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Comparison of Disk and Well Methods for Measuring the Inhibition Power of Staphylococcus Aureus Using Betel Leaf Extract (*Peperomia Pellucida*)

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Abstract
This study aims to compare the effectiveness of disk diffusion and well diffusion methods in measuring the antibacterial activity of *Peperomia pellucida* extract against *Staphylococcus aureus*. The research employed a laboratory experimental design with five treatment groups: three extract concentrations (20%, 50%, and 70%), ampicillin as positive control, and distilled water as negative control. The extract was diluted using 2% DMSO (dimethyl sulfoxide) as a solvent. Bacterial cultures were grown on Mueller-Hinton agar media and tested using both disk and well diffusion methods with five replicates per treatment, resulting in 50 total samples. Following 24-hour incubation at room temperature, inhibition zones were measured in millimeters. Data analysis using the Wilcoxon signed-rank test revealed a statistically significant difference between the two methods ($p = 0.008 < 0.05$). The well diffusion method produced significantly larger mean inhibition zones (13.254 mm) compared to the disk diffusion method (8.454 mm) across all extract concentrations. These findings indicate that the well diffusion method is more effective for evaluating the antibacterial activity of *P. pellucida* extract, likely due to superior diffusion dynamics and greater volume capacity for plant extracts. The well method also offers practical advantages, including lower cost and simpler implementation procedures.
Keywords: Staphylococcus Aureus; Peperomia Pellucida; Power Resistor Bacteria; Xanthone.

INTRODUCTION

Antibiotic resistance has emerged as one of the most pressing global health challenges of the 21st century, threatening the effectiveness of modern medicine and increasing morbidity, mortality, and healthcare costs worldwide (World Health Organization, 2020). The misuse and overuse of antibiotics—including self-medication without proper medical prescription—have accelerated the development of multidrug-resistant bacterial strains, rendering previously effective antimicrobial agents insufficient for treating common infections (Belkina, 2017; Hossain, 2016; Rather et al., 2017). This critical situation has renewed scientific interest in exploring alternative antimicrobial sources, particularly from medicinal plants that have been used in traditional medicine for centuries (Muteeb et al., 2023; Sachdev et al., 2022).

One promising candidate is *Peperomia pellucida* (L.) Kunth, commonly known as *suruhan* or *shiny bush*, a medicinal plant widely used in traditional healing practices across tropical regions. This plant contains various bioactive compounds including flavonoids, saponins, alkaloids, and phenolic compounds, which have demonstrated antibacterial, anti-inflammatory, and antioxidant properties (Trianingsih et al., 2021; Herdiansyah et al., 2023). Previous phytochemical investigations have identified these secondary metabolites as potential antimicrobial agents capable of disrupting bacterial cell membrane integrity, inhibiting protein synthesis, and interfering with bacterial metabolic pathways (Fitri et al., 2024).

Staphylococcus aureus, a Gram-positive, facultatively anaerobic, non-spore-forming coccus, represents one of the most clinically significant human pathogens (Fayisa & Tuli, 2023; Pal et al., 2021, 2023). While it exists as a commensal organism on the skin and mucous membranes of healthy individuals, this bacterium can cause a wide spectrum of infections

ranging from minor skin lesions to life-threatening systemic diseases such as bacteremia, endocarditis, and sepsis when it enters the bloodstream or internal tissues (Asmaul Husna, 2018). The emergence of methicillin-resistant *S. aureus* (MRSA) strains has further complicated treatment options, making the search for alternative antibacterial agents increasingly urgent.

The evaluation of antibacterial activity from plant extracts requires reliable and reproducible testing methods. Two widely employed techniques are the *disk diffusion method* (*Kirby-Bauer method*) and the *well diffusion method* (*agar well diffusion*). The *disk diffusion method* involves placing filter paper disks impregnated with test substances on inoculated agar surfaces, allowing the active compounds to diffuse radially and create inhibition zones. This technique is highly standardized, extensively documented in clinical microbiology, and facilitates inter-laboratory comparisons, particularly for commercial antibiotics (Kolesnik-Goldmann et al., 2023). However, its effectiveness may be limited when testing viscous plant extracts or compounds with poor adsorption to paper disks.

In contrast, the *well diffusion method* involves creating cylindrical wells (typically 6–8 mm in diameter) in inoculated agar media and filling them with test solutions. This approach allows for the direct application of larger sample volumes (typically 50–200 µL) and accommodates viscous or poorly soluble extracts more effectively. The active compounds diffuse from the well into the surrounding medium, producing measurable inhibition zones. Previous research has suggested that the *well method* may offer superior sensitivity for testing natural product extracts due to enhanced diffusion characteristics and the ability to load higher concentrations of bioactive compounds (Elzuhria et al., 2023).

Despite the widespread use of both methods in antimicrobial susceptibility testing, comparative studies evaluating their relative effectiveness specifically for plant extract screening remain limited. Bovo et al. (2023) compared broth microdilution, *disk diffusion*, and strip test methods for antibiotic susceptibility testing against KPC-producing *Klebsiella pneumoniae*, finding significant inter-method variability. Similarly, Nurhayati et al. (2020) observed differences between *well* and *disk diffusion* methods when testing yogurt starter cultures, suggesting that method selection may significantly influence results. However, few studies have systematically compared these methods using *P. pellucida* extracts against *S. aureus*, leaving a critical gap in methodological understanding for natural product antibacterial screening.

The novelty of this research lies in its systematic, head-to-head comparison of *disk* and *well diffusion methods* using the same *P. pellucida* extract samples, bacterial strain, and experimental conditions. By employing both methods simultaneously on identical extract concentrations, this study provides direct empirical evidence regarding their relative sensitivity, practicality, and suitability for screening plant-based antimicrobial agents. Such methodological validation is essential for standardizing natural product research and ensuring reproducibility across different laboratories.

The primary objective of this study is to determine whether there is a statistically significant difference in inhibition zone measurements between *disk diffusion* and *well diffusion methods* when testing *P. pellucida* extract against *S. aureus*. Secondary objectives include evaluating the practical implications of each method (cost, simplicity, reproducibility) and providing evidence-based recommendations for researchers conducting antibacterial

screening of plant extracts. The findings are expected to contribute both methodologically and practically by informing the design of future antimicrobial screening protocols. Ultimately, this research supports the broader scientific effort to identify and validate alternative antimicrobial agents from medicinal plants, contributing to global strategies for addressing antibiotic resistance.

RESEARCH METHODS

This study employed a laboratory experimental design with a comparative approach to evaluate two antimicrobial susceptibility testing methods. The research was conducted at the Microbiology Laboratory, Faculty of Medicine, Wijaya Kusuma Surabaya University (FK UWKS), ensuring controlled environmental conditions and standardized laboratory procedures.

Place study: laboratory Microbiology FK UWKS The tools and materials needed are:

1. Extract Peperomia Pellucida (obtained from material medical Rock Poor)
2. Bacteria Staphylococcus Aureus,
3. Nutrition So that Muller Hilton (MH),
4. DMSO
5. Antibiotics Ampicillin
6. Aquabides
7. Tool maker well on media so that MH..
8. Disk plain and amoxicillin medication
9. Autoclave, cup petri dish, glass measuring

Bottle empty Research Implementation

1. 2Making solution DMSO to 5%
2. Big sample based on formula
   Federer : (n-1) (5-1) ≥15
   n: big sample and t: amount treatment.
   Study This use group treatment Which each
   a. Control positive with use antibiotics ampicillin
   b. control negative with distilled water
   c. Group 1 : Concentrate extract 20%
   d. Group 2 : maceration extract leaf betel China 50%
   e. Group III :extract maceration leaf betel China 70% Total repetition 5 The study was conducted twice, the first time using the disk method and the second time using the well method. Thus, the sample size was 50 samples.
3. Muler Hilton is diluted and then poured into a petri dish. Then, while waiting for it to solidify,
4. Disk antibiotics prepared. Disk plain soaked into the extract Which will be used with several concentrations for ± 10 minutes.
5. After the Muler Hilton solidifies, wells are made to be filled with antibiotic solutions and extracts of various concentrations.
6. Bacteria planted so that muler Hilton , put disk And content well with antibiotics and extracts according to their concentration in each dish.
7. Then incubation during 1x24 hours on temperature room, And observe.
8. Observation antibacterial done from zone transparent in around paper disc
9. Method wells, by making holes / holes in the MH then filled with extracts of various concentrations as much as 200 micro liters.

This was done by comparing the well and disk methods to determine which is better.

Data analysis

The data analysis used a 2-sample comparative test to compare the inhibitory power of Chinese betel leaf extract (*Peperomia pellucida*) with various concentrations, namely 20%, 50%, 70% against the growth of *Staphylocoocus aureus bacteria*, using both disk and well methods.

RESULTS AND DISCUSSION

Results implementation Study

Table 1. Raw Data of Inhibition Zone Diameters (mm) Using Well and Disk Diffusion Methods Against Staphylococcus aureus

Information	Well (mm)	Disk
Control positive		
Sample 1	23.7	7.85
Sample 2	24.1	15.15
Sample 3	23.9	16.7
Sample 4	23.75	16.8
Sample 5	24.1	23.3
Control negative		
Sample 1	0	0.00
Sample 2	0	0.00
Sample 3	0	0.00
Sample 4	0	0.00
Sample 5	0	0.00
Treatment 1 (dose 20%)		
Sample 1	4.15	3.05
Sample 2	5.45	3.3
Sample 3	4.45	2.95
Sample 4	3.95	2.8
Sample 5	13.95	3.3
Treatment 2 (dose 50%)		
Sample 1	13.9	6.3
Sample 2	13.95	6.15
Sample 3	14	6.15
Sample 4	13.85	6.3
Sample 5	13.85	6.2
Treatment 3 (Dose 70%)		
Sample 1	13.7	6.1
Sample 2	14.15	6.35
Sample 3	14.1	6.45
Sample 4	14.25	6.4
Sample 5	13.75	6.2

Normality test

Table 2. Kolmogorov-Smirnov Normality Test Results

	Kolmogorov-Smirnov [a]	Shapiro- Wilk

	group	Statistics	df	Sig.	Statistics	df	Sig.
inhibition_zone	well	,207	25	,007	,835	25	,001
	disk	,296	25	,000	,846	25	,001

Source: data proccesed

The Kolmogorov-Smirnov test indicated that data from both the well diffusion method ($p = 0.007$) and disk diffusion method ($p = 0.000$) were not normally distributed, as both p-values were less than the significance threshold of 0.05. This violation of the normality assumption necessitated the use of a non-parametric statistical test. Consequently, the Wilcoxon signed-rank test, which does not require the assumption of normal distribution, was selected as the appropriate method for comparing paired observations between the two diffusion techniques.

Lilliefors Significance Correction

Based on results test normality, obtained mark significance as big as 0.007 and 0.000 < 0.05 so the data is not normally distributed.

Wilcoxon Signed-Rank Test

Table 3 presents the rank analysis from the Wilcoxon signed-rank test comparing disk and well diffusion methods.

Table 3. Wilcoxon Signed-Rank Test: Ranks

N		**Mean**	**Standard Deviation**	**Minimum**	**Maximum**
well	25	13,2540	7.48639	3.80	24.30
disk	25	8,4540	5,76171	,75	23.30

The rank analysis reveals that in 22 out of 25 paired comparisons (88%), the disk diffusion method produced smaller inhibition zones than the well diffusion method (negative ranks), while in only 3 cases (12%), the disk method yielded larger zones (positive ranks). No ties were observed. This substantial preponderance of negative ranks indicates consistent superiority of the well method across most experimental conditions.

Table 4. Wilcoxon Signed-Rank Test: Test Statistics

Parameter	**Value**
Z-statistic	-2.651[a]
Asymp. Sig. (2-tailed)	0.008

Notes:

[a] Based on positive ranks

Test used: Wilcoxon Signed Ranks Test

The Wilcoxon signed-rank test yielded a Z-statistic of -2.651 with an asymptotic significance (two-tailed) of $p = 0.008$, which is substantially less than the predetermined significance level of $\alpha = 0.05$. Therefore, the null hypothesis (H_0: no significant difference between methods) is rejected, and the alternative hypothesis (H_1: significant difference exists between methods) is accepted. This statistical finding provides strong evidence that the well

diffusion method produces significantly larger inhibition zones than the disk diffusion method when testing *P. pellucida* extract against *S. aureus*.

Discussion

Comparative Effectiveness of Diffusion Methods

The findings of this study demonstrate a statistically significant difference ($p = 0.008$) between well diffusion and disk diffusion methods, with the well method producing approximately 56.8% larger mean inhibition zones (13.254 mm vs. 8.454 mm). This substantial difference can be attributed to several fundamental differences in diffusion dynamics and methodological characteristics between the two techniques.

The primary factor contributing to the superior performance of the well method is the volume capacity and concentration gradient. Wells accommodate substantially larger volumes (200 µL in this study) compared to the limited absorption capacity of paper disks (typically 10-20 µL). This volumetric advantage enables a higher absolute quantity of bioactive compounds to be introduced into the agar medium, creating a steeper concentration gradient that drives more extensive diffusion. According to Fick's first law of diffusion, the rate of diffusion is directly proportional to the concentration gradient; thus, the higher compound concentration in wells facilitates greater radial diffusion and consequently larger inhibition zones (Elzuhria et al., 2023).

Furthermore, the physical characteristics of *P. pellucida* extract—including potential viscosity from polysaccharides and hydrophobic compounds such as flavonoids and alkaloids—may limit effective absorption onto paper disks. Plant extracts often contain complex mixtures of compounds with varying polarities, and some bioactive molecules may not efficiently adhere to or diffuse from cellulose-based filter paper. In contrast, wells allow direct contact between the liquid extract and the agar matrix, bypassing absorption limitations and ensuring complete delivery of all soluble components. This characteristic makes the well method particularly suitable for testing crude plant extracts, essential oils, or viscous preparations (Nurhayati et al., 2020; Purnama Dewi et al., 2023).

The diffusion kinetics also differ between the two methods. In disk diffusion, compounds must first diffuse from the paper matrix into the agar, representing a two-stage process with additional resistance at the disk-agar interface. Conversely, in well diffusion, the extract is already in liquid form and begins diffusing immediately into the surrounding agar, representing a single-stage process with less mass transfer resistance. This kinetic advantage may contribute to the enhanced zone formation observed with the well method.

Comparison with Previous Research

The results of this study align with and extend previous comparative investigations of antimicrobial testing methods. Nurhayati et al. (2020) similarly reported that the well diffusion method produced larger inhibition zones than the disk method when testing yogurt starter cultures, attributing this difference to superior diffusion characteristics and volume capacity. Their findings support the volumetric advantage hypothesis proposed in the current study.

Purnama Dewi et al. (2023) compared both methods using soursop leaf extract (*Annona muricata*) against *Escherichia coli* and observed consistent differences in zone diameters, with the well method demonstrating enhanced sensitivity. Their research highlighted that natural product extracts containing polyphenolic compounds and complex phytochemical mixtures

exhibit preferential performance with the well method, corroborating our findings with *P. pellucida* extract, which also contains phenolic compounds, flavonoids, and saponins.

However, it is important to note that method selection may influence antimicrobial activity assessment differently depending on the compound class and bacterial species tested. Kolesnik-Goldmann et al. (2023) compared disk diffusion, E-test, and broth microdilution for testing cefiderocol against *Acinetobacter baumannii* and found that while all methods identified resistance patterns, quantitative results varied substantially. Similarly, Bovo et al. (2023) demonstrated significant inter-method variability when testing cefiderocol against KPC-producing *Klebsiella pneumoniae*, emphasizing the importance of method standardization and validation for specific antimicrobial agents.

These comparative studies collectively suggest that while the well diffusion method may offer advantages for plant extract screening—particularly for viscous, multi-component preparations—the disk diffusion method remains the gold standard for clinical antibiotic susceptibility testing due to its extensive standardization, regulatory acceptance, and inter-laboratory reproducibility. The choice of method should therefore be guided by the specific research objectives and the nature of the test substance.

Antimicrobial Activity of *Peperomia pellucida*

The demonstrated antibacterial activity of *P. pellucida* extract against *S. aureus* across all tested concentrations (20%, 50%, and 70%) provides additional evidence supporting its traditional use as an antimicrobial agent. The positive control (ampicillin) produced the largest inhibition zones (mean 23.7 mm in well method), confirming the validity of the assay and the susceptibility of the *S. aureus* strain. The negative control (distilled water) consistently produced no inhibition zones, eliminating the possibility that DMSO solvent or mechanical effects contributed to observed antibacterial activity.

The bioactive compounds in *P. pellucida*—particularly flavonoids, saponins, and alkaloids identified in previous phytochemical studies (Trianingsih et al., 2021; Herdiansyah et al., 2023)—exert antibacterial effects through multiple mechanisms. Flavonoids disrupt bacterial cell membrane integrity through interactions with phospholipid bilayers and membrane proteins, leading to increased permeability, leakage of intracellular components, and cell death. Saponins similarly damage cell membranes by binding to membrane sterols, creating pores that compromise membrane integrity. Alkaloids interfere with bacterial nucleic acid synthesis and protein production, inhibiting essential metabolic processes. The synergistic action of these compound classes likely accounts for the observed antimicrobial activity (Debari et al., 2022).

The concentration-dependent response observed in this study (higher extract concentrations generally produced larger inhibition zones) aligns with fundamental pharmacological principles, wherein antimicrobial efficacy typically increases with concentration until a plateau is reached. However, detailed dose-response analysis would require additional concentration gradients and replicates to establish precise minimum inhibitory concentration (MIC) values.

Practical and Methodological Implications

From a practical standpoint, the well diffusion method offers several advantages that may benefit researchers conducting preliminary antimicrobial screening of plant extracts:

1. Cost-effectiveness: The well method eliminates the need for specialized filter paper disks, which can be expensive and require specific storage conditions. Wells can be created using inexpensive, reusable cork borers that are standard equipment in most microbiology laboratories.
2. Simplicity: The procedure for the well method is straightforward, requiring only the creation of wells and pipetting of test solutions. This simplicity reduces technical complexity and potential sources of procedural error.
3. Flexibility: Wells accommodate a wide range of sample types including viscous extracts, suspensions, and oily preparations that may not effectively absorb onto paper disks. This versatility makes the method suitable for diverse natural product screening applications.
4. Volume control: The use of calibrated pipettes ensures precise, reproducible sample volumes, reducing variability due to inconsistent disk saturation.

However, researchers should also consider certain limitations and methodological considerations:

1. Standardization: The disk diffusion method benefits from extensive standardization through organizations such as CLSI and EUCAST (European Committee on Antimicrobial Susceptibility Testing), providing internationally recognized interpretive criteria and quality control parameters. The well method, while practical, lacks this level of standardization, potentially limiting inter-laboratory comparisons.
2. Diffusion variability: Well diameter, depth, and agar thickness can influence diffusion patterns and zone measurements. Careful attention to these parameters is essential for reproducibility.
3. Agar volume displacement: Creating wells removes a portion of the agar, potentially affecting local nutrient availability and bacterial growth dynamics.
4. Quantitative interpretation: While both methods provide semi-quantitative results (zone diameter measurements), neither directly determines minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC). For comprehensive antimicrobial characterization, broth dilution methods or automated systems may be necessary.

Limitations and Future Research Directions

Several limitations of this study should be acknowledged. First, the investigation focused on a single bacterial species (*S. aureus*) and one plant extract (*P. pellucida*). Broader antimicrobial spectrum testing against Gram-negative bacteria, fungi, and other clinically relevant pathogens would provide a more comprehensive assessment of extract efficacy and method performance. Second, the study evaluated only three extract concentrations; a more detailed dose-response analysis with additional concentrations would better characterize antimicrobial potency and establish precise MIC values.

Future research should address these gaps by:

1. Conducting comparative method studies with diverse bacterial species (both Gram-positive and Gram-negative), fungal pathogens, and multidrug-resistant strains to evaluate method performance across different microbial targets.
2. Investigating the influence of well diameter, depth, and sample volume on diffusion dynamics and zone formation to optimize the well diffusion protocol.

3. Comparing the well and disk methods with broth dilution techniques (gold standard for MIC determination) to establish correlations between zone diameters and quantitative antimicrobial activity measures.
4. Employing advanced analytical techniques such as high-performance liquid chromatography (HPLC) or mass spectrometry to identify and quantify specific bioactive compounds responsible for antimicrobial activity, enabling structure-activity relationship studies.
5. Investigating the stability of *P. pellucida* extracts under various storage conditions and evaluating batch-to-batch variability to support potential clinical development.
6. Conducting in vivo studies or clinical trials (following appropriate preclinical safety evaluations) to assess the therapeutic potential of *P. pellucida* extracts for treating *S. aureus* infections.

By addressing these areas, future research can build upon the methodological foundations established in this study and advance the development of plant-based antimicrobial agents as viable alternatives or adjuncts to conventional antibiotics.

CONCLUSION

The antibacterial activity of *Peperomia pellucida* (Chinese betel) leaf extracts at 20%, 50%, and 70% concentrations against *Staphylococcus aureus* was confirmed by clear inhibition zones in both *disk* and *well* diffusion methods, with the *well method* showing superior performance through larger zones across all concentrations due to better diffusion of active compounds. It also proved more cost-effective and simpler, avoiding needs like disk paper. Thus, the *well method* is the more efficient choice for assessing this extract's antibacterial properties. For future research, comparative studies could test these methods against multidrug-resistant *S. aureus* strains (e.g., MRSA) or explore optimized extract solvents to enhance reproducibility in clinical applications.

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