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Antibacterial Property Evaluation of Ethanolic Extract of *Plantago major L. and Its Fractions*

Evaluasi Efek Antibakteri Ekstrak Etanolik Plantago major L. dan Fraksinya

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ABSTRACT

The escalating global health crisis of antibiotic resistance necessitates the search for novel antibacterial agents from natural sources. This study investigated the potential of Plantago major L., a plant used in traditional medicine, as a source of antibacterial compounds. It aimed to evaluate the antibacterial activity of its ethanol extract and its polarity-based fractions against Escherichia coli and Staphylococcus aureus. Dried P. major L. leaves were extracted with ethanol using maceration. The crude extract was then fractionated using a sequential liquid-liquid extraction with hexane, ethyl acetate, and n-butanol. The antibacterial activity of the extract and all fractions was tested against S. aureus (ATCC 25923) and E. coli (ATCC 25922) using the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC). Results revealed distinct antibacterial profiles based on fraction polarity. The ethyl acetate fraction exhibited the strongest activity against S. aureus with an MIC of 0.104 mg/mL, indicating the presence of potent semi-polar compounds like flavonoids effective against gram-positive bacteria. Conversely, the n-butanol fraction was most effective against E. coli with an MIC of 3.3 mg/mL, suggesting the role of hydrophilic compounds such as saponins in disrupting the more complex gram-negative membrane. The crude ethanol extract showed lower activity, confirming the critical role of fractionation in concentrating the bioactive compounds. Plantago major L. possesses significant antibacterial potential, with its active compounds concentrated in specific polarity-based fractions. These findings validate its traditional use and support its further development as an alternative therapy in the face of growing antibiotic resistance.

Keywords: *Plantago major L., Antibacterial Activity, Escherichia coli, Staphylococcus aureus, Phytochemical Extracts*

ABSTRAK

Resistensi antibiotik merupakan masalah kesehatan global yang mendesak, sehingga mendorong pencarian agen antibakteri baru dari sumber alam. *Plantago major* L., yang secara tradisional digunakan untuk pengobatan, berpotensi sebagai sumber senyawa antibakteri. Penelitian ini bertujuan untuk mengevaluasi aktivitas antibakteri ekstrak etanol dan fraksi-fraksi berbasis polaritas dari *P. major* L. terhadap *Escherichia coli* dan *Staphylococcus aureus*. Daun kering *P. major* L. diekstraksi menggunakan etanol melalui maserasi. Ekstrak kasar kemudian difraksinasi secara bertingkat dengan pelarut heksana,

etil asetat, dan n-butanol. Aktivitas antibakteri dari ekstrak dan semua fraksi diuji terhadap *S. aureus* (ATCC 25923) dan *E. coli* (ATCC 25922) menggunakan metode mikrodilusi untuk menentukan Konsentrasi Hambat Minimum (KHM). Hasil penelitian menunjukkan profil aktivitas antibakteri yang berbeda berdasarkan polaritas fraksi. Fraksi etil asetat menunjukkan aktivitas paling kuat terhadap *S. aureus* dengan KHM 0,104 mg/mL, yang mengindikasikan keberadaan senyawa semi-polar seperti flavonoid yang efektif melawan bakteri gram-positif. Sebaliknya, fraksi n-butanol paling efektif terhadap *E. coli* dengan KHM 3,3 mg/mL, menunjukkan peran senyawa polar seperti saponin dalam mengatasi struktur membran gram-negatif yang lebih kompleks. Ekstrak etanol kasar menunjukkan aktivitas yang lebih rendah, yang menegaskan pentingnya proses fraksinasi untuk memekatkan senyawa bioaktif. *Plantago major* L. terbukti memiliki potensi antibakteri yang kuat, dengan senyawa aktif yang terkonsentrasi pada fraksi polaritas tertentu. Temuan ini mendukung pengembangannya lebih lanjut sebagai terapi alternatif untuk mengatasi resistensi antibiotik.

Kata Kunci: *Plantago major* L., Aktivitas antibakteri, *Escherichia coli*, *Staphylococcus aureus*, Ekstrak fitokimia.

1. INTRODUCTION

Bacterial infection remains one of the most common problems of human health, particularly in tropical areas. With the progressing issue of antibiotic resistance in society, the pursuit of a new antibiotic substance is required. The common limitation for synthetic-based antibiotics is the limited synthesis route and acute toxicity. Therefore, much research tends to evaluate other readily analyzed natural organic compounds to avert the complicated synthetic pathways and minimize the human toxicity problem. This led to the research of plant-based secondary metabolites. The secondary metabolites from various plants have been a promising place for potential of medicinal substances including antibacterial compound.

Broadleaf plantain (*Plantago major* L.) is one of Indonesian native plants that possess various pharmacological benefits. It is a perennial herbaceous plant commonly found in urban areas, highly adaptable and able to grow in a range of conditions, although it prefers slightly damp and compacted soils. It is now considered a cosmopolitan species found in temperate regions worldwide (USDA, 2020). Being highly resilient and capable of growing in compacted soils, it can be a nuisance in lawns and gardens but also serves as an important species for soil stabilization and as a pioneer species in disturbed sites (Hilty, 2021).

Based on the ethnobotanical empirical evaluation, the leaves from broadleaf plantain has been used as a topical herb for wound healing and disinfectant.(Chevallier, 1996) Several works also discuss other broadleaf plantain effects such as antimicrobial, anti-inflammatory, antiviral, and antifungal (Monjd, Abd Razik, Hasan, & Murtadha, 2019; N. Z. Sahakyan, Ginovyan, Petrosyan, & Trchounian, 2019; Samuelsen, 2000; Zhakipbekov et al., 2023). Some researches have pursued this empirical evidence by using its extract to find the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) of broadleaf plantain.

The antimicrobial and disinfectant properties of *Plantago major* are attributed to several key chemical constituents identified in the plant. The primary bioactive compounds responsible for these activities include flavonoids, alkaloids, and terpenoids. Notably, aucubin, an iridoid glycoside, and baicalein, a flavonoid, have been recognized for their significant antimicrobial effects. Additionally, phenolic compounds such as caffeic acid derivatives, including plantamajoside and acteoside, contribute to the plant's ability to inhibit the growth of various pathogens. These compounds have been shown to be effective against a range of microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, highlighting the potential of *Plantago major* as a natural source of antimicrobial agents (Adom et al., 2017).

This research aims to evaluate the broadleaf plantain extract with ethanol, a less toxic solvent than methanol. We also implement a polarity-based fractionation of the ethanol extract to specify which fraction of the ethanolic extract possesses greater antibacterial effect for common bacteria such as *E. coli* and *S. aureus*.

2. METHODS

All materials used in this study are of analytical grade and were purchased from Merck and Sigma Aldrich without further purification unless stated otherwise.

Plant Material and Extraction

Dried leaves of Broadleaf plantain (*Plantago major* L.) from Central Java, Indonesia, were ground into a fine powder using a mechanical grinder. A measured quantity of the powder was subjected to maceration extraction with ethanol (1:10 w/v ratio) for 24 hours at room temperature (25±2°C) with periodic agitation. The resulting mixture was filtered, and the marc (solid residue) was re-macerated twice with fresh solvent to ensure exhaustive extraction. The combined filtrates were concentrated using a rotary evaporator (Buchi, Switzerland) under reduced pressure (175 mbar) and a controlled temperature (40°C) to obtain the crude ethanolic extract (CEE).

Liquid-Liquid Fractionation

The CEE was fractionated based on polarity using a sequential liquid-liquid extraction (LLE) protocol. Briefly, the CEE was first suspended in distilled water. This aqueous suspension was then successively partitioned with immiscible organic solvents in order of increasing polarity: hexane, ethyl acetate, and n-butanol (each at a 1:1 v/v ratio against the aqueous suspension). For each step, the mixture was vigorously shaken in a separatory funnel and allowed to stand until the two liquid layers separated completely. The organic layer (hexane, ethyl acetate, or n-butanol fraction) was carefully decanted each time. This process was repeated three times for each solvent to ensure complete partitioning. The remaining aqueous solution constituted the aqueous fraction. All four fractions (hexane, ethyl acetate, n-butanol, and water) were concentrated to dryness using a rotary evaporator (organic fractions) or a freeze-dryer (aqueous fraction). The dried fractions were stored at 4°C until further use.

Antibacterial Assay

The antibacterial activity of the four fractions was evaluated against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) using the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC). Bacterial suspensions were adjusted to a turbidity of 0.5 McFarland standard (approx. 1.5 x 10^8 CFU/mL) in Mueller-Hinton Broth (MHB). These suspensions were then diluted in MHB to achieve a final inoculum density of ~5 x 10^5 CFU/mL in each well of a sterile 96-well microtiter plate.

Each fraction was dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted with MHB to create a series of two-fold dilutions (e.g., 1000, 500, 250... µg/mL) across the plate's rows. The following test groups were included for each bacterial strain:

1. **Test Groups:** Fractions at various concentrations.
2. **Growth Control:** MHB + inoculum (to confirm bacterial growth).
3. **Sterility Control:** MHB only (to confirm media sterility).
4. **Solvent Control:** MHB + inoculum + maximum DMSO concentration used (typically ≤1% v/v, to rule out solvent toxicity).

The covered microplate was incubated at 37°C for 18-24 hours. Bacterial growth was assessed by measuring the optical density (OD) at 600 nm using a microplate reader (e.g., BioTek Instruments, USA). To visually confirm the MIC, 40 µL of resazurin indicator solution (0.015% w/v) was added to each well and incubated for 2-4 hours; a change from blue to pink indicated bacterial viability.

Statistical Analysis

The OD data from the microdilution assays were analyzed for statistical significance. Since the data were not normally distributed, the non-parametric Kruskal-Wallis test was applied first to determine if there were overall significant differences in antibacterial activity among the different fraction groups and concentrations. If a significant difference was found ($p < 0.05$), a post-hoc Dunn's test was performed for pairwise comparisons to identify which specific fractions or concentrations were significantly different from each other. All analyses were conducted using SPSS software (Version 29.0.1.0).

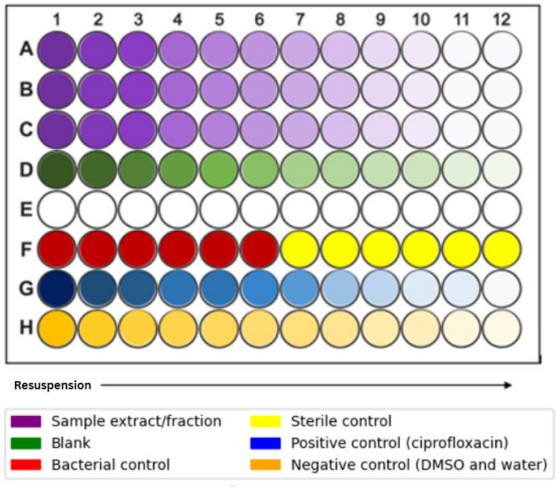

Figure 1. Microplate mapping for microdilution antibacterial activity evaluation.

3. RESULT AND DISCUSSION

The leaves of *Plantago major* L. were subjected to ethanol extraction, followed by fractionation using hexane, ethyl acetate, and n-butanol. The extraction process yielded a significant quantity of extracts, with the ethanol extraction providing a yield of 1.76% (**Table 1**). Fractionation of the ethanol extract resulted in varying quantities of each fraction: hexane (0.33 g), ethyl acetate (2.45 g), and n-butanol (0.23 g). These results highlight the varying solubility of different phytochemicals in these solvents, reflecting the chemical diversity of *Plantago major* L.

Table 1. Yields obtained from *Plantago major* L. extract and fractions.

Extract/Fraction	Yield (g)	Yield (%)
Ethanol Extract	6.16 ± 2.781	1.76
Hexane Fraction	0.33 ± 0.01	0.09
Ethyl Acetate Fraction	2.45 ± 1.043	0.70
n-Butanol Fraction	0.23 ± 0.01	0.07

The antibacterial activity of the extracts and fractions was tested against Staphylococcus aureus, a gram-positive bacterium commonly found on wounds. The optical density of the microdilution test was represented in **Table 2**. The ethyl acetate fraction demonstrated the most potent antibacterial activity with an MIC of 0.104 mg/mL, followed by the hexane fraction (MIC of 0.13 mg/mL) and the n-butanol fraction (MIC of 0.625 mg/mL). The ethanol extract showed moderate activity with an MIC of 1.6 mg/mL.

Table 2. Minimum Inhibitory Concentrations (MICs) of *P. major* L. extract and fractions against *Staphylococcus aureus* and *Escherichia coli*

Sample	MIC for *S. aureus* (mg/mL)	MIC for *E. coli* (mg/mL)
Ethanol Extract	1.6 ± 0.01	>20
Hexane Fraction	0.13 ± 0.01	>20
Ethyl Acetate Fraction	0.104 ± 0.01	>20
n-Butanol Fraction	0.625 ± 0.01	3.3

Testing against Escherichia coli, a gram-negative bacterium, revealed a different outcome of effectiveness. The optical density of the microdilution test was represented in **Table 3**. The n-butanol fraction emerged as the most effective, with an MIC of 3.3 mg/mL. The ethyl acetate and hexane fractions, as well as the ethanol extract, did not demonstrate significant inhibitory effects against E. coli at the tested concentrations.

Table 3. Comparative antibacterial activity of *Plantago major* L. extract and fractions.

Fraction/Extracts	Active Compounds (Probable)	Activity Against *S. aureus*	Activity Against *E. coli*
Ethanol Extract	Mixed compounds	Moderate	None
Hexane Fraction	Non-polar (terpenoids)	High	None
Ethyl Acetate Fraction	Semi-polar (flavonoids)	Very high	None
n-Butanol Fraction	Polar (saponins, alkaloids)	Moderate	Moderate

A comparative analysis of the MIC values highlights the distinct antibacterial profiles of each extract and fraction. The ethyl acetate fraction's effectiveness against S. aureus and the

n-butanol fraction's effectiveness against E. coli reflect the complex interplay of chemical composition and bacterial cell wall structure.

Statistical analysis using the Kruskal-Wallis test confirmed significant differences in the antibacterial activities of the various fractions. For S. aureus, the ethyl acetate and hexane fractions showed statistically significant differences compared to the ethanol extract, with p-values of 0.005 and 0.015 respectively. No statistical analysis was conducted for E. coli due to insufficient variation in the data, but the trend suggested a clear preference for the n-butanol fraction.

The effectiveness of each fraction was visually confirmed through microplate assays and resazurin staining. Clear and distinct changes in colour correlate with the bacterial inhibition, providing a qualitative measure of antibacterial activity that corroborated the quantitative MICs findings.

This study highlights the potent antibacterial properties of various fractions of *Plantago major* L. extracts against both *Escherichia coli* and *Staphylococcus aureus*, suggesting the plant's potential as a source of novel antibacterial agents. The observed differences in efficacy among the extracts and fractions can be attributed to the diverse range of bioactive compounds present in *Plantago major* L., which may act synergistically or independently to inhibit bacterial growth. These findings contribute to the broader understanding of plant-based antimicrobial strategies and spotlight the value of *Plantago major* L. in traditional medicine (Gunawan & Prismawan, 2023).

The ethyl acetate fraction showed significant antibacterial activity against *S. aureus*, with an MIC of 0.104 mg/mL. This suggests that the fraction contains potent bioactive phytoconstituents such as flavonoids and phenolic compounds, which are known for their antibacterial properties (Razik, Zain, & Bakar, 2017). The efficacy of the ethyl acetate fraction aligns with its ability to dissolve a wide range of semi-polar compounds that are particularly effective against gram-positive bacteria, which typically have simpler cell walls susceptible to disruption by these compounds (N. Sahakyan, Sargsyan, & Grigoryan, 2020).

Similarly, the n-butanol fraction demonstrated the highest activity against *E. coli*, with an MIC of 3.3 mg/mL. The polar nature of n-butanol suggests that it extracts hydrophilic compounds such as saponins and polysaccharides, which can disrupt the complex outer membrane of gram-negative bacteria such as *E. coli*, enhancing membrane permeability and interfering with cellular functions (Ozkan, Uslu, & Yildirim, 2019). This indicates that the bioactive compounds in the n-butanol fraction are particularly effective against gram-negative bacteria, which are often more resistant due to their complex cell walls (Gunawan & Prismawan, 2023).

The distinct antibacterial activities observed among the fractions can be attributed to their unique phytochemical compositions and the mechanisms by which these compounds exert their effects. The *Plantago major* L. main secondary metabolite which is acteoside and plantamajoside is noted to have certain potential as antibacterial agent (Budzianowska, Kikowska, & Budzianowski, 2022). Ethyl acetate, a semi-polar solvent, is effective in extracting these compounds and other compounds like flavonoids and phenolics, which are known to disrupt bacterial cell walls and inhibit essential enzymes, leading to increased permeability and eventual cell death (Bajpai, Kang, & Lee, 2016). The efficacy of these compounds against gram-positive bacteria highlights their potential in the development of alternatives for infectious diseases.

In contrast, the polar n-butanol fraction extracts hydrophilic compounds, including saponins and polysaccharides, which inhibit bacterial growth by interacting with cell wall components and preventing nutrient uptake or interfering with metabolic processes. This makes them effective against resistant strains of gram-negative bacteria, demonstrating the versatility of *Plantago major* L. extracts in targeting different types of bacteria (Hirani, Patel, & Trivedi, 2020).

The effectiveness of *Plantago major* L. extracts compares favourably with conventional antibiotics, particularly against antibiotic-resistant strains. *S. aureus*, which has shown increasing resistance to antibiotics such as methicillin (MRSA), responded well to the ethyl acetate fraction with low MIC values, indicating that *Plantago major* L. could be a valuable

alternative treatment in cases where conventional antibiotics are less effective (Kagan, Friedman, & Shapiro, 2018). The n-butanol fraction's notable efficacy against *E. coli*, a bacterium known for its resistance mechanisms such as efflux pumps and β-lactamase production, suggests that compounds in this fraction may offer a new approach to treating gram-negative bacterial infections (Gunawan & Prismawan, 2023).

Further research should focus on isolating and identifying the specific compounds responsible for the observed antibacterial activity. Advanced techniques such as high-performance liquid chromatography (HPLC) in tandem with mass spectrometry (MS) could be employed to analyse the active components in the ethyl acetate and n-butanol fractions. *In vivo* studies are essential to evaluate the efficacy and safety of these extracts in treating bacterial infections, ensuring that they are not only effective in a laboratory setting but also in the clinical settings (Gunawan & Prismawan, 2023). Additionally, exploring the potential synergistic effects of *Plantago major* L. extracts with conventional antibiotics could provide valuable insights into enhancing antibacterial efficacy and overcoming antibiotic resistance, a critical challenge in modern medicine (N. Sahakyan et al., 2020).

4. CONCLUSION

This study successfully demonstrates that the ethanolic extract of *Plantago major* L., prepared using a less toxic solvent, possesses significant antibacterial properties. Furthermore, the critical step of polarity-based fractionation precisely identified which fractions held greater antibacterial effects against the tested bacteria. Specifically, the semi-polar ethyl acetate fraction was revealed to be the most potent against *Staphylococcus aureus*, while the polar n-butanol fraction was the most effective against *Escherichia coli*. These findings confirm that the antibacterial compounds in Plantain are polarity-specific, with semi-polar flavonoids and phenolics likely targeting gram-positive bacteria and more polar compounds like saponins acting against gram-negative strains. Thus, this research not only validates the empirical use of Broadleaf plantain but also successfully pinpoints the specific fractions that should be prioritized for the future development of targeted plant-based antibacterial agents.

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