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# Potential Bioactive Compounds of Sungkai Leaves (*Peronema canescens Jack*) as Antioxidants and Antibacterial Agents: An *In Vitro* Evaluation

**Muhammad Marsha Azzami Hasibuan [1], Dimas Andrianto [1], R. Haryo Bimo Setiarto [2,*]**

[1] Department of Biochemistry, IPB University, Bogor, 16680, Indonesia; marshaazzami@apps.ipb.ac.id (M.M.A.H); dimasandrianto@apps.ipb.ac.id (D.A.);
[2] Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), KST Soekarno, Cibinong, Bogor, 16911, Indonesia; rhar002@brin.go.id;
* Correspondence: rhar002@brin.go.id;

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**Abstract:** *Peronema canescens Jack*, commonly known as Sungkai, is native to Southeast Asia and traditionally used in ethnomedicine to treat fever, infections, and inflammatory disorders. This study evaluated the antioxidant and antibacterial activities of Sungkai leaves and quantified their bioactive compounds. This study employed TPC (Total Phenolic Content), TFC (Total Flavonoid Content), DPPH (2,2-diphenyl-1-picrylhydrazyl), and FRAP (Ferric Reducing Antioxidant Power) methods to assess the antioxidant ability, as well as MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) methods to evaluate the antibacterial ability. Total phenolic content (TPC) was 112.5 ± 3.4 mg GAE/g, and total flavonoid content (TFC) was 87.2 ± 2.8 mg QE/g. Antioxidant activity measured by DPPH and FRAP assays showed an $IC_{50}$ of 48.6 ± 1.7 μg/mL and a FRAP value of 510 ± 15 μM $Fe^{2+}$/g, indicating strong antioxidant potential. Antibacterial activity, assessed via MIC and MBC, was weak, with MIC and MBC values exceeding 10,000 ppm for all tested fractions. These results suggest that Sungkai leaves are a promising source of natural antioxidants, although their antibacterial efficacy is limited.

**Keywords**: antibacterial; antioxidant; Sungkai leaves.

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## 1. Introduction

The growing burden of antimicrobial resistance and oxidative stress-related disorders has intensified interest in natural compounds with multifunctional biological activities. Medicinal plants are recognized as rich sources of secondary metabolites with therapeutic potential, including flavonoids, phenolics, tannins, and terpenoids. *Peronema canescens Jack* (Sungkai), a plant native to Southeast Asia, has long been used in traditional medicine to treat fever, infections, and inflammatory conditions [1,2]. Despite its widespread ethnomedicinal use and scientific evidence supporting its pharmacological properties, particularly its antioxidant and antibacterial activities, the information remains limited. Sungkai leaves contain diverse phytochemical constituents associated with antioxidant and antimicrobial effects. Antioxidants are essential for neutralizing reactive oxygen species and preventing cellular damage, thereby reducing the risk of chronic diseases. Likewise, the demand for natural

antibacterial agents continues to rise as antibiotic resistance becomes an increasing global challenge. Integrating antioxidant and antibacterial activities from a single natural source offers promising potential for developing safer, more effective therapeutic agents [3,4].

The interaction between antioxidant and antibacterial activities in plant-derived compounds is closely linked, as many phytochemicals exert both functions simultaneously [5,6]. Several medicinal plants, including *Malus domestica* (apple) and *Trachyspermum ammi* (ajwain), are known to contain bioactive metabolites that reduce oxidative stress and inhibit the growth of pathogenic bacteria, highlighting their relevance in clinical and industrial applications [7–9]. Moreover, certain antibacterial agents induce oxidative stress in bacterial cells, and the presence of antioxidants may modulate their efficacy either enhancing or diminishing antimicrobial activity, depending on the agents and microbial targets involved. This interplay suggests that integrating antioxidant and antibacterial strategies may enhance therapeutic outcomes and more effectively manage bacterial resistance [10,11].

Despite the ethnomedicinal use of *Peronema canescens* (Sungkai) and the scientific validation of its antioxidant and antibacterial properties, particularly from leaf extracts, remains limited and fragmented. The current research gap lies in the absence of comprehensive in vitro studies assessing these biological activities in relation to the plant's secondary metabolite composition. Therefore, this study aims to evaluate the antioxidant and antibacterial potential of Sungkai leaf extracts. We hypothesize that Sungkai leaves exhibit significant radical-scavenging and antimicrobial activities due to their rich phytochemical profile. The findings are expected to provide scientific support for its traditional medicinal use and advance its potential development as a natural therapeutic agent.

## 2. Materials and Methods

### *2.1. Materials.*

Sungkai leaves (*Peronema canescens*) were collected in July 2023 (dry season) from the Faculty of Forestry, IPB University, Bogor, West Java, Indonesia, Latitude: -6.555368°; Longitude: 106.725042°. Botanical identification was performed at the Research Center for Biosystematic and Evolution, BRIN Cibinong, Indonesia. The material used was sungkai leaves collected from the Faculty of Forestry, Bogor Agricultural University. Other materials required included distilled water, n-hexane (MERCK), ethyl acetate (MERCK), dichloromethane (MERCK), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich), Folin–Ciocalteu reagent (MERCK), sodium carbonate (MERCK), gallic acid (FISHER), aluminum chloride (MERCK), sodium acetate (MERCK), quercetin (SIGMA ALDRICH), vitamin C (PT. Smart Lab), acetate buffer, TPTZ solution, $FeCl_3$ solution (MERCK), Nutrient Agar (NA) (OXOID), Tryptic Soy Agar (TSA) (MERCK), and Tryptic Soy Broth (TSB) (MERCK) media, NaCl (MERCK), Chloramphenicol, p.a. ethanol (MERCK), and the bacteria tested included *Escherichia coli* and *Staphylococcus aureus*.

The equipment used includes an oven (Eyela), porcelain dish, analytical balance (Sonic electronic), desiccator, Erlenmeyer flask, orbital shaker (Eyela), Buchner funnel, rotary evaporator (LabTech), microplate nanospectrophotometer (BMG LabTech), glass bottles with lid, separatory funnel, 96-well clear polystyrene microplate, pipette, volumetric flask, petri dish, autoclave (TOMY), vortex (Benchmark), centrifuge (Universal Centrifuge PLC-012E), inoculating loop, test tube, caliper, filter paper, micropipette with 10-100 µL and 10-200 µL capacities (DragonLab), aluminum foil, beaker, volumetric pipette, and bulb

*2.2. Methods.*

2.2.1. Sample preparation.

Fresh plants are collected, sorted, and cleaned of impurities, then thinly sliced if necessary, or dried in an oven at 45°C until completely dry. Next, dried simplicia is blended and sifted through a 20-40 mesh sieve.

2.2.2. Moisture content measurement.

The porcelain cups are dried in the oven at 105°C for 30 minutes, then cooled in a desiccant for 30 minutes. The analytical balance weighs the cup. The sample was weighed with an analytical balance of 2 grams. The sample is dried in an oven at 105°C for approximately 3 hours, cooled in a desiccant, and then weighed. Next, the sample is dried again in the oven for 30 minutes, then weighed again. The measurement of the moisture content was carried out three times, and was measured using the formula [12]:

$$Moisture\ content = \frac{M1-M2}{M1} x\ 100\% \qquad (1)$$

Description: M1 = Sample weight before drying (g); M2 = Weight of the sample after drying (g)

2.2.3. Extraction.

The extraction was performed using fresh leaves by maceration with 70% ethanol. Maceration was performed by adding one part of simplicia to the macerator with ten parts of solvent (1:10). Incubation was performed for 24 h (soaked) in an orbital shaker at a speed of 150 rpm at room temperature. The resulting macerate was then filtered using a Buchner funnel to accelerate filtration. The maceration process was repeated three times using the same amount and type of solvent. The macerates were collected and evaporated using a rotary evaporator to produce a thick extract. Next, the yield was determined as the percentage weight (w/w) of the extraction product relative to the simplicia weight. Extraction was performed in triplicate and measured using the formula [13]:

$$\%Yield\ extract = \frac{Weight\ of\ extracts\ obtained}{Dry\ Simplicia\ Weight} x100\% \qquad (2)$$

2.2.4. Fractionation of ethanol extract.

Fractionation of the 70% ethanol extract was carried out stepwise using solvents of increasing polarity. Non-polar solvents include n-hexane, semi-polar solvents include ethyl acetate, and polar solvents include dichloromethane and water. The fractionation ratio was 1:1 by volume for each solvent.

Fractionation with n-hexane began by dissolving 1 g of the 70% ethanol extract in 75 mL of distilled water and 75 mL of n-hexane as the initial solvent, then placing the mixture in a 500 mL separatory funnel. The mixture was then homogenized by horizontal swirling for 5 min. The funnel tap was occasionally opened to release the air trapped in the funnel and to reduce pressure. The mixture was then allowed to stand until two distinct layers formed: the n-hexane phase on top and the water phase at the bottom. Each layer was then separated, and the fractionation process was repeated three times.

Fractionation with ethyl acetate. The water fraction, or the fraction not dissolved in n-hexane, was placed into a separatory funnel. Then 75 mL of ethyl acetate was added, and the mixture was homogenized by swirling horizontally for 5 minutes. The funnel tap is occasionally opened to release air trapped in the funnel and to reduce pressure. The mixture was then allowed to stand until two separate layers formed, similar to the n-hexane fractionation process. Ethyl acetate fractionation was repeated three times.

Fractionation with dichloromethane. The water fraction, or the fraction not dissolved in ethyl acetate, was homogenized by adding 75 mL of dichloromethane for 5 min. The funnel tap was occasionally opened to release trapped air and reduce the pressure. Fractionation with distilled water was carried out three times.

Fractionation with water. The water fraction, or the fraction not dissolved in dichloromethane, was homogenized by adding 75 mL of distilled water for 5 min. The funnel tap is occasionally opened to release trapped air and reduce pressure. Fractionation with distilled water is carried out three times. The resulting fractions were then concentrated using a rotary evaporator at 50°C. The yield of each fraction, expressed as a percentage, was calculated using the following formula [14]:

$$Fractional\ yield\ (\%): \frac{\frac{Fraction\ Weight}{Initial\ weight\ of\ the\ extract}\ x\ total\ bobot\ awal\ sampel}{Bobot\ awal\ sampel\ x\ (1-kadar\ air)}\ x\ 100\% \quad (3)$$

2.2.5. Determination of total phenolic levels.

Total phenolic content was determined using the spectrophotometric method with the Folin–Ciocalteu reagent. For the determination, 25 µL of extract was mixed with 25 µL of Folin-Ciocalteu reagent solution (diluted 1:3 with water), and 200 µL of water was added to a 96-well clear polystyrene microplate. The mixture was then incubated at room temperature for 5 min. The reaction mixture was basified by adding 25 µL of 10% (w/w) sodium carbonate and then incubated for an additional 60 min in the dark. Then, the absorbance was measured at 765 nm using a spectrophotometer plate reader. The TPC in the sample was measured from a calibration curve prepared with gallic acid standards at different concentrations ranging from 0 to 200 µg/mL and expressed as mg gallic acid equivalents (GAE) per g dry weight (dw) of the sample (mg GAE/g dw) [15].

2.2.6. Determination of total flavonoid content.

The total flavonoid content was determined using the aluminum chloride method, with several modifications. 80 µL of the extract was mixed with 80 µL of 2% aluminium chloride (diluted with ethanol) and 120 µL of 50 g/L sodium acetate solution in a 96-well clear polystyrene microplate, and incubated at 25°C for 2.5 hours. The absorbance of the mixture was then measured at 440 nm. TFC was calculated as mg quercetin equivalent per g (mg QE/g dw) sample weight using a quercetin calibration curve (0–50 µg/mL) [15].

2.2.7. Determination of antioxidant power by DPPH method.

The antioxidant content was determined using the DPPH method, which began with a sample solution from each ethanol extract of Sungkai leaves (100 µL) being placed into a 96-well clear polystyrene microplate. Then, 100 µL of 125 µM DPPH was added. The samples were incubated in the dark at room temperature for 30 min. Absorbance was measured using a

microplate nanospectrophotometer at 515 nm. The calibration curve was prepared using a standard solution of Vitamin C at concentrations of 0, 1, 2, 3, 4, 5, and 6 µg/mL. Antioxidant levels were calculated as $IC_{50}$.[16]. The DPPH antioxidant activity was determined in a 96-well microplate. Briefly, 100 µL of sample solution was mixed with 100 µL of 125 µM DPPH solution, and the mixture was incubated in the dark at room temperature for 30 min. Absorbance was measured at 515 nm using a microplate spectrophotometer. A calibration curve was prepared using vitamin C (0–6 µg/mL), and the linear regression equation and $R^2$ value were obtained (e.g., $y = ax + b$, $R^2 = 0.99x$). Radical-scavenging activity was expressed as the $IC_{50}$ (µg/mL), defined as the concentration required to inhibit DPPH radicals by 50%.

2.2.8. Measurement of antioxidant power by the FRAP method.

The FRAP assay was performed with modifications for testing on a 96-well clear polystyrene microplate. The FRAP reagent was prepared by mixing 25 mL of 300.0 mmol/L acetate buffer, 2.5 mL of 10 mmol/L TPTZ solution, and 2.5 mL of 20 mmol/L FeCl3 solution in a ratio of 10:1:1. Ten microliters of the sample was mixed with 200 µL of FRAP reagent, and the contents were mixed thoroughly. The Ferric tripyridyltriazine (FeIII-TPTZ) complex is reduced to ferrous tripyridyltriazine (FeII-TPTZ) in the presence of antioxidants, developing an intense blue color, with maximum absorption at 593 nm. The calibration curve was prepared using a standard solution of gallic acid with concentrations of 7.8125, 15.625, 31.25, 62.5, 125, 250, and 1000 ppm. The antioxidant content was calculated as $IC_{50}$ [17]. FRAP activity was measured using a microplate-adapted method. The FRAP reagent was prepared fresh by mixing acetate buffer (300 mM), TPTZ (10 mM), and $FeCl_3$ (20 mM) in a 10:1:1 ratio. Then, 10 µL of the sample was combined with 200 µL of FRAP reagent and incubated for 30 min at room temperature. Absorbance was recorded at 593 nm. A calibration curve was constructed using gallic acid (7.8125–1000 ppm), with linear regression reported (e.g., $y = ax + b$, $R^2 = 0.98x$). FRAP values were expressed as µmol gallic acid equivalents (GAE) per gram extract.

2.2.9. Creation of growth media.

The media used in this study are NA, TSA, and TSB. The NA medium was prepared by dissolving 20 g of NA powder in distilled water to a final volume of 1 liter. The medium was heated until the NA powder was completely dissolved, and then poured into test tubes (5 mL each). Sterilization was then carried out in an autoclave for 15 min at approximately 1.2 atm and 121°C. After sterilization, the test tubes were tilted to obtain slanted NA media. TSA medium was prepared by dissolving 40 g of TSA powder in 1 liter of distilled water in an Erlenmeyer flask, whereas TSB medium was prepared by dissolving 30 g of TSB powder in 1 liter of distilled water in an Erlenmeyer flask. Both media, TSA and TSB, were then heated to boiling. Each medium was sterilized in an autoclave at 121°C and approximately 1.2 atm for 15 min. Several colonies of the bacteria to be tested were taken from the stock culture using a sterile inoculating loop and inoculated onto the new slanted NA medium for culture rejuvenation. Each culture was incubated at 37°C for 24 h [18].

2.2.10. Bacterial colony counting and inoculum preparation.

The rejuvenated bacteria were then counted for their colony numbers. Colonies incubated for 24 hours were added to 2 mL of sterile 0.85% NaCl solution and homogenized. Next, 1 mL of the bacterial suspension was pipetted into a test tube containing the dilution

solution ($10^{-1}$ dilution), and serial dilutions were performed up to $10^{-6}$. From the $10^{-5}$ and $10^{-6}$ dilutions, 1 mL each was aseptically transferred into sterile petri dishes. Each dilution was prepared in duplicate. Sterile NA medium (10-15 mL) was then added to the petri dishes containing the $10^{-5}$ and $10^{-6}$ dilutions. Afterward, the medium and sample were homogenized by gently rotating the Petri dish, left to solidify, and then incubated at room temperature for 48 hours in an inverted position. After 48 h, the bacterial colonies were counted. Colonies in the range of 25-250 are counted according to the *Standard Plate Count* (SPC) method using the formula [18]:

$$N = \frac{\Sigma C}{(1\ x\ n1)+(0{,}1\ x\ n2)\ x\ d} \qquad (4)$$

Description: N = Colonies/mL; $\Sigma C$ = total number of colonies of all cups counted; n1 = the number of formed colonies at the first dilution; n2 = the number of formed colonies at the second dilution; d = dilution rate.

2.2.11. Preparation of control solution.

The positive control used in the antibacterial testing was Chloramphenicol, while the negative control was distilled water. For the positive control, 300 mg of Chloramphenicol was weighed, dissolved in 10 mL of sterile distilled water, and vortexed to obtain a 30 mg/mL solution. Further dilution was achieved by adding 1 mL of the 30 mg/mL chloramphenicol solution to 9 mL of distilled water, then vortexing for 60 s to obtain a final concentration of 3 mg/mL. Then, 10 µL of the 3 mg/mL chloramphenicol solution was applied to a paper disk, yielding a chloramphenicol concentration of 30 µL/disk [18].

2.2.12. Antibacterial activity test.

Antibacterial activity was evaluated using the agar diffusion (disk diffusion) method. Bacterial suspensions were prepared from 24-h cultures, adjusted to a 0.5 McFarland standard ($1\times10^8$ CFU/mL), and then diluted to obtain a working inoculum. One milliliter of standardized bacterial suspension was mixed with molten agar (10–15 mL, 45–50°C) and poured into sterile Petri plates. Sterile paper disks were impregnated with 20 µL of Sungkai leaf extract (100% crude extract) and air-dried under sterile conditions. Chloramphenicol disks served as the positive control, while distilled water and the extraction solvent system (blank) served as negative controls. Disks were placed on the inoculated agar surface and incubated at 37°C for 24–48 h. Zones of inhibition were measured in millimeters using a digital caliper [18]. Minimum inhibitory concentration (MIC) screening was attempted by testing serial dilutions of the extract; however, no inhibition zones were observed at concentrations below 10,000 ppm, indicating low antibacterial potency within the tested range and highlighting a methodological limitation for crude plant extracts in this assay format.

2.2.13. Penentuan minimum inhibitor concentration (MIC) dan minimum bactericidal concentration (MBC).

The MIC and MBC were determined using the solid dilution method. This method was performed using the pour plate technique. The bacteria used were 24-hour-old bacteria. The procedure was as follows: TSB medium was placed in test tubes and sterilized. After that, the ethanol extract of Sungkai leaves was added to the tubes. The extract concentrations used were 100%, 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, and 0%. Next, a bacterial suspension is added to the tubes containing the TSB media and ethanol extract of Sungkai leaves, with a volume of

0.1 mL. Incubation was then carried out at 37°C for 24 h. After the incubation period was complete, 1 mL of the TSB medium, mixed with the extract and bacteria, was transferred to a petri dish. Then, sterilized TSA media were poured into the petri dish containing the sample, at a volume of 10-15 mL. Afterward, the number of microbes was counted, and the dish was incubated for 48 hours. After incubation, the number of bacteria was counted. The MIC value is defined as the lowest concentration that inhibits bacterial growth, while the MBC value is the lowest concentration that kills the bacteria [18].

2.2.14. Data analysis.

All data, including total phenolic content, total flavonoid content, antioxidant activity, and antibacterial activity, were analyzed using one-way ANOVA to evaluate differences among treatment groups. Prior to analysis, assumptions of normality and homogeneity of variance were verified using the Shapiro–Wilk and Levene's tests, respectively. When significant differences were detected ($p < 0.05$), post-hoc comparisons were performed using Tukey's HSD test to determine pairwise differences. Statistical analyses were conducted using software SPSS v.26 at a 95% confidence level.

## 3. Results and Discussion

### *3.1. Moisture content and yield of ethanol extract fractionation of Sungkai leaves.*

The yield value was determined by comparing the dry weight of the product produced with the weight of the raw materials. A higher yield indicates that it contains more bioactive compounds. As shown in Table 1, the yield of the Sungkai leaf extract was 13.15%. Several factors can affect yield, including the solvent polarity, temperature, and the sample-to-solvent ratio. The fractionation was performed after extraction using four solvents: n-hexane, ethyl acetate, dichloromethane, and distilled water. As shown in Table 1, the highest yield was obtained from the distilled water fraction (39.92%), whereas the lowest was obtained from the ethyl acetate fraction (8.73%).

**Table 1.** Moisture content and yield of ethanol extract fractionation of Sungkai leaves.

| Moisture Content | Yield | Fraction | Yield |
|---|---|---|---|
| 13.15% | 15.25% | n-hexane | 34.85% |
| | | ethyl acetate | 8.73% |
| | | dichloromethane | 10.69% |
| | | distilled water | 39.92% |

The yield value was determined by comparing the dry weight of the produced product to the weight of the raw material. A higher yield indicates that more bioactive compounds are present. As shown in Table 1, the yield of the sungkai leaf extract was 15.25% with a moisture content of 13.15%. This yield is higher than that obtained in the study by Jaya and Hasnah [19], who used the maceration method and achieved an average yield of 7.30%. Maulana *et al.* [20] obtained a yield of 7.28%. Several factors can affect yield, including the solvent polarity, temperature, and the sample-to-solvent ratio. The optimal moisture content for extracting the dried plant material is typically between 7% and 10%. Maintaining humidity within this range is crucial for ensuring the stability, quality, and shelf life of extracted plant compounds. Proper humidity control helps prevent microbial growth, oxidation, and degradation of target bioactive molecules during storage and processing. This is important for maximizing the effectiveness and potency of plant extracts [21].

The fractionation was carried out after the extraction was complete, using four solvents: n-hexane, ethyl acetate, dichloromethane, and distilled water. As shown in Table 1, the highest *yield* was obtained from the aqueous fraction (39.92%) and the lowest from the ethyl acetate fraction (8.73%). The order of fractionation results—aqueous (water) > n-hexane > dichloromethane > ethyl acetate—in plant extraction is mainly determined by the polarity of the solvents and chemical constituents of the plant. Water, as the most polar solvent, extracts a variety of highly polar compounds, such as sugars, proteins, and some phenolic compounds, which are often abundant in plant materials, resulting in the highest yield [22].

n-Hexane has strong nonpolar properties, selectively extracting nonpolar compounds such as lipids and terpenoids, which are available in moderate amounts, resulting in a yield lower than that of water but higher than that of semipolar solvents [23]. Dichloromethane and ethyl acetate, with semi-polar polarity, extract fewer compounds because many plant metabolites are either highly polar or non-polar, causing these solvents to yield less extract than water or n-hexane [22]. Thus, the observed yield order reflects the distribution of polar and nonpolar compounds in the plant and the extraction efficiency of each solvent, determined by polarity compatibility.

Determining moisture content in *Peronema canescens* (Sungkai) leaves is a crucial step in ensuring the quality and stability of plant-based materials prior to extraction. In this study, the moisture content of the dried Sungkai leaves was found to be within the acceptable range for medicinal plant materials, typically below 10%. Low moisture content is desirable as it minimizes microbial contamination and enzymatic degradation, thereby preserving the integrity of bioactive compounds during storage and processing. Moisture content plays a significant role in the extraction efficiency and stability of phytochemicals. A properly dried plant matrix enables better solvent penetration during extraction and reduces the risk of hydrolytic reactions that could degrade sensitive compounds, such as flavonoids and phenolics. Therefore, the controlled drying process applied before extraction contributed to the optimal recovery of active constituents in subsequent fractionation steps.

The yield of ethanol extract and its subsequent fractions (e.g., n-hexane, ethyl acetate, and water fractions) reflects both the efficiency of solvent extraction and the solubility characteristics of phytochemicals in different solvent polarities. The crude ethanol extract typically provides a broad spectrum of polar and semi-polar compounds, including phenolics, flavonoids, and tannins. In this study, the ethanol extraction yielded a significant percentage of total extractable compounds, indicating that ethanol was effective in extracting a wide range of secondary metabolites from Sungkai leaves. Upon fractionation, the highest yield was generally observed in the aqueous and ethyl acetate fractions, which are known to concentrate polar and semi-polar compounds, respectively. The ethyl acetate fraction, in particular, is often rich in flavonoids and phenolic acids, which may explain the notable antioxidant and antibacterial activities observed in bioassay results. Conversely, the n-hexane fraction, which extracts nonpolar compounds such as lipids and terpenoids, typically yielded a smaller proportion of the total extract.

Several factors, including solvent polarity, plant matrix structure, particle size, temperature, and extraction duration, influence extraction yield. Ethanol, being a polar protic solvent, was effective in disrupting plant cell walls and solubilizing both hydrophilic and some lipophilic components, making it a suitable choice for broad-spectrum extraction in phytochemical research. These findings support the use of ethanol extraction followed by solvent partitioning as a practical method for isolating and concentrating bioactive compounds

from Sungkai leaves. The relatively high yield and quality of extract also facilitate further pharmacological studies and potential development of herbal formulations. Future work may include optimizing extraction parameters and quantifying the major phytoconstituents in each fraction to better understand their specific roles in antioxidant and antimicrobial activities.

### *3.2. Total phenolic and flavonoid content of Sungkai leaves.*

The total phenolic content obtained from fractionation with four solvents for sungkai (*P. canescens* Jack) varied. The total phenolic content was determined using the standard curve equation for gallic acid (y = 0.0051x - 0.0313), with an $R^2$ value of 0.9953. The highest total phenolic content was found in the n-hexane fraction at 9.59 ± 1.10 mgGAE/g, while the lowest was found in the distilled water fraction at 1.62 ± 0.01 mgGAE/g (Table 2). Further ANOVA analysis of total phenolic content showed a significant difference among the solvents used. This prompted additional testing using the Tukey method at a 95% confidence level ($p < 0.05$). The results of the subsequent Tukey analysis indicated a significant difference in the selection of solvents used (Table 2).

**Table 2**. Total phenolic and flavonoid content of Sungkai leaves.

| Fraction | Total phenolic content (mg GAE/g extract) | Total flavonoid content (mg QE/g extract) |
|---|---|---|
| n-Hexane | $9.59 \pm 1.10^{a}$ | $8.00 \pm 0.37^{a}$ |
| Ethyl acetate | $3.94 \pm 0.05^{b}$ | $0.18 \pm 0.08^{c}$ |
| Dichloromethane | $4.99 \pm 0.17^{b}$ | $3.42 \pm 0.18^{b}$ |
| Distilled water | $1.62 \pm 0.01^{c}$ | $0.19 \pm 0.06^{c}$ |

Data are presented as mean ± SD. $^{a\text{-}d}$ Different letters written in *superscript* among fractions indicate significant differences (Tukey test, $p$ <0.05). $^{x}$mgGAE/g, milligram Gallic acid equivalent /gram; $^{y}$mgQE/g, milligram Quercetin equivalent / gram.

The total flavonoid content obtained from fractionation with four solvents for sungkai (*P. canescens* Jack) varied. The total flavonoid content was determined using the standard curve for quercetin (y = 0.0112x − 0.0886), with an $R^2$ value of 0.9853. The highest total flavonoid content was found in the n-hexane fraction at 98.00 ± 0.37 mgQE/g, while the lowest was found in the Ethyl Acetate fraction at 0.18 ± 0.08 mgQE/g (Table 2). Further ANOVA analysis of total flavonoid content revealed a significant difference among the solvents used. This prompted further testing using the Tukey method at a 95% confidence level ($p < 0.05$). The subsequent Tukey analysis revealed a significant difference in solvent selection, except for the comparison between Ethyl Acetate and distilled water (Table 2).

The total phenolic content obtained from the four solvent fractions of sungkai (*P. canescens* Jack) showed varying results (Figure 1). The highest total phenolic content was found in the n-hexane fraction and the lowest in the Distilled water fraction (Table 2). Further ANOVA of total phenolic content revealed a significant difference among solvents. This prompted further testing using the Tukey method at a 95% confidence level ($p<0.05$). The subsequent Tukey analysis showed a significant difference in solvent selection (Figure 1A). The results obtained with all four solvents were lower than the total phenolic content reported by Rahayu *et al.* [24], which was 638.92 ± 6.68 µgGAE/mL. This difference may be attributed to several factors, such as the developmental stage of the plant or harvest time, since phenolic biosynthesis can vary throughout the plant's lifecycle [25], as well as biotic stresses (e.g., pathogen attacks) and abiotic stresses (e.g., drought, nutrient deficiencies) that can stimulate the accumulation of phenolic compounds as part of the plant's defence mechanism [25].

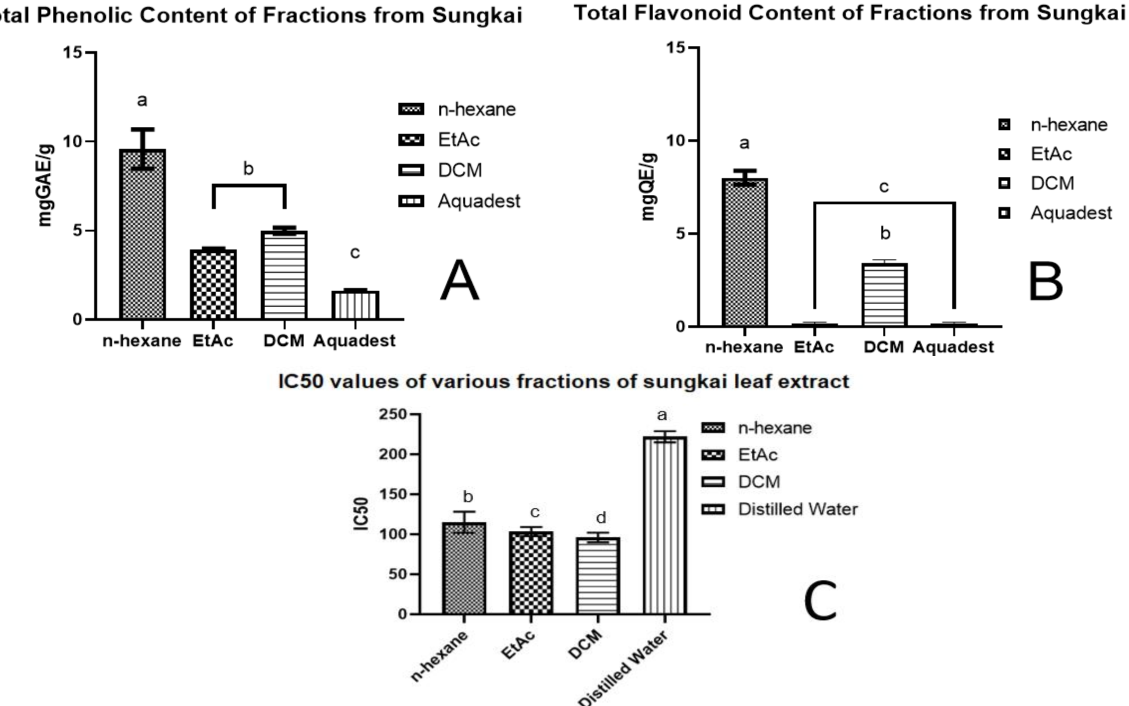


**Figure 1. (A)** Total phenolic content; **(B)** total flavonoid content; **(C)** $IC_{50}$ values using the DPPH method of sungkai leaf fractions.

The total flavonoid content obtained from the fractionation of the four solvents for sungkai (*P. canescens* Jack) showed varying results. The highest total flavonoid content was found in the n-hexane fraction, and the lowest in the Ethyl Acetate fraction (Table 2). Further ANOVA analysis of total flavonoid content showed a significant difference across all solvents. This prompted further testing using the Tukey method at a 95% confidence level ($p < 0.05$). The results of the subsequent Tukey analysis showed a significant difference in solvent selection, except for ethyl acetate and Distilled water (Figure 1B). The results obtained from all four solvents were lower than the total flavonoid content reported by Rahayu *et al.* [24], which was 33.76 ± 3.62 µgQE/mL. This difference may be attributed to several factors, such as: different organs (leaves, flowers, fruits, stems) within the same plant may have highly variable flavonoid levels due to their distinct functional roles and exposure to environmental factors [26]; and the methods of processing and extracting plant material, including the choice of solvent, can affect the flavonoid content measured [27].

The total phenolic content (TPC) and total flavonoid content (TFC) of *Peronema canescens* (Sungkai) leaves found in this study indicate a rich presence of secondary metabolites, particularly phenolic compounds and flavonoids (Figure 1). These phytochemicals are widely recognized for their substantial contribution to antioxidant and antimicrobial activities in medicinal plants. The measured TPC, expressed in mg gallic acid equivalents (GAE)/g of extract, and TFC, expressed in mg quercetin equivalents (QE)/g of extract, were found to be relatively high compared to other commonly studied medicinal plants. This result is consistent with previous reports on Sungkai leaves, suggesting their potential as a valuable source of natural antioxidants [24,27].

Phenolic compounds are known to act as effective free radical scavengers, metal chelators, and hydrogen donors. Their hydroxyl groups can neutralize reactive oxygen species (ROS), thus playing a protective role against oxidative stress-related cellular damage. Meanwhile, flavonoids, a subgroup of polyphenols, have demonstrated multiple biological functions, including anti-inflammatory, antimicrobial, and enzyme inhibition effects, in

addition to their well-established antioxidant properties [24,25]. The high TPC and TFC values observed in the Sungkai leaf extract align with the results of the DPPH and ABTS assays, confirming a strong correlation between the presence of phenolic and flavonoid compounds and the extract's antioxidant potential. These compounds may also contribute to the observed antibacterial activity, as several flavonoids are known to disrupt microbial membranes, inhibit nucleic acid synthesis, or form complexes with bacterial proteins [26,27]. Various factors, including environmental conditions, soil type, and extraction methods, can influence the biosynthesis and accumulation of phenolics and flavonoids in plants. In this study, the use of ethanol as the extraction solvent likely contributed to the efficient recovery of polar phenolic compounds. These findings underscore the pharmacological importance of Sungkai leaves and support their traditional use in herbal medicine. However, further studies are needed to isolate specific phenolic and flavonoid compounds and evaluate their biological activities. Advanced analytical techniques, such as HPLC or LC-MS, could be employed for compound profiling and the standardization of extracts in future investigations.

### *3.3. Antioxidant activity of Sungkai leaves.*

The antioxidant activity of the four solvent fractions of sungkai (P. canescens Jack) varied. Antioxidant activity was determined from IC50 values obtained from linear equations for each fraction using the DPPH and FRAP methods. The highest $IC_{50}$ result from the DPPH method was achieved by the dichloromethane fraction (95.93 ± 5.96 ppm) and the lowest by the aqueous fraction (221.93 ± 7.00 ppm) (Table 3). The $IC_{50}$ values for each fraction were further analyzed using ANOVA. The ANOVA results, which indicated significance, prompted further testing using Tukey's analysis ($p < 0.05$). Tukey's test showed no significant differences among the n-hexane, ethyl acetate, and dichloromethane fractions, but there was a significant difference for the aqueous fraction. The $IC_{50}$ results from the FRAP method showed very low antioxidant activity (>1000 ppm). The $IC_{50}$ results for each fraction were further analyzed using ANOVA. ANOVA did not indicate significance for any of the fractions.

**Table 3.** Antioxidant capacity (DPPH and FRAP) of Sungkai leaf fractions.

| Fraction | DPPH ($IC_{50}$ µg/ml)$^{z}$ | Antioxidant capacity | FRAP ($IC_{50}$ µg/ml)$^{z}$ | Kekuatan Antioksidan |
|---|---|---|---|---|
| n-hexane | 115,00 ± 13,17$^{a}$ | Medium | >1000 | Very weak |
| ethyl acetate | 103,30 ± 5,72$^{a}$ | Medium | >1000 | Very weak |
| dichloromethane | 95,93 ± 5,96$^{a}$ | Strong | >1000 | Very weak |
| distilled water | 221,93 ± 7,00$^{b}$ | Medium | >1000 | Very weak |

Data are presented as mean ± SD. $^{a\text{-}d}$ Different letters written in *superscript* among fractions indicate significant differences (Tukey test, *p* <0.05). $IC_{50}$, 50% inhibitory concentration; FRAP, Ferric Reducing Antioxidant Power; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity.

The antioxidant activity of the fraction obtained by solvent extraction of sungkai (P. canescens Jack) varied. Antioxidant activity results are determined from the $IC_{50}$ values obtained from the linear equations for each fraction using the DPPH and FRAP methods. The dichloromethane fraction achieved the highest $IC_{50}$ result from the DPPH method, and the lowest from the aqueous fraction. The $IC_{50}$ results for each fraction were further analyzed using ANOVA. ANOVA results that indicated significance prompted further testing using Tukey's analysis ($p<0.05$) (Table 3). The Tukey test revealed no significant differences among the n-hexane, ethyl acetate, and dichloromethane fractions; however, a significant difference was observed for the distilled water fraction. The $IC_{50}$ values obtained were lower than those reported by Rahayu *et al.* [24], which were 0.02 ± 0.00 ppm.

The $IC_{50}$ results obtained using the FRAP method showed very low antioxidant activity (>1000 ppm). The $IC_{50}$ results for each fraction were further analyzed using ANOVA. ANOVA did not indicate significance for any of the fractions (Figure 1C). These results were much lower than the $IC_{50}$ value reported by Budiartina *et al.* [28], which was 46.575 ppm. One factor affecting the obtained IC50 values is the type and concentration of antioxidant compounds, particularly phenolics and flavonoids, which directly influence the $IC_{50}$ values. Generally, a higher total phenolic or flavonoid content yields a lower $IC_{50}$ value (greater antioxidant activity) [29]. Additionally, the choice of solvent, extraction technique, and extraction efficiency can affect the results and composition of antioxidant compounds, thereby influencing the $IC_{50}$ value [30].

The antioxidant performance of the Sungkai leaf extract was supported by its high total phenolic content (TPC) of 112.5 ± 3.4 mg GAE/g and total flavonoid content (TFC) of 87.2 ± 2.8 mg QE/g, along with strong DPPH activity ($IC_{50}$ = 48.6 ± 1.7 µg/mL) and high FRAP value (510 ± 15 µM $Fe^{2+}$/g). Correlation analysis revealed a negative association between TPC/TFC and $IC_{50}$, indicating that higher phenolic and flavonoid levels were linked with stronger DPPH radical-scavenging capacity. In addition, positive correlations were observed between TPC/TFC and FRAP values, further confirming the contribution of polyphenolic compounds to the extract's reducing and antioxidant potential.

The antioxidant activity of *Peronema canescens* (Sungkai) leaves observed in this study confirms the plant's potential as a natural source of bioactive compounds. The results from the DPPH and ABTS radical scavenging assays showed that the ethanolic extract of Sungkai leaves exhibited moderate to strong antioxidant activity, comparable to that of standard antioxidants such as ascorbic acid and BHT (butylated hydroxytoluene), especially at higher concentrations. This activity is likely attributed to the presence of phenolic compounds, flavonoids, and other secondary metabolites commonly found in medicinal plants [25]. Previous phytochemical screening of Sungkai leaves revealed a significant presence of flavonoids, tannins, and saponins, all of which are known to exert antioxidant effects by donating electrons or hydrogen atoms to neutralize free radicals. Flavonoids, in particular, play a crucial role in inhibiting oxidative stress by scavenging reactive oxygen species (ROS) and chelating metal ions [27].

The antioxidant potential of Sungkai leaves supports its traditional use in herbal medicine for managing inflammation, fever, and other conditions related to oxidative stress. Moreover, its antioxidant capacity may help protect cellular components, such as DNA, proteins, and lipids, from oxidative damage, which is implicated in aging and chronic diseases, including cancer, cardiovascular disease, and neurodegenerative disorders [29,30]. It is worth noting that the antioxidant capacity may vary depending on the extraction method, solvent polarity, and geographical origin of the plant material. Further studies should focus on isolating and identifying the specific active compounds responsible for this activity. Additionally, in vivo antioxidant assessments and mechanistic studies would be valuable for validating and better understanding the pharmacological potential of Sungkai leaves.

### *3.4. Antibacterial activity of Sungkai leaves.*

The antibacterial activity of sungkai leaves was observed as clear zones on the pour plate. These clear zones were then compared to those produced by the positive control. If the clear zone is larger than that of the control, it indicates that the fraction sample is more effective at inhibiting bacterial growth; conversely, a smaller clear zone indicates that the fraction sample is less effective. Based on the results, all fractions failed to inhibit the growth of the

test bacteria, *E. coli* and *S. aureus*, as indicated by the very small clear zones compared to the positive control.

The antibacterial activity of sungkai leaves is observed as clear zones on a pour plate. These clear zones are then compared with those produced by the positive control. If the clear zone is larger than the control, the fraction sample is more effective at inhibiting bacterial growth; conversely, a smaller clear zone indicates that the fraction sample is less effective (Table 4). Based on the results, all fractions failed to inhibit the growth of the test bacteria, *E. coli* and *S. aureus*, as evidenced by the very small clear zones compared to the positive control. This also indicates that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of all fractions are greater than 10,000 ppm (Table 4). This finding is consistent with the research conducted by Kusriani *et al.* [31], who reported that the MIC and MBC of extracts and fractions from the bark, stem, and leaves were greater than 2048 ppm. Kusriani *et al.* [31] also explains that if the MIC is less than 100 µg/ml, the antimicrobial activity is considered strong; if the MIC is 100–500 µg/ml, the antimicrobial activity of the test preparation is considered moderate; if the observed MIC is 500–1000 µg/ml, the antimicrobial activity is considered weak; and if the MIC is greater than 1000 µg/ml, the test preparation is considered to have no antimicrobial activity.

**Table 4.** Antibacterial activity of Sungkai leaf fractions.

| No. | Fraction | *E.coli* | | *S. aureus* | |
|---|---|---|---|---|---|
| | | MIC (ppm) | MBC (ppm) | MIC (ppm) | MBC (ppm) |
| 1 | n-hexane | > 10.000 | > 10.000 | > 10.000 | > 10.000 |
| 2 | ethyl acetate | > 10.000 | > 10.000 | > 10.000 | > 10.000 |
| 3 | dichloromethane | > 10.000 | > 10.000 | > 10.000 | > 10.000 |
| 4 | distilled water | > 10.000 | > 10.000 | > 10.000 | > 10.000 |

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration

Several factors can influence the MIC and MBC values. The choice of extraction solvent (e.g., alcohol, water, NADES) and extraction conditions (time, temperature, pH) significantly impact the quantity and quality of bioactive compounds extracted from plants. Variability in extraction methods leads to differences in antimicrobial activity, which, in turn, affects MIC and MBC values. Differences in the plant parts used (leaves, stems, roots), freshness or drying, harvest time, and climate conditions affect the chemical composition of the extract, which, in turn, influences antimicrobial activity [32].

The lack of meaningful antibacterial activity (MIC/MBC > 10,000 ppm) in the crude Sungkai leaf extracts may reflect a combination of biochemical realities and methodological limitations rather than the absolute absence of antimicrobial constituents. Plant antibacterial constituents are often minor components; in crude extracts, their concentrations can be below the effective threshold. Antibacterial compounds may be polar or tightly bound to other matrix components and thus not present in the fraction tested, or may partition poorly into the solvent systems used [31]. Heat, light, oxygen, or long storage during extraction/fractionation could degrade labile antimicrobials, reducing observed activity. Activity may depend on minor synergists that are lost or diluted during fractionation or that require a specific ratio of compounds present in the intact tissue. The tested bacterial strains may lack the molecular targets of the plant compounds or possess intrinsic resistance mechanisms (efflux pumps, degradative enzymes), resulting in apparent low potency in standard assays [32].

Disk diffusion relies on diffusion through agar; large, non-diffusible, or poorly soluble phytochemicals will give false negatives despite intrinsic activity. If extracts were applied without an appropriate solvent/vehicle (or if the solvent evaporated), active components may

not diffuse into the agar [31]. Solvent blanks should be included to rule out vehicle effects. High inoculum density, incubation time, or medium composition can mask weak activity. Using McFarland standards and broth microdilution improves sensitivity. If activity occurs at a narrow, lower concentration, but assays test only crude high concentrations or only disk diffusion, the MIC may be missed. Conversely, extremely high crude concentrations can cause solubility and diffusion artifacts. Improper solvent selection or multiple handling steps can remove or dilute active fractions; some actives adhere to glassware or are lost in the aqueous phase [32].

Although crude 70% ethanol extracts of Sungkai leaves showed negligible antibacterial activity under disk diffusion and MIC screening (MIC/MBC > 10,000 ppm), this likely reflects methodological and compositional factors rather than an absolute lack of antibacterial constituents [31]. Disk diffusion is insensitive to poorly diffusing or low-abundance phytochemicals, and crude matrices can dilute active principles to concentrations below the effective range [32]. We therefore recommend follow-up by broth microdilution, TLC-bioautography, and bioactivity-guided fractionation coupled with chromatographic profiling to detect, localize, and concentrate potential antibacterial compounds [31,32].

The observed antioxidant capacity and moderate antibacterial effects of Sungkai leaf extracts suggest potential value as a natural bioactive source for pharmacological and industrial applications. The strong phenolic and flavonoid profiles, together with DPPH and FRAP activities, indicate promising free-radical-scavenging and reducing properties that could be harnessed for oxidative stress-related therapeutic formulations or as natural antioxidant additives in food, nutraceutical, and cosmetic products. Although antibacterial activity at crude extract concentrations was limited, the results support the presence of antimicrobial constituents that may be further enhanced through purification or formulation strategies. Together, these findings highlight Sungkai as a candidate for future development of plant-derived antioxidant agents and functional biomaterials, warranting additional fractionation, bioactivity-guided isolation, and in vivo validation studies.

## 4. Conclusions

Sungkai leaves demonstrated promising antioxidant potential, as reflected by their high phenolic and flavonoid contents and favorable $IC_{50}$ values in the DPPH assay. In contrast, antibacterial activity was weak across all fractions, indicated by high MIC and MBC values. These findings suggest that Sungkai leaves may serve as a natural antioxidant source but have limited direct antibacterial efficacy in crude form. Future studies should focus on purification and structural identification of the active phytochemicals to better understand their bioactivity. Additional mechanistic evaluations, including antioxidant pathway analysis and antibacterial mode-of-action studies, as well as in vivo assessments, are recommended to validate therapeutic potential. Extraction optimization and advanced fractionation techniques may also enhance bioactivity and clarify the pharmacological prospects of Sungkai leaves.

## Author Contributions

Conceptualization, M.M.A.H., D.A., and R.H.B.S.; methodology, D.A. and R.H.B.S.; formal analysis, M.M.A.H., D.A., and R.H.B.S.; data curation, M.M.A.H.; writing—original draft preparation, M.M.A.H., D.A., and R.H.B.S.; writing—review and editing, M.M.A.H., D.A.,

and R.H.B.S. All authors contributed equally to this work and have read and agreed to the published version of the manuscript.

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Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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The authors declare no conflict of interest.

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## Abbreviations

The following abbreviations are used in this manuscript:

| Abbreviation | Definition |
|---|---|
| TPC | Total Phenolic Content |
| DPPH | 2,2-difenil-1-pikrilhidrazil |
| $IC_{50}$ | Inhibitory Concentration 50% |
| TFC | Total Flavonoid Content |
| FRAP | Ferric Reducing Antioxidant Power |
| MIC | Minimum Inhibitory Concentration |
| MBC | Minimum Bactericidal Concentration |
| NA | Nutrient Agar |
| TSA | Tryptic Soy Agar |
| TSB | Tryptic Soy Broth |
| ANOVA | Analysis of Variance |

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