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Antioxidant Activity and Inhibition of HMG CoA Reductase Enzyme by Bay Leaf (*Syzygium polyanthum* Wight) Extract as a Treatment for Hyperlipidemia

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ABSTRACT

The prevalence of hyperlipidemia in Indonesia is increasing, due to the intake of high-fat foods, the demand for physical activity, and advancing age. Flavonoids are antioxidant compounds with anti-hyperlipidemic potential. Bay leaf (*Syzygium polyanthum*) is a flavonoid-rich plant that has been examined as a therapy for hyperlipidemia, but its mechanism of action is unknown. This study was aimed at determining the antioxidant potential and *in vitro* mechanism of action of bay leaf extract. The bay leaves were extracted with ethanol as a solvent, and the total flavonoid content was determined. Furthermore, the antioxidant activity was evaluated with a spectrophotometer using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. An enzyme-linked immunosorbent (ELISA) assay was used to measure the activity of the HMG CoA reductase enzyme inhibition and compared it to simvastatin. The flavonoid concentration of quercetin in the bay leaf extract was found to be 2.852%, and the IC_{50} value of the bay leaf extract's antioxidant activity was 12.05 µg/mL. The IC_{50} value of bay leaf extract in the HMG-CoA reductase enzyme inhibitory test was 22.74 µg/mL. The findings of this study indicate that the bay leaf extract is a very strong antioxidant with the ability to inhibit the HMG CoA reductase enzyme.

***Keywords*:** Antioxidant, Bay leaf extract, HMG CoA reductase enzyme, Hyperlipidemia, *Syzygium polyanthum* .

Introduction

According to the World Health Organization (WHO), coronary heart disease (CHD) and stroke are the leading causes of death in the world.[1] In addition to hypertension, smoking, blood sugar abnormalities, and a lack of physical activity, high blood cholesterol levels are major risk factors for coronary heart disease and stroke.[1] Plaque buildup in the coronary arteries causes coronary heart disease, which begins with atherosclerosis caused by excessive LDL density.[2] The oxidation of fatty acids may allow cholesterol to move freely through arterial walls. The presence of antioxidants stabilizes free radicals by compensating for the absence of electrons and limiting the chain reaction of the free radical arrangement. Excessive fatty acid oxidation raises the level of cholesterol in the blood. One of the components of repressing the arrangement of cholesterol is by restraining cholesterol amalgamation through the enzyme 3-hydroxy-3-methylglutaryl Coenzyme A (HMG CoA) reductase.[3]
After Brazil, Indonesia has the highest level of biodiversity. Indonesia's tropical timberlands cover almost 143 million hectares and are home to nearly 80% of the world's therapeutic plants. It is estimated that 28,000 plant species grow in Indonesia's tropical forests. One of the potential therapeutic plants that developed in Indonesia is the bay leaf (*Syzygium polyanthum* Wight), which is widely known as a kitchen spice and is frequently used by the public for alternative medicine as a hyperlipidemia herbal medicine.[5]

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Bay leaves contain the active ingredient quercetin (flavonoid), flavonoids, tannins, saponins, and vitamins C, B3, A, and E, which have been linked to body fat in some studies.[6] In Indonesia, bay leaves are a well-known alternative medicine that is also commonly used as a spice food complement. Bay leaves are used as a medicinal herb to treat high cholesterol, diabetes, stomach ulcers, diarrhea, and weight reduction. In addition, bay leaves can also help with gout, stroke, cholesterol, blood circulation, and stomach inflammation.[7-8] Wirawan *et al*.,[9] noted in a previous study that the findings of the preclinical test of the bay leaf fraction are efficient in lowering hyperlipidemia levels in male white rats due to the flavonoid compounds, tannins, and polyphenols in bay leaves. The fraction is an antioxidant that has been shown to lower LDL (low-density lipoprotein) and triglycerides, thereby preventing LDL formation on blood vessel walls. Flavonoid compounds and tannins at the dose used can inhibit the HMG CoA reductase enzyme.[9] Several other preclinical studies on the effects of bay leaf extract conducted on experimental animals showed that the bay plant has the potential to be used as a drug for hyperlipidemia, but the mechanism of action remains unknown. *In vitro* studies are needed to determine the mechanism of the bay leaf plant as a treatment for hyperlipidemia to strengthen scientific evidence.
The present study was therefore conducted to investigate the antioxidant activity of bay leaf (*Syzygium polyanthum* Wight) extract and its inhibitory effect on the HMG CoA reductase enzyme as a treatment for hyperlipidemia.

Materials and Methods

Source of plant material
Bay leaves were obtained from South Tangerang District, Banten in May 2022. The leaves were air-dried, and the dried leaves were stored at ambient temperature before use.

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Equipment and chemical materials

The types of equipment used include: a spectrophotometer (Spectroquant Pharo 300), an oven (Memert), a rotary evaporator (Buchi), a water bath (Memert), a grinder (Fritsch), a muffle furnace (Thermo Scientific), a moisture balance (Sartorius), an analytical balance (Mettler toledo), a sonicator (GB-928 Ultrasonic Cleaner), and an ELISA (Accuris MR9600). The materials used were 70% ethanol (Merck), methanol (Merck), DPPH (Sigma), vitamin C (Sigma-Aldrich), aluminum chloride (Sigma-Aldrich), quercetin standard (Sigma-Aldrich), simvastatin (Sigma-Aldrich), and an HMG CoA reductase kit (BioVision).

Preparation of bay leaf extract

Bay leaf was collected from South Tangerang District in May 2022. A total of 335 grams of dried bay leaves were macerated with 3.35 L of 70% ethanol for 48 hours. The resultant macerate was filtered, and the residue was macerated again with the same amount of 70% ethanol three times. Then, the filtered macerate was evaporated using a rotary evaporator at 60°C. The extract was concentrated in a water bath at 60°C to obtain a thick bay leaf extract. The maceration process was used for the extraction because it is easy and quick. It was carried out at room temperature to avoid damage or loss of the extracted active ingredients.[10]

Determination of the total flavonoid content of bay leaf extract

The concentration series of the quercetin standard solution was prepared to obtain a standard solution with concentrations of 2, 4, 6, 8, 10, and 12 µg/mL in 5 mL of ethanol p.a.[11] The concentration series of 6 µg/mL was taken up to 1000 µL, 200 µL, 10% $AICI_3$, 200 µL and sodium acetate were added in a 5 mL volumetric flask. Then ethanol p.a was added to the limit. The UV-Vis spectrophotometer was used for scanning, with the wavelength set to 400-500 nm. The maximum wavelength of quercetin is defined as the wavelength that provides the most absorption.[11] A 1000 µL solution of bay leaf extract with an 800 µg/mL concentration was collected and mixed with 200 µL of 10% $AlCl_3$ reagent, 200 µL of sodium acetate, and 5 mL of ethanol p.a. The absorption was measured with a UV-Vis spectrophotometer at the maximum wavelength and an operating time of 30 minutes.[11] The quercetin content was then estimated using a linear regression equation based on the calibration curve of the UV-Vis spectrophotometric values. The measurements' absorption data were entered into the linear regression equation. The absorbance of the sample is y, and the concentration is x.

$$\text{Total flavonoid content} = \frac{\text{Concentration x sample volume/sample weight)}}{1000} \text{ x } 95\%$$

Evaluation of the antioxidant activity of bay leaf extract

The evaluation of the antioxidant activity of bay leaf extract was conducted by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The concentration of the bay leaf extract was 100 µg/mL for initial screening at 5, 10, 25, 50, and 100 µg/mL. Vitamin C (0.5, 1, 2, 4, and 8 µg/mL) was utilized as a standard for comparison, and 0.4 mM DPPH was used as a control. The test solution, control, and vitamin C were all incubated at 37˚C. It was then pipetted onto a UV-Vis spectrophotometer cuvette and incubated at 37 ˚C for 30 minutes. The control blank was made from ethanol and compared to vitamin C. A UV-Vis spectrophotometer with a 515 nm wavelength was also used to measure absorption. The IC_{50} value, which represents the sample's ability to inhibit 50% of the oxidation process, was used to estimate antioxidant activity. It was calculated by plotting a linear relationship between the concentration of the test solution (x-axis) and the percentage antioxidant activity (y-axis).[12]

$$Antioxidant\ activity\ (\%) = \frac{\text{Ab} - \text{As}}{\text{Ab}} x\ 100\%$$

Ab is the absorbance of the blank (without sample) and As is the absorbance of the sample.

HMG CoA reductase inhibition assay

The standard control was made in series of 1, 2, 3, 4, and 5µL of HMG CoA reductase. An aliquot of 2 µL of inhibitor was added to the well. Fifty micrograms (50 µg) of bay leaf extract were mixed with a reaction mixture containing 400 µM of nicotinamide adenine dinucleotide phosphate, 400 µM of HMG CoA substrate, and 100 mM potassium phosphate buffer (pH 7.4; containing 120 mM of potassium chloride, 1 mM of ethylenediamine-tetraacetic acid, and 5 mM of dithiothreitol), followed by the addition of 12 µL HMG CoA reductase. The reaction was incubated at 37°C, and the absorbance in 340 nm wavelength was measured. Simvastatin was used as the positive control, while aquabidestilata was the negative control. The percentage of inhibition was calculated by the following formula:[12]

$$\%\ \text{inhibition} = \frac{\Delta\text{Control absorbance} - \Delta\text{Sample absorbance}}{\Delta\text{Control Absorbance}} x\ 100\%$$

Results and Discussion

The extraction was carried out using the maceration method with 70% ethanol as the solvent because ethanol is safe, non-toxic, and capable of extracting more compounds from bay leaf.[13] A total of 62.9 grams of bay leaf extract was obtained from 335 grams of bay leaf powder (Figure 1). The results are consistent with previous findings by Puspitasari *et al*.[14] The characterization of bay leaf extract carried out in this study ensured that the extract had a particular parameter with a consistent value. Based on the characterization results (Table 1), the extract contains 0.92% water, which meets the Indonesian Herbal Pharmacopoeia II standards of less than 10%. The extract contains 2.72% total ash, as required by the Indonesian Herbal Pharmacopoeia II of less than 10.1%.[15] The ash content determination is intended to determine the physiological ash, which is the internal mineral content originating from the plant tissue itself, as well as the non-physiological ash that is residue from the surrounding environment such as sand and soil contained in the sample. The smaller the ash content, as well as the impurities in the bay leaf extract.[16]

The total flavonoid (quercetin) concentration of bay leaf extract was determined first, followed by the wavelength. The wavelength obtained was 425 nm. Then, a standard curve for comparative analysis was made. To produce the linear regression equation and the relationship curve between concentration and absorbance, a 50 mL standard solution was divided into a series of 2, 4, 6, 8, 10, and 12 µg/mL.[17] Figure 2 depicts the quercetin standard curve. The purpose of developing a curve is to determine the levels of flavonoid compounds (quercetin) in a sample using a linear regression equation from the flavonoid standard curve.[17]

From the curve, the linear regression equation is y = 0.0761x - 0.0166, where y = absorbance, x = concentration and the value of the correlation coefficient (R^2) is 0.9998.

Figure 1: Bay leaf extract

Table 1: Characterization results of bay leaf extract

Characterization	Result
Shape	Thick
Color	Blackish green
Yield	18.7 %
Moisture Content	0.92 %
Total Ash Content	2.72 %

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The results of the determination of the quercetin content of bay leaf extract in percentage are presented in Table 2. The quercetin content in the bay leaf extract is 2.852% (Table 2). It is possible to deduce that the quercetin content in bay leaf extract meets the standards for total flavonoid content based on the Indonesian Herbal Pharmacopoeia II, which is not less than 1.14%.[15]

The antioxidant inhibition of bay leaf extract was determined using the free radical scavenging action of DPPH. The purple color change in DPPH to yellowish and colorless indicates that atomic antioxidants neutralize DPPH free radicals. The linearity test determines whether or not the examination of the substance's concentration using the UV-Vis spectrophotometer method has a linear relationship.[18] The linearity parameter is used to confirm the ability of the standard to ensure that there is a directly proportional relationship between the substance concentration and the detector responses. For a good analytical procedure, the relationship coefficient is expected to be close to 1 or over 0.9900.[19] To determine the relationship between concentration and inhibition, linearity and range tests were performed by making a standard curve. The linear regression analysis result (Figure 3) yielded a linear equation, y = 4.7613 x-7.3854 in a 0.9994 coefficient value suggests that the measured detector signals have a linear relationship. As a result, the confirmation of antioxidant action using UV-Vis spectrophotometry has high linearity.[18] The IC_{50} value can be evaluated from the linear regression equation obtained by substituting 50 in the equation. The IC_{50} indicates the concentration of the test sample (µg/mL) that inhibits 50% of the oxidation process.

The DPPH free radical method is an antioxidant assay based on hydrogen atom donation as the presence of an antioxidant molecule, which leads to a color change from purple to yellow.

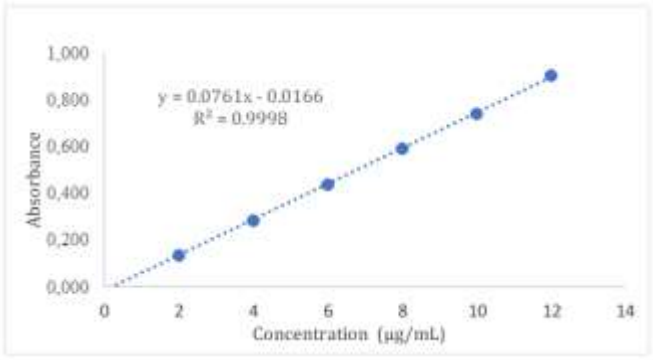

Figure 2: Quercetin standard linearity curve.

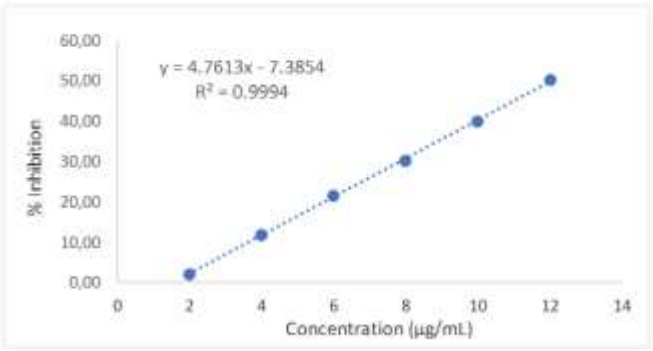

Figure 3: Antioxidant activity linearity curve.

Table 2: Quercetin measurement results

Abs	µg/mL	% Quercetin content
0.477	6.486	2.852

This is then detected at a wavelength of 515 nm. The reduced color of the DPPH solution may suggest that hydrogen atoms released by the test material react with the DPPH radical molecule to form the yellow compound 1,1-diphenyl-2-picrylhydrazil.[2] The IC_{50} of a compound can be classified as "very strong" if the IC_{50} is less than 50 µg/mL; "strong" if the IC_{50} is between 50 and 100 µg/mL; "moderate" if the IC_{50} is between 100 and 150 µg/mL; and "weak" if the IC_{50} is between 150 and 200 µg/mL.[20] Based on this classification, the IC_{50} of bay leaf extract (12.05 µg/mL) could be classified as very strong. However, the value is still below that of vitamin C, with an IC_{50} value of 9.70 µg/mL. The maceration process of bay leaf extraction has a higher antioxidant potential. The observation is in agreement with the previous research. According to the findings, the ethanol extract obtained by the maceration method exhibits antioxidant activity that differs from the soxhlet extraction procedure (18.73%) and the infusion extraction method (40.26 µg/mL).[21]

Table 4 shows the results of *in vitro* testing of bay leaf ethanolic extract and simvastatin for suppressing the activity of the HMG-CoA reductase enzyme. Simvastatin was employed as a reference compound in this investigation because it was a first-generation statin for the treatment of hyperlipidemia. Flavonoids act by preventing LDL oxidation through H^+ donation and the activity of 3-hydroxy-3-methylglutaryl Coenzyme-A reductase (HMG-CoA reductase), preventing foam cell formation and lipid degradation.[12] Flavonoids can inhibit the activity of the HMG CoA reductase enzyme, resulting in decreased mevalonate arrangement from HMG CoA reductase. Flavonoids' ability to bind cholesterol inside the lumens of the duodenum and jejunum resulted in a reduction in exogenous cholesterol intake.[2] Quercetin is a group of flavonoids that have a strong antioxidant activity and inhibit the formation of human LDL levels *in vitro*. The flavonoid quercetin serves as an antioxidant in decreasing the incidence of LDL oxidation as a result of inflammatory events, where these substances inhibit the formation of reactive O_2 radicals, preventing endothelial damage by inhibiting oxidation reactions from initiating. Antioxidants also reduce the tonicity of oxidized LDL for endothelial cells and reduce oxidative degradation due to nitric oxide.[22]

The HMG CoA reductase inhibition test revealed that bay leaf extract had a higher IC_{50} than simvastatin for inhibiting the HMG CoA reductase enzyme. This suggests that bay leaf extract has a lower inhibitory effect on HMG-CoA reductase than simvastatin.[23] With an IC_{50} value of 22.74 µg/mL, bay leaf extract has the potential to inhibit the enzyme HMG CoA reductase. The quercetin in bay leaf extract inhibits the activity of the HMG CoA reductase enzyme, resulting in a reduction in the mevalonate arrangement produced by HMG CoA reductase. Flavonoids may bind cholesterol within the lumen of the duodenum and jejunum, reducing the need for exogenous cholesterol.[22] A few more studies have also looked into the effectiveness of plant extracts in inhibiting HMG-CoA reductase. The IC_{50} for *Vernonia condensata* extract was 271.7 mg/ml.[24] Grapefruit peels have an IC_{50} value of 0.11 mg/ml on HMG-CoA reductase activity, according to Ademosun *et al*.[25] The study's comparator was simvastatin, the first-line medication for hyperlipidemia disorders. Simvastatin was the most effective inhibitor of the HMG CoA reductase enzyme in the treatment of hyperlipidemia. It acts by preventing the development of cholesterol. Previous research has indicated that the flavonoids in bay leaves function as antioxidants, potentially lowering cholesterol via decreasing the activity of HMG CoA reductase.[26]

Table 3: IC_{50} value of antioxidant activity of samples and comparison

Sample Name	IC_{50} (µg/mL)
Bay leaf extract	12.05
Vitamin C	9.70

Table 4: IC_{50} value of samples and comparison of the HMG CoA reductase enzyme

Sample Name	IC_{50} (µg/mL)
Bay leaf extract	22.74
Simvastatin	5.26

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Conclusion

The findings of this study reveal that *Syzygium polyanthum* (bay leaf) extract has anti-hyperlipidemic activity and acts as an antioxidant by inhibiting the production of cholesterol. Bay leaf extract may play a significant role in the prevention of diseases caused by free radicals. The research helps to understand the mechanism of action of bay leaf extract as a hyperlipidemia treatment by inhibiting the HMG CoA reductase enzyme. The bay leaf extract is a very strong antioxidant and has the potential to inhibit the HMG CoA reductase enzyme.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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