatment had no significant effect on body weight, with an average final weight of 238.79±46.45 g. Blood was collected from the rats via the lateral tail vein at weeks 0, 2, and 4 and intracardially at week 6 to test cholesterol and MDA levels. Termination was performed under ketamine:xylazine (95:5 mg/kg) anesthesia using exsanguination via

cardiac puncture after six weeks of treatment. There was non-experimental death in one normal control rat. The study received ethical approval (number: 265/KEH/SKE/X/2024) from the Unit Pengelola Hewan Laboratorium IPB (UPHL) and was conducted in accordance with ethical guidelines for handling laboratory animals.

Table 1. Experimental design of the animal study.

Groups	Adaptation (1 week)	Treatment (6 weeks)
Normal (n=5)	S	S + Aq
Hypercholesterol (n=15)	S	C + PTU
Extract (n=5)	S	C + PTU + E

Aq = water; C = cholesterol feed; E = combined extract; PTU = propyl-thiouracil; S = standard feed.

2.4 High-cholesterol diet preparation

A high-lipid diet was prepared using chicken eggs obtained from a local commercial egg provider. Eggs were separated into yolk and white. The yolk was steamed, mashed using a blender, dried in an oven at 80 °C, and the cholesterol content was analyzed using the Lieberman-Burchard method, as described by Waliyuddin *et al.* (2013) [8]. The cholesterol content of dried egg yolk was 11.59 mg/g. Cholesterol feed was prepared by mixing dried egg yolk to obtain a final cholesterol concentration of 3%, which was then mixed with 6% vegetable oil, 5% goat fat, and standard feed of up to 100% (w/w). The feed was mixed, molded into pellets, and stored in a refrigerator at -4 °C until use [21].

2.5 Serum preparation and animal necropsy

Blood sampling was performed at weeks 0, 2, and 4 via the lateral tail vein, and at week 6 via intracardial necropsy. Rats were fasted for 14–16 h before blood sampling. For intravenous tail blood sampling, the rats were placed in a restrainer, and the tail was dipped in warm water at 37 °C for 1 min. Blood samples were collected from the tail vein using a syringe. Blood was then prepared into serum by centrifugation at 2000 × g for 15 min. Serum was used for blood cholesterol and MDA tests.

Necropsy was performed at the end of treatment, end of week 6th. Rats were fasted for 14–16 h before necropsy. Rats were anesthetized intraperitoneally with ketamine and xylazine (95:5 mg/kg body weight). Termination was performed by collecting as much blood as possible intracardially (exsanguination via cardiac puncture). Blood samples were collected, and serum was prepared to measure cholesterol and MDA levels. The cadavers were then cremated at the crematorium of the Veterinary and Biomedical School (SKHB) of IPB.

2.6 Serum cholesterol analysis

Serum cholesterol levels were determined as described by Waliyuddin *et al.* (2013) using a cholesterol oxidase-peroxidase aminoantipyrine or CHOD-PAP kit (Dumo Lab, Austria), according to the accompanying procedure [8]. A standard cholesterol curve was prepared in the range 40–240 mg/dL. The highest concentration standard was measured for absorbance over the visible wavelength range to determine the maximum wavelength (λmax). A 10 µL sample was reacted with 1 mL of reagent and incubated at 37 °C for 10 min. The pink complex formed was read for absorbance at λmax —498 nm.

2.7 Serum MDA analysis

Serum MDA levels were measured using the TBA method as described by D'souza *et al.* (2012) [9]. A TMP standard curve was constructed at concentrations ranging from 1–25 ppm. A total of 100 µL of the test solution was reacted with 500 µL TBA and 500 µL TCA. The mixture was heated at 95 °C for 15 min and then centrifuged at ~2000 g for 15 min. The absorbance of the supernatant was measured at λmax of 532 nm. The λmax was obtained using a method similar to that used for cholesterol analysis.

2.8 Data analysis

A Completely Randomized Design (CRD) was used in this study. The statistical analysis used was one-way analysis of variance (ANOVA), followed by Tukey's B test at a confidence level of 95% and $\alpha = 0.05$.

3 Results and discussion

3.1 Leaf powder water content and extract yield

The water content of papaya leaf and pulai leaf powder was 8.61% and 8.63% (w/w), respectively. Water content below 10% is considered favorable for maintaining the stability of active compounds by preventing microbial contamination and undesirable chemical reactions driven by enzymatic activity [10]. After extraction and drying, the papaya leaf water extract yield was 5.26% (w/w). The result was comparable to previous study reported by Hasimun *et al.* (2018) as the yield was 6.24% [2]. Meanwhile, the pulai leaf water extract yield was 12.16% (w/w). The obtained yield was higher than that reported in a previous study by Pratiwi *et al.* (2023), where the methanol extract of pulai leaf yielded 5.7% [6], suggesting an optimum result.

3.2 Serum cholesterol level

The cholesterol level of normal, hypercholesterolemic, and extract-treated control were shown in Figure 1. The Hypercholesterolemia group was administered induction treatment to create a hypercholesterolemia animal model. A high-fat, high-cholesterol diet combined with PTU successfully induced hypercholesterolemia, evidenced by a significant, more than two-fold increase in blood cholesterol levels (173.72 ± 5.6 mg/dL) compared to the normal control group (α=0.05) as shown is Figure 1. These findings align with those of a previous study that reported hypercholesterolemic rats with blood cholesterol levels of 156 mg/dL [2].

The cholesterol-lowering effect of the extract combination was quantified using the area under the curve of the average cholesterol levels throughout the six-week treatment period. Administration of the extract significantly decreased blood cholesterol by 27% compared with that in the hypercholesterolemia group (α=0.05). Previous research has shown that papaya leaf has a cholesterol-lowering effect. Papaya leaf ethanol extract was able to reduce cholesterol 63% (57.8 mg/dL), equivalent to simvastatin of 66% (53.6 mg/dL), with induction using high fructose feed [2]. Pulai is also known to have cholesterol-lowering effects. Bandawane *et al.* (2011) reported that pulai leaf water extract significantly reduced liver cholesterol, serum LDL-c, and VLDL-c levels in alloxan-induced diabetic rats [11].

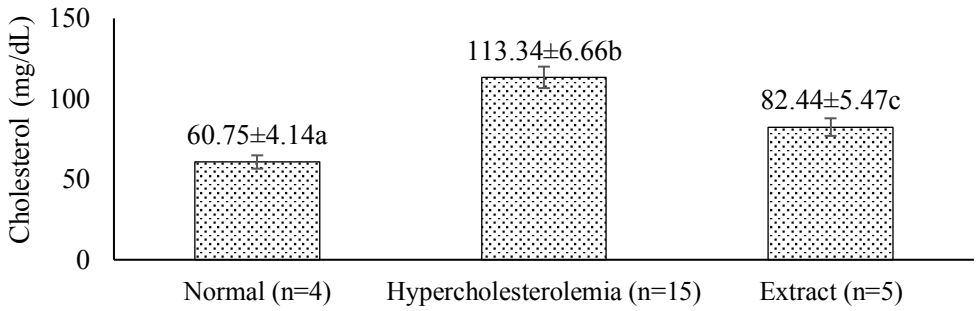

Fig. 1. Average area under the curve of the cholesterol level during the 6-week treatment period. Values sharing the same letter are not significantly different according to Tukey's B test (α = 0.05).

This effect may be attributed to several mechanisms. Papaya leaf extract inhibits HMGCR to lower cholesterol synthesis, similar to statin drugs. Papaya leaf water extract inhibits HMGCR by 27.98% *in vitro* [7]. The indole alkaloid fraction reduced LDL cholesterol by 25% and total triglycerides by 39% *in vivo* in high-fat diet rats [5]. Papaya leaf water extract also contains six HMGCR inhibitor compounds *in silico* [12]. In addition, papaya leaves contain phytosterols that structurally resemble cholesterol, displacing it from bile salt micelles in the intestines and reducing absorption [13].

3.3 Serum MDA level

Hypercholesterolemia induction significantly increased serum MDA levels by more than two-fold compared to the normal control, reaching 14.44 nmol/mL (α=0.05) as shown in Figure 2. Hypercholesterolemia induces oxidative stress through several mechanisms. In hypercholesterolemia, LDL-c enhances NADPH oxidase activity, a significant source of superoxide radicals in vascular cells. Excess cholesterol and radicals also disrupt mitochondrial function, generating ROS and activating pro-inflammatory pathways. The hypercholesterolemia-induced oxidative stress characterized by increased inflammatory mediators, decreased endogenous antioxidant enzyme activity, and increased oxidative stress biomarkers. High-cholesterol feeding increased TNF-α by more than two-fold and MDA and significantly decreased GSH levels in the hearts of rats with hypercholesterolemia induced by a 1% high-cholesterol diet [14].

Administration of a combination of extracts significantly reduced blood MDA by 52% compared to hypercholesterolemic control as shown in Figure 2. The blood MDA levels in the extract-treated group were still significantly higher than those in the normal

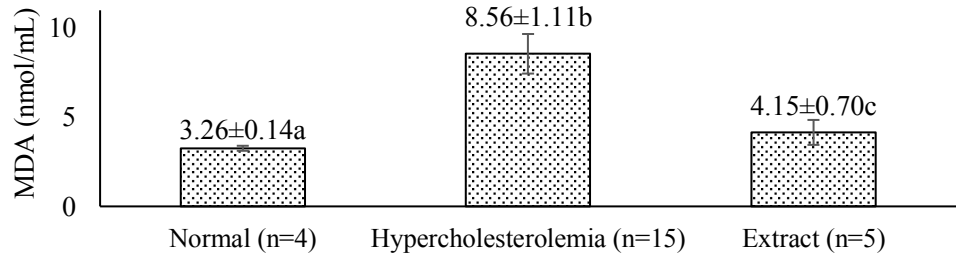

Fig. 2. Average area under the curve of the MDA level during the 6-week treatment period. Values sharing the same letter are not significantly different according to Tukey's B test (α = 0.05).

control group, at 4.15±0.7 and 3.26±0.14 nmol/dL (α=0.05). These results indicate that the combined administration of the extracts exerted an antioxidant effect by inhibiting the lipid peroxidation. Previous research has shown that papaya leaf water extract contains phenolic compounds (p-coumaric acid), flavonoids (3-hydroxyflavone, kaempferol), and vitamins (ascorbic acid, alpha-tocopherol) [12]. Similarly, pulai leaf water extract also contains phenolics (coumarin, isatin bis-cresol, 2-tert-butyl-4-methoxyphenol) and flavonoids (luteolin, cianidanol, quercetin, procyanidin B2, kaempferol-3-glucoside-2"-p-coumaroyl) [12]. Both phenolic compounds and flavonoids act as antioxidants by directly scavenging free radicals. In addition, some phenolics exhibit antioxidant activity through metal chelation. Papaya leaf ethanol extract has demonstrated significant antioxidant activity with an ABTS IC_{50} value of 45.5 µg/mL *in vitro* [15]. This same extract has also been shown to reduces blood MDA level and increases blood SOD level in rats subjected to high-intensity work [13]. Furthermore, administration of the indole alkaloid fraction of pulai leaf to hyperlipidemic rats helps regulate fatty acid oxidation [5].

4 Conclusion

The combined water extract of papaya and pulai leaves demonstrated significant antihypercholesterolemic and antioxidant activities in the experimental model. Administration of the combined extract effectively prevented the elevation of serum cholesterol levels, indicating its potential to modulate lipid metabolism in rats. Additionally, the treatment significantly inhibited the increase in MDA concentration, suggesting a protective effect against lipid peroxidation and oxidative stress.

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