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Combination of Pulai Leaf (*Alstonia scholaris* (L.) R. Br.) and Papaya Leaf (*Carica papaya* L.) Water Extract as HMG-CoA Reductase Inhibitor

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ABSTRACT

Heart disease has become the leading cause of death in the world in the last 15 years. This disease has several primary risk factors, one of which is hypercholesterolemia. Cholesterol biosynthesis in the liver is influenced by the activity of the rate-limiting enzyme, HMG-CoA reductase. Traditional herbal medicine provides alternative treatment for hypercholesterolemia. Both pulai and papaya leaves were reported as HMG-CoA reductase inhibitors. There has been very limited study however, on the efficacy of a mixture of aqueous extracts of the two in inhibiting HMG-CoA reductase. This study aims to determine the activity of a mixture of water extracts of both pulai and papaya leaves. The activity of the enzyme HMG-CoA reductase was measured by Abcam® kit No. Ab204701 by spectrophotometric method at 334 nm. The results showed that the three extracts of the combination of pulai and papaya leaves had four times better inhibitory action than the single extract (p0.05). The results of the phytochemical literature study showed that the aqueous extract contained flavonoids, alkaloids, tannins, and saponins

Keywords: combination, HMG-KoA reductase, inhibitors, papaya leaves, pulai leaves

1. INTRODUCTION

Data from the World Health Organization (WHO) (2018) states that out of 56.9 million deaths worldwide in 2016, more than half (54%) were caused by the top ten leading causes, with heart disease ranking first. Heart disease was the leading cause of death globally, resulting in 15.2 million deaths in 2016. Heart disease has remained one of the leading causes of death over the past 15 years. Meanwhile, findings from the Basic Health Research (Riskesdas) by the Ministry of Health of the Republic of Indonesia (2018) reported that the prevalence of degenerative diseases such as stroke has increased over the past 5 years from 7.0% to 10.9%. One of the primary risk factors for the increased incidence of deaths due to heart disease is hypercholesterolemia. Hypercholesterolemia, or high cholesterol levels, can accumulate in the blood vessel walls and become a risk factor for coronary artery narrowing and cerebral blood vessels leading to heart disease and stroke (Ministry of Health of RI 2014).

Several synthetic drugs from the statin group (fluvastatin, lovastatin, pravastatin, simvastatin, etc.) can lower cholesterol levels. Statins can inhibit the activity of the enzyme

HMG-CoA reductase (a key enzyme in cholesterol biosynthesis) competitively (Burg and Espenshade 2011). However, long-term use of these drugs can cause side effects. Side effects include liver dysfunction indicated by increased levels of SGPT and SGOT or increased blood pressure (Marinetti 1990). Therefore, there is a need for the development of drugs with similar efficacy but without side effects. This alternative can be achieved by utilizing bioactive compounds found in herbal plants.

One herbal plant that can be used is the Pulai tree (*Alstonia scholaris* (L.) R. Br.). The Pulai tree has been widely used as a traditional remedy for various diseases (Dalimartha 1999). Parts of the Pulai tree commonly used as traditional medicine are the bark and leaves. The bark of the Pulai tree is effective in treating fever, malaria, dyspepsia, and productive coughs. Meanwhile, the leaves of the Pulai tree are effective in treating swelling, boils, ulcers, diabetes, asthma, and rheumatism (Heyne 1987; Wiart 2002; Pratyush et al. 2011). Its medicinal properties are influenced by the secondary metabolites it contains. Pulai bark extract is known to contain flavonoids, alkaloids, saponins, tannins, and terpenoids (Saxena et al. 2013). Meanwhile, Pulai leaf extract contains secondary metabolite compounds such as flavonoids, alkaloids, tannins, saponins, phenolics, terpenoids, and steroids (Swastiratu 2015).

The potential of Pulai as an anti-hypercholesterolemia agent has been extensively studied. According to Usman (2000), Pulai has the potential as an anti-hypercholesterolemia agent. In his research, Usman (2000) reported that water fraction and chloroform fraction of Pulai bark could reduce total cholesterol levels by 61.57% and 37.02% in hypercholesterolemic rat groups. Another study was conducted by Sulistiyani et al. (2017), reporting that the flavonoid fraction of Pulai leaves at a concentration of 20 ppm had an activity of 73.79%, while the water-soluble alkaloid fraction (polar) of Pulai leaves at the same concentration (20 ppm) showed an activity of 50%. Zuraida (2018) also reported that the ethanol fraction of 9 μg/mL of Pulai bark was an effective concentration capable of demonstrating inhibitory mechanisms against LDL oxidation in macrophages, with its ability equivalent to estrogen as a positive control.

The efficacy of herbal plants can be enhanced through mixing or combination. This can be done to determine the synergistic or antagonistic effects of herbal plant mixtures. Another plant that has been reported to have similar efficacy is papaya leaves. According to Hasimun et al. (2018), ethanol extract of papaya leaves containing alkaloids, flavonoids, quinones, tannins, steroids, and triterpenoids at a dose of 200 mg/kg body weight in vivo can inhibit HMG-CoA reductase enzyme with an inhibition power of 40.6%. Research conducted by Duniya *et al.* (2018) also reported that ethanol extract of papaya leaves can reduce cholesterol, LDL, triglycerides, and increase HDL in Wistar rats induced with hyperlipidemia. Another study was also conducted by Zetina-Esquivel *et al.* (2015), reporting that chloroform extract of papaya leaves can reduce cholesterol, LDL, triglycerides, and increase HDL in rats given a high cholesterol diet. Furthermore, Kolimon (2017) in his research mentioned that the combination of cinnamon bark ethanol extract and papaya leaf water extract with doses of 0.19 grams/kgBW:1.875 grams/kgBW showed a decrease in triglyceride levels in the serum of Wistar strain white rats by 70.2 mg/dL.

2. METHODOLOGY

Materials

The main ingredients used are Pulai leaves (*Alstonia scholaris* (L.) R. Br.) obtained from the Dramaga Research Forest, Center for Forest Research and Development (Puslitbang

Hutan), Bogor, and papaya leaves (*Carica papaya* L.) obtained from a papaya plantation in Sibanteng Village, Leuwisadeng, Bogor.

The samples used in this study were harvested during the rainy season (January 2020). The materials used for extracting Pulai leaves and papaya leaves are distilled water. The materials used for the HMG-CoA reductase inhibition activity test are HMG-CoA reductase assay kit Abcam® ab20470 (Shanghai, China), 5% dimethyl sulfoxide (DMSO), and sterile distilled water (ddH_2O).

Sample Preparation and Extraction (Modified from Aprilia 2019)

Pulai leaves samples (*Alstonia scholaris* (L.) R. Br.) were obtained from the Dramaga Research Forest, Center for Forest Research and Development (Puslitbang Hutan), Bogor, while papaya leaves samples (*Carica papaya* L.) were obtained from a papaya plantation in Setu Village, Jasinga, Bogor. Both samples were first identified at the Bogor Botanical Herbarium, Indonesian Institute of Sciences (LIPI), Cibinong. The preparation of 20 mesh simplicia and moisture content measurement were performed by a service at the Research Institute for Spice and Medicinal Plants (Balittro). Leaf drying was carried out using the oven blower method, and moisture content was measured using the toluene distillation method.

Extraction was carried out by maceration method at 100°C with a ratio of 1:20 (w/v). The simplicia was placed into an Erlenmeyer flask containing water solvent at 100°C until it reached half of the initial solvent volume, which is 250 mL, and the required time was 30 minutes. Then, the solvent extraction was evaporated using a freeze dryer until the solvent completely evaporated.

Phytochemical Meta-Aggregation (Siswanto 2010)

Phytochemical analysis, both qualitative and quantitative, was conducted by summarizing and reviewing several relevant research data through a portal. The criteria for selecting phytochemical data were based on sample similarity (Pulai and papaya leaf simplicia) and solvent (water). Ten literature sources were found using the keywords qualitative phytochemical analysis on Pulai and papaya leaves with five types of solvents: water, methanol, ethanol, butanol, and ethyl acetate. From these ten sources, six literature sources that used water as a solvent were selected. Additionally, five literature sources for quantitative phytochemical analysis of Pulai and papaya leaves with water, ethanol, and methanol solvents were also obtained. These eleven pieces of data were obtained by searching journals on portals such as garuda.ristekbrin.go.id, repository.ipb.ac.id, and googlescholar.com (Table 1 and Table 2).

Table 1 The source of phytochemical analysis data for papaya leaf extract

Researcher	Sample Collection Location	Solvent
Kolimon (2017)	Pengging Village, Boyolali, Cental Java	Water
Maniyar and Bhixavatimah (2012)	Nijalingappa Medical College, Bagalkot, Karnataka, India	Water
Kining (2015)	IPB University	Water
Nugrahani *et al*. (2020)	Lekong Village, Sukamulia, East Lombok	Water, Ethanol 96 %
Vuong *et al*. (2013)	Central Coast, New South Wales, Australia	Water, Methanol, Ethanol

Table 2 The source of phytochemical analysis data for pulai leaf extract

Researcher	Sample Collection Location	Solvent
Antony *et al*. (2011)	Universitas VIT, Vellore, India	Water
Misra *et al*. (2011)	Universitas VIT, Vellore Tamilnadu, India	Water

Dhruti *et al*. (2016)	India	Water
Purnama (2015)	Biopharmaca, Bogor	Ethanol 96 %

HMG-CoA Reductase Activity Inhibition Test (Abcam 2019)

The testing of HMG-CoA reductase inhibition activity refers to the HMG-CoA reductase assay kit from Abcam® ab204701 (Shanghai, China). This kit contains buffer solution, NADPH as a coenzyme, HMG-CoA substrate, HMG-CoA reductase enzyme (0.7 mg/mL), and atorvastatin inhibitor at 10 mM ≈ 5586 µg/mL concentration. Measurements consist of blanks, negative controls, positive controls (inhibitor standards), and treatment groups. The positive control (inhibitor standards) consists of the atorvastatin kit, lovastatin, and quercetin (flavonoid standard). The treatment groups consist of a mixture of water extracts from Pulai leaves and papaya leaves in ratios of 1:0, 3:1, 2:1, 1:1, and 0:1. Each measurement group is performed in duplicate (n=2).

Sample and Reagent Preparation. Mixed samples of water extracts from Pulai leaves and papaya leaves were prepared as a stock solution with a concentration of 10000 µg/mL for the combinations of 1:0, 3:1, 2:1, 1:1, and 0:1, then diluted to a final concentration of 50 µg/mL for each treatment group. Lovastatin and quercetin stocks were prepared at a concentration of 5000 µg/mL, then diluted to final concentrations of 50 µg/mL and 5 µg/mL. Samples were dissolved using 5% DMSO. HMG-CoA reductase, HMG-CoA, and NADPH were dissolved in ddH_2O with volumes of 550 µL, 1300 µL, and 440 µL, respectively. All samples were kept in a cool box with ice during the process.

Determination of Maximum Wavelength. The maximum wavelength was measured by recording the absorbance of NADPH substrate without enzyme within the range of 320-360 nm at one nm intervals.

Enzyme Activity Measurement. Enzyme kit components were thawed in a desiccator, except for the enzyme, which was kept in an ice bath to maintain a temperature of ≤ 4°C. Measurements were conducted using a microplate at a final concentration of 50 µg/mL in a final reaction volume of 200 µL. A mixed reaction consisting of 12 µL HMG-CoA, 4 µL NADPH, and 174 µL buffer was prepared beforehand. Samples and reagents were added to the microplate according to the volumes specified in Appendix 2. The reaction results were read for absorbance every 2 minutes for 10 minutes at a wavelength of 334 nm and a temperature of 37°C. Each sample was tested in duplicate (n=2).

Table 3 Volume of reagent in the test for inhibiting HMG-CoA reductase activity

Sample	Buffer (µL)	Inhibitor (µL)	Enzyme (µL)	Total (µL)
Blanko	10	-	-	190
Control negative	5	-	5	190
Control positive	3	2	5	190
Treatment	3	2	5	190

Enzyme activity and percentage inhibition are determined using the following equation:

$$\text{Enzyme activity (U/mgP)} = \frac{\left[\left(-\frac{\Delta\, A\ sample}{\Delta t}\right) - \left(-\frac{\Delta\ \text{A control}}{\Delta \text{t}}\right)\right] \times 0{,}2}{12{,}44 \times \text{V} \times [\text{enzyme}] \times 0{,}55}$$

$$\%\ \text{Inhibiton} = \frac{\left[\left(-\frac{\Delta\, A\ control}{\Delta t}\right) - \left(-\frac{\Delta\ \text{A sample}}{\Delta \text{t}}\right)\right]}{\left(-\frac{\Delta\ \text{A control}}{\Delta \text{t}}\right)} \times 100\ \%$$

Note:
0,2 = reaction volume (0,2 mL)
12,44 = NADPH extinction coefficient at 340 nm
V = Enzyme volume (mL)
[enzyme] = Enzyme concentration (0,7 mgP/mL)
0,55 = light path (cm)
Δ A control = difference in absorbance of control
Δ A sample = difference in absorbance of sample
Δt = difference in time (minutes)

Data Analysis

Data analysis was conducted statistically to observe the influence of combinations on the inhibition activity of HMG-CoA reductase. Statistical analysis was performed using IBM SPSS Statistics 22 software. The method used was a one-factor completely randomized design (CRD), namely One-Way ANOVA. Duncan's test was conducted if the results showed a significant difference at the 95% significance level. A p-value of <0.05 indicates a significant difference. The significant effect of the treatments given is indicated by different letter codes in the data presented in the results section.

3. RESULTS

Moisture Content of Pulai Leaves and Papaya Leaves

Measurement of moisture content in simplicia aims to standardize the simplicia for testing. The moisture content measurements of Pulai leaves and papaya leaves obtained from Balittro are 6.85% (v/w) and 6.90% (v/w), respectively. These results meet the standard for good moisture content, which is <10% (Indonesian Ministry of Health 2014).

Phytochemical Components of Pulai and Papaya Leaf Water Extracts

The results of phytochemical component analysis, both qualitatively and quantitatively, in water extracts of Pulai and papaya leaves were obtained through a search of several literature sources. Seven types of secondary metabolite compounds were measured qualitatively, namely alkaloids, flavonoids, phenolics, tannins, saponins, triterpenoids, and steroids. Two of these compounds, alkaloids and flavonoids, will also be quantitatively measured.

A. Qualitative Phytochemistry

Qualitative phytochemical analysis of Pulai leaf extract with water solvent indicates the presence of alkaloids, flavonoids, phenolics, tannins, saponins, triterpenoids, and steroids in the water solvent (Table 4). The results of qualitative phytochemical analysis on papaya leaf extract show that alkaloids, flavonoids, tannins, saponins, and steroids can be found in the water solvent. However, triterpenoids cannot be found in the water solvent (Table 5).

Table 4 Phytochemical component of pulai leaf water extract

Component	Antony *et al.* (2011)	Misra *et al.* (2011)	Dhruti *et al.* (2016)
Flavonoid	√	X	√
Alkaloid	√	√	√
Fenolic	√	-	-
Tannis	√	X	√
Saponnin	√	√	√
Terpenoid	X	√	√
Steroid	√	√	√

Note: √: detected; -: not detected; X: not tested

Table 5 Phytochemical component of papaya leaf water extract

Component	Kolimon (2017)	Kining (2015)	Maniyar and Bhixavatimah (2012)
Flavonoid	√	X	√
Alkaloid	√	√	√
Fenolic	X	X	X
Tannis	√	√	√
Saponnin	√	-	√
Terpenoid	X	-	-
Steroid	X	√	-

Note: √: detected; -: not detected; X; not tested

B. Quantitative Phytochemistry

Quantitative phytochemical analysis aims to determine the total content of secondary metabolites present in simplicia or extracts. Two types of secondary metabolites were tested, namely total alkaloids and total flavonoids in Pulai and papaya leaf extracts with water, methanol, and ethanol solvents.

The data obtained indicate that the alkaloid content of Pulai leaf extract with water solvent is higher than that of methanol solvent (Table 6). Meanwhile, the total alkaloids in papaya leaf extract with 96% ethanol solvent are higher than with water solvent (Table 6).

Total flavonoid analysis was also conducted on Pulai and papaya leaf extracts. Total flavonoid analysis of Pulai and papaya leaf extracts was obtained from three types of solvents, namely water, methanol, and 96% ethanol. The results obtained show that the highest flavonoid content is found in methanol solvent and the lowest in 96% ethanol solvent, at 76.23 ± 0.02 mg and 1.72 ± 0.25 mg, respectively (Table 7). Meanwhile, the results of total flavonoid analysis of papaya leaf extracts indicate that the highest flavonoid content is found in ethanol solvent and the lowest in water solvent, at 17.07 ± 2.37 mg and 6.44 ± 0.14 mg, respectively (Table 7).

Table 6 Analysis of total alkaloids in pulai leaf and papaya leaf extracts

Sample	Solvent	Alkaloid Total	Researcher
Pulai leaf	Water	3,94 ± 0,003 mg	Dhruti *et al.* (2016)
	Methanol	2,82 ± 0,003 mg	Dhruti *et al.* (2016)
Papaya Leaf	Water	8,27 ± 1,84 %	Nugrahani *et al.* (2020)
	Ethanol 96%	26,12 ±5,72 %	Nugrahani *et al.* (2020)

Table 7 Analysis of total flavonoids in pulai leaf and papaya leaf extracts

Sample	Solvent	Flavonoid Total	Researcher
Pulai leaf	Water	68,73 ± 0,003 mg	Dhruti *et al.* (2016)
	Methanol	76,23 ± 0,02 mg	Dhruti *et al.* (2016)
	Ethanol 96 %	55,49 ± 1,26 mg	Purnama (2015)
Papaya Leaf	Water	6,44 ± 0,14 mg	Vuong *et al.* (2013)
	Methanol	11,96 ± 0,85	Vuong *et al.* (2013)
	Ethanol	17,07 ± 2,37 mg	Vunog *et al.* (2013)

HMG CoA Reductase Inhibition Activity

The inhibition activity of HMG CoA reductase was conducted to determine the potential of water extracts from Pulai leaves and papaya leaves, as well as their combination, in inhibiting HMG CoA reductase activity. This HMG CoA reductase inhibition assay used a positive control from the statin group, namely atorvastatin from the available kit and commercial lovastatin. Atorvastatin (55 µg/mL) exhibited an inhibition activity of 97.48%, while lovastatin (50 µg/mL) showed an inhibition activity of 85.77%. Single extracts of Pulai leaves (50 µg/mL) and papaya leaves (50 µg/mL) each showed inhibition activity four times lower than lovastatin with values that were not significantly different. Meanwhile, the combination of Pulai leaf extract with papaya leaf extract showed inhibition activity four times higher compared to single extracts (Figure 1).

Statistical analysis indicates a significant difference ($p < 0.05$) between single extracts and combinations. However, there is no significant difference ($p > 0.05$) among the three combinations (samples C, D, and E). This means that adding compositions to the Pulai leaf extract does not affect its inhibitory power. Additionally, it can also be noted that combinations C, D, and E have the same effectiveness as the positive control lovastatin ($p > 0.05$).

Quercetin at 5 µg/mL is capable of inhibiting HMG-CoA reductase enzyme activity by 40.62%. These results indicate that quercetin at 5 µg/mL produces inhibitory power that is not significantly different from single extracts at a concentration of 50 µg/mL ($p > 0.05$). Quercetin at 50 µg/mL can inhibit HMG-CoA reductase enzyme activity by 33.18%. This result is smaller compared to quercetin (5 µg/mL).

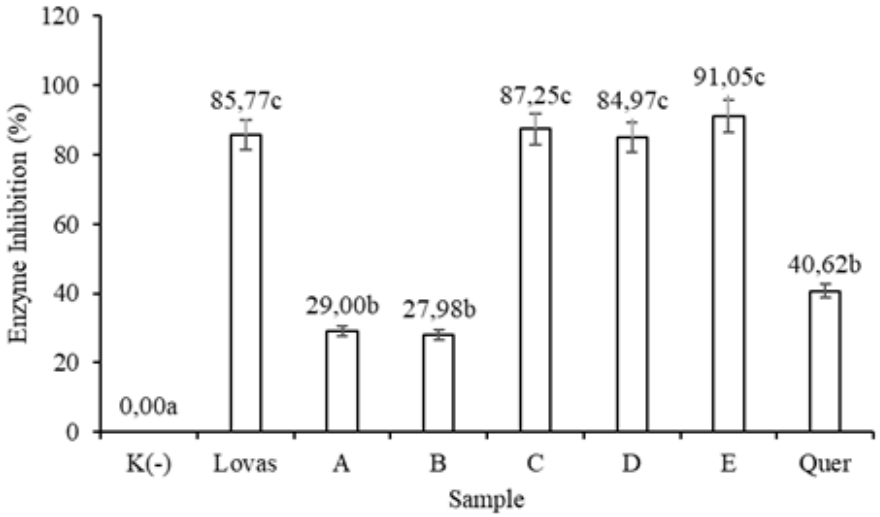

Figure 1 Inhibition of HMG CoA reductase activity

Note: K(-): negative control; Lovas: lovastatin; A: pulai leaf extract; B: papaya leaf extract; C: pulai leaf extract: papaya leaf 1:1; D: pulai leaf extract: papaya leaf 2:1; E: pulai leaf extract: papaya leaf 3:1; Kuer: quercetin. Vertical lines on the data bars indicate standard error. Numbers followed by different letters indicate significant differences at the 95% confidence level.

4. DISCUSSION

Moisture Content of Pulai Leaves and Papaya Leaves

One of the quality standardization parameters for simplicia is moisture content. The moisture content value is used to describe the storage resistance of simplicia (Indonesian National Agency of Drug and Food Control 2014). The moisture content of Pulai and papaya leaf simplicia obtained is not more than 10%. This value meets the standard moisture content set by the Indonesian Pharmacopoeia, which is <10% (Indonesian Ministry of Health 2014). High moisture content can lead to microbial growth on simplicia and activate certain enzymes (Pranowo *et al*. 2016). According to Batubara *et al.* (2017), moisture content of less than 10% means low microbial activity in the material. Based on this, the low moisture content of simplicia in this study can slow down microbial growth, thus allowing for longer storage. Rahmawati (2015) stated in her writing that moisture content values <10% also affect the extraction process. The lower the moisture content value, the easier it is for solvents to bind active compounds within it.

The moisture content obtained in this study is lower than that in the study by Tambunan *et al.* (2016), which was 8.42%. This difference may be due to the drying process. The drying method used in this study is oven blower, while Tambunan *et al.* (2016) used air drying method, which has much lower temperatures than oven blower. According to Syafrida *et al.* (2018), the drying temperature causes vapor pressure inside the sample to be higher than the vapor pressure of water outside the sample, causing water molecules to diffuse from inside to outside. The higher the temperature used, the faster the transpiration process and the lower the moisture content value.

Phytochemical Components of Pulai Leaf and Papaya Leaf Water Extracts

The phytochemical components of the pulai leaf water extract were analyzed both qualitatively and quantitatively. Qualitative phytochemical analysis was conducted to determine the types of secondary metabolite

compounds, while quantitative measurements were performed to determine the amount of secondary metabolite compounds present. These tests were used to speculate which compounds have the potential to be HMG-CoA reductase inhibitors.

A. Qualitative Phytochemistry

Secondary metabolites play a crucial role in the plant defense system against pathogens and environmental stress. Genetic, ontogenic, morphogenic, and environmental variations are factors that can influence the biosynthesis and accumulation of secondary metabolites. The accumulation of secondary metabolites is strongly influenced by various environmental factors such as light, temperature, soil moisture, soil fertility, and salinity (Yang *et al*. 2018). According to Achakzai (2009), differences in the age of plant parts also affect the content of secondary metabolites within them.

Phytochemical analysis results report that alkaloids can be found in both pulai leaves and papaya leaves with water as the solvent. According to Prayoga *et al.* (2019), alkaloids generally dissolve in organic solvents, but alkaloids in the form of salts as well as pseudo and proto alkaloids can dissolve in water and other polar solvents. Therefore, it can be inferred that alkaloids in pulai and papaya leaves are the type of alkaloids that can dissolve in polar solvents. Flavonoid compounds are also found in pulai and papaya leaves with water as the solvent. According to Kemit *et al*. (2016), flavonoids contain several hydroxyl groups, making them soluble in polar solvents such as water, acetone, ethanol, and methanol.

Additionally, phenolic compounds, tannins, saponins, and steroids were found in pulai and papaya leaves, and triterpenoids were found in pulai leaves. However, among the three references used, phenolics, saponins, and steroids were only detected in one of them. This difference could be attributed to the differences in the plants' growing locations. According to Katuuk *et al.* (2019) and Safrina and Priyambodo (2018), the altitude of the growing location can affect the content of secondary metabolites. Lower growing locations result in greater light intensity received by the plants. This light intensity is used for photosynthesis, which is the key process in secondary metabolism in plants. Therefore, plants grown at lower altitudes will produce more secondary metabolite compounds. This is consistent with the research findings of Radusiene *et al.* (2013), which stated that the total phenolic content in Hypericum perforatum L. increases with increasing light intensity and temperature.

Furthermore, an environmental factor that can affect the content of phytochemical compounds is nutrient elements. According to Salim *et al.* (2017), macro-nutrient elements (nitrogen, potassium, and calcium) are linearly related to the formation of secondary metabolite compounds. Soil nutrient content is directly proportional to the variety of secondary metabolites and inversely proportional to the production of secondary metabolites.

B. Quantitative Phytochemistry

The major compound contained in all parts of the pulai plant is alkaloid. There are approximately 70 types of alkaloids in the pulai plant, with the majority reported to be in the pulai leaves (Dey 2011; Khyade *et al*. 2014). Alkaloids are chosen for quantitative testing because, according to Antony et al. (2011), they are compounds of great importance in the development of new drugs. This is because alkaloids have various chemical structures and have been identified as compounds with pharmacological properties in medicinal plants.

The total alkaloid results in pulai leaves show that the total alkaloid in water is higher than in methanol. This means that there are more types of alkaloids soluble in water compared to methanol. Meanwhile, in papaya leaves, the total alkaloid in 96% ethanol solvent

is higher than in water. This suggests that there are more types of alkaloids soluble in 96% ethanol compared to water. Thus, it can be assumed that the alkaloid compounds in pulai and papaya leaves are alkaloids in the form of their salts or pseudo and proto alkaloid groups (Prayoga *et al*. 2019).

Sulistiyani *et al*. (2017) reported in their study that the alkaloid fraction was able to inhibit HMG-CoA reductase by 50%. However, according to Usman (2000), hypercholesterolemia effects due to the administration of pulai alkaloid extracts in rat models were not related to their activity as HMG-CoA reductase inhibitors.

Flavonoids are compounds classified as polyphenols with the highest distribution rate in all plants (Puspitasari and Desrita 2019). Flavonoids have been shown to inhibit HMG-CoA reductase activity (Ademosun *et al*. 2015). Therefore, flavonoids were chosen to determine their presence quantitatively.

The total flavonoid analysis reported that in pulai leaves, the highest flavonoid content was found in methanol solvent. Meanwhile, the total flavonoid analysis in papaya leaves showed that the highest flavonoid content was in ethanol solvent. This could be because the polarity of the solvent affects the ability and effectiveness of the solvent in extracting flavonoids. Flavonoids are divided into various types that have different polarities depending on the number and position of hydroxyl groups (Verdiana *et al*. 2018). The high total flavonoid content in methanol and ethanol extracts explains that the characteristics of flavonoid compounds in pulai leaves have the same polarity as methanol, and the characteristics of flavonoid compounds in papaya leaves have the same polarity as ethanol, thus, extracting pulai leaves with ethanol results in the highest flavonoid content.

The lowest total flavonoid content was found in water solvent. According to Septiana and Asnani (2012), water is a highly polar solvent compared to methanol and ethanol. Its high polarity can bind other polar components such as carbohydrates, resulting in a low flavonoid content per sample weight. However, Rohadi and Wahjuningsih (2019) stated in their research that the extraction of compounds with water extracts can be improved by increasing the extraction temperature. This was evidenced by their findings, where the flavonoid content of white tea (*C. sinensis* Linn.) water extracts increased with increasing temperature up to 120°C with an incubation time of 3 minutes.

Flavonoid compounds are suspected to play a role in inhibiting HMG-CoA reductase activity. This statement is supported by several previous studies, such as in silico testing through molecular docking showing that flavonoid compounds like quercetin, quercetagetin, and quercimeritrin found in bay leaf and jati belanda leaf polyphenol fractions can competitively inhibit HMG-CoA reductase activity (Ramdayanti 2020). Kimura *et al.* (2015) reported that flavonoids, specifically flavonol O-glycosides identified from indigo leaves (*Polygonum tinctorium* Lour), can inhibit HMG-CoA reductase activity. Ademosun *et al.* (2015) also reported that phenolic grape skin extracts containing flavonoid compounds such as catechin, epicatechin, rutin, quercetin, and kaempferol can competitively inhibit HMG-CoA reductase activity with HMG-CoA by binding to the enzyme's active site, i.e., interaction between the hydroxyl (-OH) group of the cinnamoyl ring and the carboxylic acid (-COOH) group of the HMG-CoA.

Effect of HMG-CoA Reductase Inhibition from Combination Extracts of Pulai Leaves and Papaya Leaves

The enzyme inhibition activity test aims to determine the ability of a sample to inhibit enzyme activity expressed in percentage (%). The inhibition activity test against HMG-CoA reductase was conducted with atorvastatin kit

and commercial drug lovastatin as positive controls. The inhibitory effects produced by atorvastatin kit and lovastatin were 97.48% and 85.77%, respectively. This research has a better effect compared to Maulana's study (2019) reporting an inhibition activity of atorvastatin at 92.45% and also Rinto *et al.* (2015) reporting a lovastatin inhibition activity of 66.67%.

Statin drugs competitively inhibit HMG-CoA reductase activity. Statins and HMG-CoA structurally share similarities, thus competing to bind to the enzyme's active site. When the statin concentration exceeds that of the HMG-CoA substrate, the enzyme tends to react with statin, reducing the production of cholesterol (Nelson and Cox 2013). The addition of statin groups reduces the ability of HMG-CoA reductase activity, as evidenced by measurable NADPH at λ334 nm. This result indicates that the enzyme kit used functions effectively. NADPH serves as an indicator because it readily absorbs UV light and is easily oxidized under acidic or basic conditions. The greater the inhibitor's ability to inhibit HMG-CoA reductase activity, the less NADPH is consumed or the more residual NADPH remains compared to without the addition of an inhibitor (Perchellet *et al.* 2009).

Testing of single crude extracts of pulai leaves and papaya leaves resulted in insignificantly different inhibitory activities of 29% and 27.98%, respectively. These results are four times smaller compared to the positive control lovastatin. The inhibitory effect produced by the papaya leaf extract in this study is lower compared to Hasimun *et al.* (2018). That study reported that ethanol extract of papaya leaves can inhibit HMG-CoA reductase enzyme by 40.6% in vivo. The difference in results could be due to the difference in extract concentrations used. That study used a dose of 200 mg/kg BW or equivalent to ±2900 µg/mL, which is much higher than in this study. Nevertheless, the inhibition of single crude extracts of pulai leaves and papaya leaves in this study still falls within the moderate inhibition range as stated by Andrianto *et al.* (2015), who classified inhibition as strong if it exceeds 50%, moderate if it exceeds 25%, and low if the inhibition value is ≤ 25%.

This study also shows the combined effects on the inhibition of HMG-CoA reductase enzyme. Pulai leaves combined with papaya leaves in three formulations, namely 1:1, 2:1, and 3:1. These three combinations resulted in insignificantly different inhibitory activities. The inhibitory activities produced were 87.25%, 84.97%, and 91.05%, respectively. This means that adding compositions to pulai leaves did not affect the inhibitory activities produced. When pulai leaves are combined with papaya leaves, there is a fourfold increase in enzyme inhibitory activity compared to their individual components. This fact indicates that the combination of these two samples produces a synergistic interaction. This is in line with the research by Mutalik and Sanghavi (2014), stating that a synergistic interaction occurs when the action of one drug is facilitated or enhanced by another drug.

The inhibitory activity of HMG-CoA reductase produced by the combination extract of pulai leaves and papaya leaves in this study is better compared to the inhibitory activity of HMG-CoA reductase produced by Sulistiyani *et al*. (2017). In their research, Sulistiyani *et al*. (2017) used flavonoid fraction and alkaloid fraction with a concentration of 20 ppm as HMG-CoA reductase inhibitors with inhibitory activities of 73.79% by the flavonoid fraction and 50% by the alkaloid fraction. The inhibition of combined extracts in this study is classified as strong inhibition.

The positive control for the flavonoid group tested is quercetin, and this test shows an enzyme inhibition value of 40.62%. This value is much lower than the inhibition by the statin group ($p<0.05$). Quercetin is used for comparison because it is one of the flavonoids

that has a protective effect against hepatotoxicity by reducing triglyceride levels, total cholesterol, alanine transaminase (ALT), aspartate aminotransferase (AST), low-density lipoprotein (LDL) in plasma (Ganeshpurkar and Saluja 2016).

In conclucion combining pulai leaf and papaya leaf water extracts can enhance the ability of single extracts to inhibit HMG-CoA reductase enzyme with inhibitory potency that is not significantly different from the commercial positive control lovastatin. Adding components to the pulai leaf extract does not significantly affect the inhibition of HMG-CoA reductase, as indicated by the inhibitory ability of the combination of pulai leaf and papaya leaf extracts at ratios of 2:1 and 3:1, which are generally as effective as the 1:1 combination.

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