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Evaluation of Total Phenolic Content, Total Flavonoid Content, and Free Radicals Scavenging Activity of *Syzygium polyanthum* Wight. Leaves

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Abstract: Bay leaves plant (*Syzygium polyanthum* Wight), from the *Myrtaceae* family, are traditionally used for their medicinal properties due to bioactive compounds like phenolics, flavonoids, and steroids with antioxidant activity. This study aimed to evaluate the antioxidant potential of bay leaf extract by measuring total phenolic and flavonoid content and assessing free radical scavenging activity. Fresh leaves were collected in Yogyakarta in December 2024, dried, and extracted via methanol maceration, followed by fractionation with n-hexane, ethyl acetate, and aqueous solvents. The Folin-Ciocalteu method was used to measure total phenolic content, using gallic acid as a reference standard, revealing high phenolic levels, particularly in the ethyl acetate fraction (198.20 ± 0.42 mg GAE/g). The Folin-Ciocalteu method was used to measure total phenolic content, using gallic acid as a reference standard, showing significant flavonoid levels, with the ethyl acetate fraction at 62.06 ± 1.76 mg QE/g. The DPPH assay was utilized to assess antioxidant activity, revealing that the ethyl acetate fraction had the lowest IC50 value (41.71 ± 0.617 µg/mL), demonstrating potent free radical scavenging ability similar to ascorbic acid (9.61 ± 0.138 µg/mL) The ethyl acetate fraction contains a significant amount of phenolics and flavonoids correlated strongly with its antioxidant capacity, highlighting bay leaves as a promising source for herbal medicine development. These findings validate the traditional use of bay leaves and offer updated quantitative data on phenolic/flavonoid levels and antioxidant capacity, highlighting their promise for natural health product development.

Keywords: antioxidant; DPPH; total flavonoid; total phenolic

1. INTRODUCTION

The bay leaves (*Syzygium polyanthum* Wight), belonging to the *Myrtaceae* family, are widely known among the community as a traditional medicine for various diseases [1]. Bay leaves contain bioactive components such as phenolic compounds, flavonoids, and steroids that have antioxidant activity. To validate its bioactive potential, a study was performed by assessing the total phenolic content through spectrophotometric techniques utilizing the Folin-Ciocalteu reaction [2], [3]. This method allows for the quantitative determination of phenolic compound concentrations employing gallic acid as a reference. The results showed that bay leaf extract contains significant phenolic content, which contributes to its antioxidant capacity [4].

In addition to phenolic content, research was also conducted to measure total flavonoids in bay leaf extract using a colorimetric method with aluminum chloride reagent. Flavonoids were measured based on the formation of colored complexes with quercetin as a standard [5],[6]. The analysis results

showed that bay leaves have a sufficiently high flavonoid content, supporting their pharmacological activity as a natural antioxidant. These flavonoids play a role in capturing free radicals and preventing oxidative damage to cells. This test reinforces the evidence that bay leaves can be used as a source of bioactive compounds for the development of herbal medicines [7].

To evaluate antioxidant activity, the DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used as an in vitro approach. This method measures the ability of bay leaf extract to scavenge DPPH free radicals, indicated by a color change in the solution from purple to pale yellow [8]. The IC50 value, which is the concentration of extract required to inhibit 50% of DPPH radicals, was calculated to determine antioxidant potential [9]. The test results showed that bay leaf extract has a low IC50 value, indicating strong antioxidant activity. Although most previous studies have focused on crude ethanol or methanol extracts of bay leaves, comparative evaluations of solvent fractions particularly the semi-polar ethyl acetate fraction remain limited. The present study addresses this gap by demonstrating that the high phenolic and flavonoid content in the ethyl acetate fraction is the primary factor contributing to its superior antioxidant capacity, thereby positioning bay leaves as a promising candidate for the development of herbal-based health products [10], [11].

2. MATERIALS AND METHODS

2.1. Materials

Analytical balance (Ohaus PJ1003, Japan), laboratory glassware, maceration vessel, oven (Memmert, Germany), micro pipettes (Scorex, Switzerland), 96-well clear polystyrene microplates (Biologix), vacuum rotary evaporator (Buchi R-220 Pro, Switzerland), microplate reader (Corona SH-1000lab).

2.2. Methods

2.2.1. Sample preparation and extraction

Fresh mature leaves of *S. polyanthum* were collected from nearby Yogyakarta in December 2024. Only fully expanded mature leaves (approximately the 4th–6th leaf blades from the shoot tip) were selected to ensure consistency in leaf age and minimize variations in secondary metabolite content. This plant was identified by an expert taxonomist from the Faculty of Pharmacy, Gadjah Mada University. After identification, 1.5 kg of fresh leaves were sorted, cleaned, and dried using an oven for 24 hours at 50°C and then followed by grinding and stored in an airtight container. The extraction process was carried out through maceration with a ratio of 1:10, namely 500 g of ground bay leaf mixed in 5 L of methanol in a closed glass chamber for 5 days, which was divided into two stages. In the first stage of maceration, 500 g of bay leaf powder was put into a closed glass chamber protected from sunlight, then soaked in 3.75 L of methanol (75% of the total solvent) for 3 days. During this period, the mixture was stirred twice a day for 15 minutes, then filtered to separate the filtrate from the dregs. In the second stage of maceration, the dregs from the previous stage were soaked again with 1.25 L of methanol (25% of the total solvent) for 2 days, followed by filtration to separate the filtrate and dregs. The filtrate from both stages was then combined in one container. The extract obtained was then concentrated using a rotary vacuum evaporator (Buchi R-220 Pro, Swiss) at a temperature of 50°C.

2.2.2. Fractionation

The methanol extract 20.31 g was dissolved in 300 mL of aquadest. The solution was then partitioned gradually with n-hexane (3x100 mL) until the n-hexane phase was clear. The remaining aqueous phase of the n-hexane partition was then partitioned with ethyl acetate (3x100 mL) until the ethyl acetate phase was clear. The aqueous phase is the result of separation from ethyl acetate. The three fractions obtained were then concentrated using a rotary evaporator at a temperature of 45°C until a thick fraction was obtained.

2.2.3. Total phenolic and total flavonoid content

Total phenolic and total flavonoid content was assayed following the protocol outlined by a previous study with minor modifications [7]. In this method, total phenolic assay was carried out

using the microdilution method with gallic acid as a standard, with dilution series at concentration ranges of 10, 20, 30, 40, 50, and 60 ppm. A complete volume of 20 µL of the diluted sample (extract/fraction/gallic acid) was combined with 20 µL of Folin-Ciocalteu reagent, then shaken for sixty seconds. The blend was set aside for 5 minutes, then 200 µL of 7% sodium carbonate (Na_2CO_3) solution and 10 µL of deionized water were added. Furthermore, the mixture was shaken at medium speed continuously for 1 minute. After incubation in the dark at room temperature for 120 minutes, the absorbance was measured at a wavelength of 750 nm using a microplate spectrophotometer (Corona SH-1000 lab, Japan).

The total flavonoid content was determined using the microdilution technique with quercetin as a reference, employing a series of dilutions at concentrations of 30, 40, 50, 60, 70, 80, and 90 ppm. Each well was filled with 50 µL of the sample solution, which could be an extract, fraction, or quercetin, along with 100 µL of methanol. One well was reserved as a blank control for measuring absorbance and contained 150 µL of methanol. Then, 20 µL of a 10% AlCl3 solution was added to each well, and the plate was gently shaken five times, both vertically and horizontally. The plate was left at room temperature for three minutes. After this incubation period, 20 µL of 1 M CH3COONa solution was added, followed by 60 µL of methanol. The plate underwent another incubation in the dark at room temperature for 40 minutes, and absorbance was measured at a wavelength of 430 nm using a microplate spectrophotometer (Corona SH-1000 lab, Japan).

2.2.4. Free radical scavenging activity

The scavenging activity of free radicals (DPPH) in extracts and fractions of bay leaves was conducted using the microdilution technique with minor adjustments as outlined by [12]. Each well received 100 µL of DPPH solution, then 100 µL of the test solution (extract, fraction, or ascorbic acid) at a specified concentration was added. Ascorbic acid served as a standard with dilution series at concentrations of 2, 4, 6, 8, 10, and 12 ppm. Negative controls were created by combining 100 µL of DPPH with 100 µL of methanol, whereas blank controls were made up of 200 µL of methanol. The microtiter plates were subsequently kept in the dark at a room temperature of 25°C for 30 minutes. Absorbance was recorded at a wavelength of 517 nm with a microplate spectrophotometer (Corona SH-1000 lab, Japan). The activity of scavenging free radicals was determined using the formula below:

$$\%\ Inhibition\ = \frac{A_b - A_s}{A_b} \times\ 100\%$$

3. RESULTS AND DISCUSSION

3.1. Total Phenolic Content

The quantification of total phenolic content is achieved by employing a gallic acid standard curve, which enables results to be reported as milligrams of gallic acid equivalents per gram (mg GAE/g). The connection between gallic acid concentration and its absorbance is described by the equation y = 0.0137x – 0.0212, with a high correlation coefficient of (r = 0.9978) as depicted in Figure 1a. The varying concentrations of total phenolic content for the extract and bay leaf fractions are displayed in Figure 1b. The total phenolic content amounts for the methanol extract and its fractions (ethyl acetate, n-hexane, and aqueous) measured 217.42 ± 0.42, 198.20 ± 0.42, 46.13 ± 0.73, and 182.14 ± 0.42, respectively (Figure 1B).

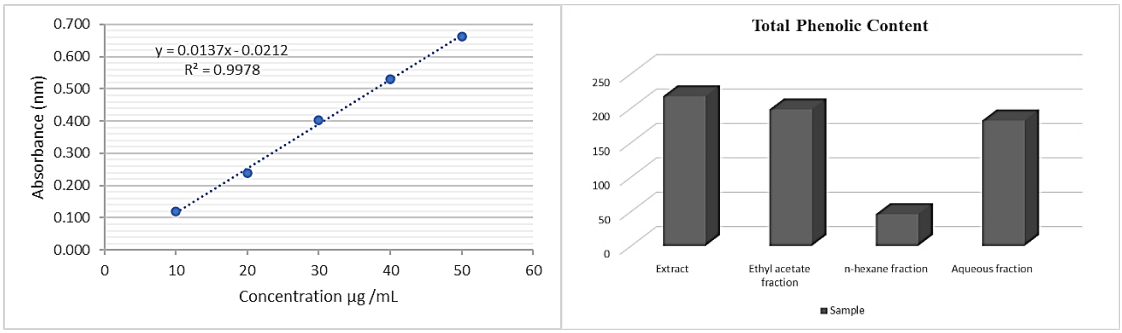
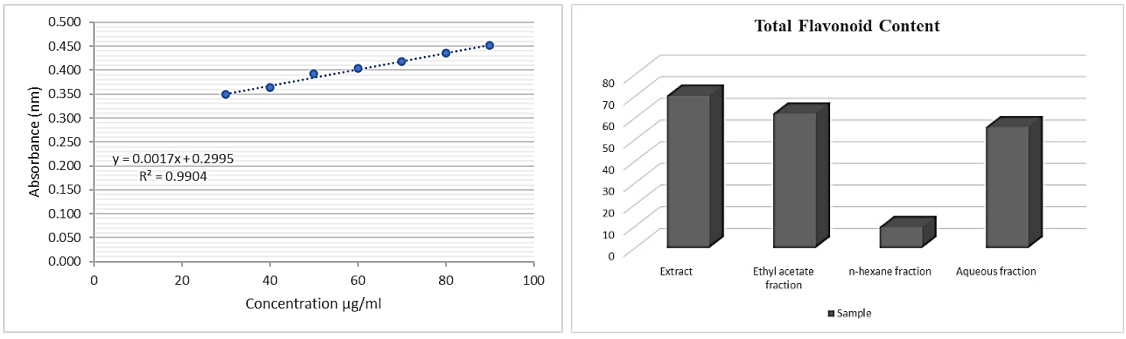


Figure 1. Gallic acid standard curve (a) and total phenolic content of bay leaves extract and fraction

The ethyl acetate fraction exhibited the highest total phenolic content, consistent with its semi-polar nature (polarity index 4.4), which efficiently extracts phenolic compounds such as polyphenols and flavonoids, suggesting a higher concentration of bioactives post-fractionation compared to the crude extract [13], [14]. The aqueous fraction, characterized by lower phenolic content, contained polar phenolic glycosides due to its high polarity (index 10.2). However, this fraction had fewer phenolics compared to the ethyl acetate fraction. On the other hand, the n-hexane fraction exhibited the lowest phenolic content, attributed to its non-polar nature (index 0.1), which favors the extraction of non-phenolic substances such as lipids and terpenoids rather than polar or semi-polar phenolics. These observations underscore the ethyl acetate fraction as the richest source of phenolic compounds, illustrating successful purification during fractionation [15].

3.2. Total Flavonoid Content

The total flavonoid concentration is determined using a quercetin standard curve, and the results are expressed as milligrams of quercetin equivalents per gram (mg QE/g). The relationship between quercetin concentration and absorbance is represented by the equation y = 0.0017x – 0.2995, with a correlation coefficient of r = 0.9904, as depicted in Figure 2a. The methanol extract and its fractions, namely ethyl acetate, n-hexane, and aqueous, showed flavonoid content percentages of 70.29 ± 0.59, 62.06 ± 1.76, 9.71 ± 2.56, and 55.78 ± 0.59, respectively, as seen in Figure 2b.



(**a**) (**b**)

Figure 2. Quercetin standard curve (a) and total flavonoid content of bay leaves extract and fraction

Figure 2b illustrates the distribution of flavonoid compounds, with the ethyl acetate fraction (polarity index 4.4) and water solvent (polarity index 10.2) exhibiting higher flavonoid content than the n-hexane fraction (polarity index 0.1), aligning with the polar to semi-polar nature of flavonoids. These findings are consistent with previous reports showing wide variation in flavonoid content of bay leaf extracts, depending on solvent polarity, plant origin, and analytical conditions. For instance, studies have reported values ranging from approximately 15–166 mg QE/g for ethanol-based extracts [16,17], highlighting the influence of extraction parameters and underscoring the need for

standardized methods in comparative evaluations. The observed variations likely stem from differences in extraction solvents (methanol vs. 96% ethanol), plant conditions (e.g., geographical location and harvest time), and analytical methods (e.g., sample concentration and quercetin calibration).

3.3. Free Radical Scavenging Activity

Figure 3 shows the DPPH assay results for the free radical scavenging activity of the bay leaves extract and its fractions. The activity of scavenging free radicals is documented as the inhibitory concentration (IC50). The inhibitory concentrations (IC_{50}) of the methanol extract and fractions (ethyl acetate, n-hexane, and aqueous) were 55.01 ± 0.186, 41.71 ± 0.617, 78.74 ± 0.827, 60.57 ± 0.268, respectively. The ethyl acetate fraction had an average IC_{50} of 41.71 ± 0.617 μg/mL, indicating the highest free radical scavenging activity among the fractions and extracts, comparable to that of ascorbic acid (9.61 ± 0.138 μg/mL), a strong standard antioxidant. This is consistent with the semi-polar nature of ethyl acetate, which is effective in extracting phenolic and flavonoid compounds, as indicated by the high total phenolic content of 198.20 mg GAE/g and flavonoids of 62.06 mg QE/g in this study, both of which are positively correlated with free radical scavenging activity.

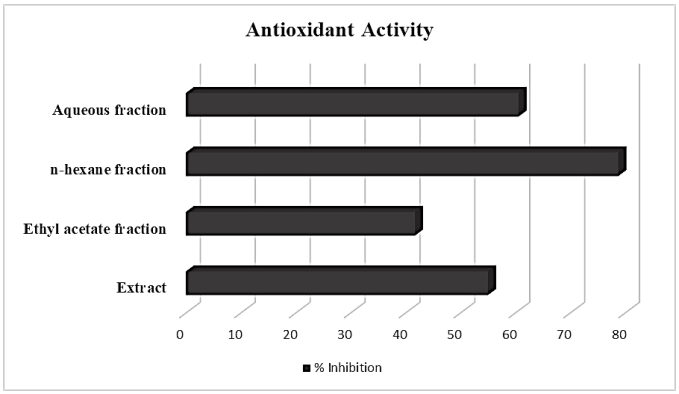


Figure 3. DPPH activity of bay leaves extract and fraction

The ethyl acetate fraction of bay leaf in this study exhibited an IC_{50} value of 41.71 μg/ml, indicating slightly better antioxidant activity compared to a previous study reporting an IC50 of 47.77 μg/ml [18]. This difference may stem from variations in extraction methods, environmental growth conditions, and bioactive compound content, particularly phenolic and flavonoid levels, which are key contributors to antioxidant effects. However, the relatively high IC_{50} value suggests that the fraction's antioxidant potential remains limited compared to standard compounds like vitamin C, which typically exhibit much lower IC_{50} values [19], [20], [21].

Table 1. Total phenolic, total flavonoid, and antioxidant activity

Sample	Total Phenolic (mgGAE/g)	Total Flavonoid (mgQE/g)	DPPH IC50 (μg/ml)
Extract	217.42 ± 0.42	70.29 ± 0.59	55.01 ± 0.186
Ethyl acetate fraction	198.20 ± 0.42	62.06 ± 1.76	41.71 ± 0.617
n-hexane fraction	46.13 ± 0.73	9.71 ± 2.56	78.74 ± 0.827
Aqueous fraction	182.14 ± 0.42	55.78 ± 0.59	60.57 ± 0.268

4. CONCLUSION

Research on the extract and fractions of *Syzygium polyanthum* Wight demonstrates that bay leaves are a significant source of bioactive compounds with substantial antioxidant capacity. Quantitative analysis revealed a high total phenolic content, particularly in the ethyl acetate fraction (198.20 ± 0.42 mg GAE/g), alongside a considerable flavonoid content (62.06 ± 1.76 mg QE/g). Evaluation of antioxidant activity through the DPPH assay indicated that the ethyl acetate fraction exhibited the

lowest IC50 value (41.71 ± 0.617 μg/mL), reflecting robust free radical scavenging ability, approaching the efficacy of ascorbic acid (9.61 ± 0.138 μg/mL). The predominant phenolic and flavonoid content in the ethyl acetate fraction showed a positive correlation with its antioxidant activity, underscoring the pivotal role of these compounds in mitigating oxidative damage. These findings validate the pharmacological potential of bay leaves as a prime candidate for the development of antioxidant-based phytopharmaceutical formulations. In particular, the present work provides reliable and updated data on the ethyl acetate fraction enriched in phenolic and flavonoid compounds with superior antioxidant capacity thereby supporting its further exploration for future pharmacological or phytopharmaceutical applications.

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