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Research Article

Determination of MIC and MBC of *Peperomia pellucida* Extract Against *Aggregatibacter actinomycetemcomitans* Using Serial Dilution and Colony Count Assay

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KEYWORDS

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ABSTRACT

Introduction: Periodontitis is an infectious disease that damages periodontal tissues and may lead to tooth loss. *Aggregatibacter actinomycetemcomitans* is one of the main pathogenic bacteria associated with periodontitis. *Peperomia pellucida* (L.) Kunth contains flavonoids, alkaloids, and tannins with antibacterial potential. This study evaluated its effectiveness against *A. actinomycetemcomitans* and determined its Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Materials and Methods: This laboratory experimental study used a post-test-only control group design. Antibacterial testing was performed using the serial dilution method in eight groups: extract concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%, 0.2% chlorhexidine as the positive control, and distilled water as the negative control. MIC was assessed by bacterial growth on Mueller–Hinton Agar, while MBC was determined by colony counts (CFU/mL). Data were analyzed using Kruskal–Wallis and Mann–Whitney tests.

Results and Discussions: *Peperomia pellucida* extract showed antibacterial activity against *A. actinomycetemcomitans*. MIC was achieved at 6.25% concentration with 92.5% inhibition, while MBC was achieved at 12.5% concentration with 100% bactericidal effect.

Conclusion: *Peperomia pellucida* extract effectively inhibited and killed *A. actinomycetemcomitans*, with greater antibacterial activity at higher concentrations.

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INTRODUCTION

Oral and dental health play a vital role in the overall well-being of every individual. However, public awareness in Indonesia regarding its importance remains low. According to the 2018 Basic Health Research Survey (*Riset Kesehatan Dasar*, RISKESDAS), the prevalence of oral and dental diseases in the Indonesian population remains relatively high at 57.6%, with periodontitis accounting for 74.1% of cases.[1]

Periodontitis is an inflammatory infection of the tooth-supporting tissues that can lead to damage of the periodontal ligament and alveolar bone, accompanied by loss of tooth attachment. This results in the formation of a gingival sulcus or periodontal pocket, which may present with bleeding on probing (BOP) and increased tooth mobility. It is a chronic, multifactorial infectious disease associated with dental plaque accumulation and characterized by progressive tissue destruction.[2–5]

Dental plaque, a biofilm composed of colonizing microorganisms, plays a central role in initiating and exacerbating periodontal infections. Among these microorganisms, *Aggregatibacter actinomycetemcomitans* is one of the primary pathogens, with a reported prevalence of up to 90% in periodontitis cases.[6] This Gram-negative anaerobic bacterium can damage periodontal attachment and bone due to its ability to form biofilms, particularly in subgingival plaque.[7–10]

The primary goal of periodontitis treatment is to halt the progression of periodontal lesions. Mechanical therapy, such as scaling and root planning, aims to produce biologically acceptable root surfaces and reduce periodontal pocket depth.[11] As an adjunct, chlorhexidine (CHX) mouthwash is commonly used as a topical disinfectant and antimicrobial agent in dentistry. However, prolonged use of CHX may cause adverse effects, including unpleasant taste, tooth staining, and oral mucosal ulcerations.[12–17]

Extensive studies have been conducted on herbal plants for their medicinal potential. *Peperomia pellucida (L.) Kunth (P. pellucida)*, locally known as "daun sirih cina" in Indonesia, is a common wild plant thriving in humid environments. It has been reported to possess analgesic, antipyretic, anti-inflammatory, antibacterial, and antimicrobial activities. Its phytochemical profile includes alkaloids, flavonoids, and tannins.[18–23]

Previous studies have demonstrated the antibacterial effect of *P. pellucida* extract against *Porphyromonas gingivalis* at concentrations of 25%, 50%, 75%, and 100%, with significant inhibition observed using the disc diffusion method.[12] Another approach for antibacterial testing is the serial dilution method, which enables the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).[24] To date, no studies have been reported on the antibacterial activity of *P. pellucida* extract against *A. actinomycetemcomitans* using the serial dilution method. Based on these considerations, this study aimed to evaluate the antibacterial effectiveness of *Peperomia pellucida (L.) Kunth* extract against *Actinomycetemcomitans* using the serial dilution method to determine its MIC and MBC values.

MATERIAL AND METHODS

This study was laboratory-based experimental research with a *post-test only control group* design, consisting of eight treatment groups to assess both the inhibitory and bactericidal activities of the test material. The preparation of *Peperomia pellucida (L.) Kunth* extract was conducted at the Phytochemistry Laboratory, Faculty of Pharmacy,

Universitas Jenderal Achmad Yani, Cimahi, in November 2024. Antibacterial testing was carried out at the Research Center Laboratory, Faculty of Dentistry, Universitas Airlangga, Surabaya, from November to December 2024. The study materials included *Aggregatibacter actinomycetemcomitans* (ATCC 43718) obtained from the Microbiology Laboratory, Faculty of Dentistry, Universitas Airlangga, and *P. pellucida* extract prepared by maceration using 96% ethanol.

Fresh *P. pellucida* leaves were selected based on inclusion criteria, namely bright green color, absence of physical damage, and no signs of wilting. The extract was obtained through maceration for 72 hours with solvent replacement every 24 hours, followed by filtration, solvent evaporation using a rotary evaporator, and concentration using a water bath.[25] *A. actinomycetemcomitans* suspension was prepared by inoculating pure cultures into Brain Heart Infusion Broth (BHIB) and incubating anaerobically in an anaerobic jar at 37°C for 2×24 hours until reaching McFarland standard 0.5 (1.5 × 10^8 CFU/mL).[26]

Antibacterial testing was conducted using the serial dilution method to determine the Minimum Inhibitory Concentration (MIC), following the Clinical and Laboratory Standards Institute (CLSI) M7-A9 guidelines.[27] Serial dilutions were prepared in 2 mL Eppendorf tubes, with the first tube containing 1 mL of 100% extract, and each subsequent tube containing 0.5 mL of BHIB. A 0.5 mL aliquot from the first tube was transferred into the second tube and homogenized, followed by consecutive dilutions until the final tube, where the last 0.5 mL was discarded to maintain a consistent volume. This procedure yielded extract concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.12%, along with a positive control (0.2% chlorhexidine) and a negative control (distilled water). A 0.1 mL bacterial suspension was added to each treatment tube, followed by anaerobic incubation at 37°C for 2×24 hours. MIC was determined by visual assessment of turbidity, while the Minimum Bactericidal Concentration (MBC) was determined by inoculating 0.1 mL from each tube onto Mueller–Hinton Agar (MHA) using the spread plate technique and counting colony-forming units (CFU/mL) with a colony counter. The MIC was confirmed at the lowest concentration that inhibited ≥90% bacterial growth compared with the negative control, and the MBC at the lowest concentration that killed ≥99.9% of bacteria based on CFU counts.[28]

Data analysis was performed using SPSS. The Shapiro–Wilk test was applied to assess data normality, and the Levene test for homogeneity. Normally distributed and homogeneous data were analyzed using one-way ANOVA, while non-normal or heterogeneous data were analyzed using the Kruskal–Wallis test, followed by the Mann–Whitney *post hoc* test to determine significant differences between treatment groups.

RESULTS AND DISCUSSIONS

The antibacterial effectiveness of *P. pellucida* extract against *A. actinomycetemcomitans* was tested at the Research Center Laboratory, Faculty of Dentistry, Universitas Airlangga, Surabaya. The study comprised eight groups: *P. pellucida* extract at concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%, a positive control (0.2% chlorhexidine), and a negative control (distilled water). Data were obtained by counting the CFU/mL of *A. actinomycetemcomitans* colonies grown on MHA plates for each treatment group, with four replicates per group. The serial dilution method was used to determine the MIC and MBC of *P. pellucida*

extract against *A. actinomycetemcomitans*. MIC values were determined based on the lowest concentration that visibly inhibited bacterial growth, while MBC values were determined from the lowest concentration at which no bacterial colonies were observed on MHA. A comparative analysis of the antibacterial effectiveness among different extract concentrations was conducted. The number of colonies for each treatment was visually assessed to evaluate the inhibitory effect of *P. pellucida* extract against *A. actinomycetemcomitans*. The results are presented in Figure 1, while the quantitative CFU/mL counts are shown in Table 1.

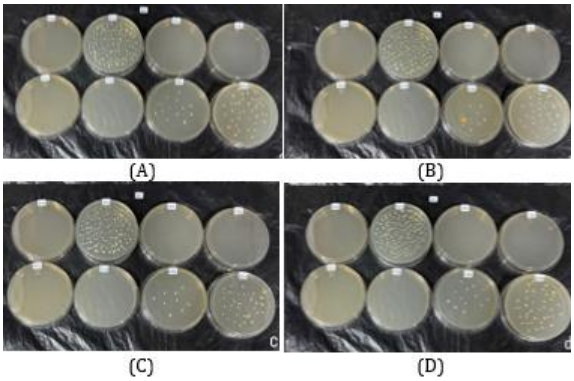

Figure 1. Research results and bacterial colony counts using the serial dilution method: (A) Replication 1, (B) Replication 2, (C) Replication 3, (D) Replication 4.

Table 1. Mean colony count of *A. actinomycetemcomitans* for each treatment group

Groups	Colony Count (CFU/mL)				Mean±SD	Min-Max
	1	2	3	4		
Control (+) CHX	0.00	0.00	0.00	0.00	0.00 ± 0.00	0.00
Control (-) aquades	174	172	165	157	167.00 ± 7.70	157±174
Extract 3,125%	35	26	37	36	33.50 ± 5.06	26±37
Extract 6,25%	13	10	9	14	11.50 ± 2.38	9±14
Extract 12,5%	0.00	0.00	0.00	0.00	0.00 ± 0.00	0.00
Extract 25%	0.00	0.00	0.00	0.00	0.00 ± 0.00	0.00
Extract 50%	0.00	0.00	0.00	0.00	0.00 ± 0.00	0.00
Extract 100%	0.00	0.00	0.00	0.00	0.00 ± 0.00	0.00

Based on Table 1, the highest mean number of *A. actinomycetemcomitans* colonies grown on MHA in the presence of *P. pellucida* extract was observed at the 3.125% concentration, with an average count of 33.50 CFU/mL. In contrast, no bacterial colonies (0 CFU/mL) were detected in the 12.5%, 25%, 50%, and 100% extract concentration groups, as well as in the positive control group (chlorhexidine), across all four replicates. The negative control group (distilled water) showed the highest average colony count at 167.00 CFU/mL.

The collected data were first analyzed for normality using the Shapiro–Wilk test, followed by a homogeneity assessment using the Levene test, prior to conducting statistical analysis.

Table 2. Normality and homogeneity tests of the colony counts of *A. actinomycetemcomitans*

Group	Normality Test (p-value)	Homogeneity Test (p-value)
Control (+) CHX	–	–
Control (−) Distilled Water	0.579*	–
Extract 3.125%	0.065*	–
Extract 6.25%	0.488*	–
Extract 12.5%	–**	0.000**
Extract 25%	–**	–
Extract 50%	–**	–
Extract 100%	–**	–

Notes:

1. * Normal distribution (Shapiro–Wilk test, $p > 0.05$).
2. ** Non-normal distribution or non-homogeneous variance ($p < 0.05$).
3. Homogeneity was assessed using Levene's test.
4. CHX = chlorhexidine positive control.
5. Distilled water served as the negative control.

Table 3. Comparison test of antibacterial effectiveness across all groups

Groups	Mean (CFU/mL)	Range (min-max)	p Value
Control (+) CHX	0.00	0.00	0.000*
Control (-) aquades	167.00	157±174	
Extract 3.125%	33.50	26±37	
Extract 6.25%	11.50	9±14	
Extract 12,5%	0.00	0.00	
Extract 25%	0.00	0.00	
Extract 50%	0.00	0.00	
Extract 100%	0.00	0.00	

Note: Non-parametric Kruskal–Wallis test, p < 0.05 (significant difference)

Based on the results of the normality and homogeneity tests shown in Table 2, the majority of data from all groups were not normally distributed, with probability values less than 0.05 (p < 0.05). The homogeneity test results also indicated that the data were not homogeneous, as all groups showed p-values less than 0.05 (p < 0.05). Given these results, statistical analysis was performed using the non-parametric Kruskal–Wallis test (*k* independent samples) to determine whether there were significant differences among the treatment groups, followed by pairwise comparisons using the Mann–Whitney test.

Based on Table 3, the data analysis results on the antibacterial effectiveness of each treatment group with different extract concentrations, as well as the negative and positive control groups, showed significant differences among the tested groups, with probability values less than 0.05 The analysis was subsequently continued with pairwise comparisons using the Mann–Whitney test to determine and compare the antibacterial effectiveness between two different concentrations of *Peperomia pellucida* (L.) Kunth extract, as presented in Table 4.

Table 4. Pairwise comparison test between treatment groups

Kelompok (n=4)	P Value *Mann-Whitney* Test							
	CHX (+)	Aquades (-)	SC 3.12%	SC 6.25%	SC 12.5%	SC 25%	SC 50%	SC 100%
Control (+) CHX		0.014*	0.014*	0.014*	1.000	1.000	1.000	1.000
Control (-) aquades	0.014*		0.021*	0.021*	0.014*	0.014*	0.014*	0.014*
Extract 3,125%	0.014*	0.021*		0.021	0.014*	0.014*	0.014*	0.014*
Extract 6,25%	0.014*	0.021*	0.021*		0.014*	0.014*	0.014*	0.014*
Extract 12,5%	1.000	0.014*	0.014*	0.014*		1.000	1.000	1.000
Extract 25%	1.000	0.014*	0.014*	0.014*	1.000		1.000	1.000
Extract 50%	1.000	0.014*	0.014*	0.014*	1.000	1.000		1.000
Extract 100%	1.000	0.014*	0.014*	0.014*	1.000	1.000	1.000	

Note: Mann–Whitney test p < 0.05 (statistically significant difference)

Based on Table 4, the results of the pairwise comparison between treatment groups using the Mann–Whitney test showed that all *Peperomia pellucida* (L.) Kunth extract concentrations—3.125%, 6.25%, 12.5%, 25%, 50%, and 100%—exhibited statistically significant differences compared with the negative control group (distilled water), with p< 0.05 Furthermore, Table 4 indicated no significant differences among the extract concentrations of 12.5%, 25%, 50%, and 100%, with p> 0.05 specifically 1.000.

MIC and MBC testing of *P. pellucida* extract against *A. actinomycetemcomitans* was performed using the serial dilution method with eight test groups: extract concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%, along with a positive control (chlorhexidine) and a negative control (distilled water). Each treatment was replicated four times. The MIC was defined as the lowest concentration of extract that inhibited ≥90% of bacterial growth compared with the negative control (distilled water), based on colony count observations.

The MBC was defined as the lowest concentration of extract that achieved ≥99.9% bacterial killing compared with the negative control.

The percentage inhibition for each treatment was calculated using the following formula:

$$\%\ Inhibition = \frac{N_c - N_t}{N_c} \times 100$$

N_c = colony count of the negative control group
N_t = colony count of the treatment group.

Table 5. Results of MIC and MBC testing of *Peperomia pellucida* (L.) Kunth extract against*A. actinomycetemcomitans*

Group	Inhibisi (%)				Mean	Int
	1	2	3	4		
Kontrol (+)	100	100	100	100	100	
Kontrol (-)	0	0	0	0	0	
Sirih Cina 3,125%	80	85	77	77	80	
Sirih Cina 6,25%	92	93	94	91	92.5*	KHM
Sirih Cina 12,5%	100	100	100	100	100*	KBM
Sirih Cina 25%	100	100	100	100	100	
Sirih Cina 50%	100	100	100	100	100	
Sirih Cina 100%	100	100	100	100	100	

Note:

1. Int (interpretation)
2. Growth inhibited by 90% (MIC), bacterial death by 99.9% (MBC)

Based on Table 5, the antibacterial effectiveness of *P. pellucida* extract against *A. actinomycetemcomitans* was observed to begin at a concentration of 3.125%, while the highest inhibitory activity occurred at concentrations of 12.5%, 25%, 50%, 100%, and in the positive control group. In contrast, the negative control group showed no antibacterial effect of *P. pellucida* extract against *A. actinomycetemcomitans*.

From the calculated results, the extract concentration of 6.25% was determined as the Minimum Inhibitory Concentration (MIC), as it inhibited *A. actinomycetemcomitans* growth by more than 90% (92.5%) compared to the negative control. The treatment group with an extract concentration of 12.5% was determined as the Minimum Bactericidal Concentration (MBC), as it achieved complete bacterial killing (>99.9%), with 100% bactericidal activity compared to the negative control.

The findings of this study indicate a clear concentration-dependent antibacterial response of *P. pellucida* leaf extract against *Aggregatibacter actinomycetemcomitans*. All tested concentrations were capable of suppressing bacterial viability, with higher concentrations demonstrating progressively stronger inhibitory and bactericidal effects. This pattern aligns with the expected behavior of phytochemical-rich extracts, in which increasing levels of active compounds enhance antimicrobial potency. The positive activity observed across the extract groups, including at lower concentrations, also suggests that *P. pellucida* contains bioactive molecules with substantial efficacy comparable to chlorhexidine. These results support the potential of *P. pellucida* as a natural antibacterial candidate against periopathogens. Several factors can influence the composition of plant extracts, including biological factors such as plant species, growing location, harvest time, plant age, and storage method. Chemical factors include the type of compounds and active constituents present, extraction methods, and solvents used.[29–32]

Antibacterial testing using the serial dilution method allows for the determination of bactericidal capacity at different antimicrobial concentrations. This method provides an objective quantitative assessment and is considered the gold standard, offering higher levels of automation compared with the diffusion method. Unlike the diffusion method, serial dilution enables the determination of both the MIC and MBC. However, its accuracy depends on factors such as the solubility of the tested compounds—poorly water-soluble compounds may not disperse evenly in liquid growth media. Additionally, turbidity and extract

coloration can complicate visual determination of bacterial growth endpoints.[33]

Based on the bacterial colony count results in Table 1, the highest bacterial growth was observed at 3.125% extract concentration, with a mean colony count of 33.50 CFU/mL. In contrast, no bacterial growth was detected at concentrations of 12.5%, 25%, 50%, and 100%, indicating that antibacterial effectiveness increased with higher extract concentrations. This finding aligns with previous studies reporting that higher concentrations enhance antibacterial potency and that increased extract concentration corresponds with stronger bacterial inhibition due to higher levels of active compounds.[12,34]

The present study also showed significant differences in bacterial growth between all extract concentrations and the negative control group (distilled water), but no significant differences compared with the positive control group (CHX). This finding is consistent with Nasution et al. (2024), who found that *P. pellucida* extract significantly differed from negative controls but not from CHX in inhibiting *A. actinomycetemcomitans* growth using the disc diffusion method.[35]

In this study, the MIC was defined as the lowest extract concentration that inhibited ≥90% of bacterial growth compared with the negative control. The MIC was determined to be 6.25%, which inhibited bacterial growth by 92.5% (mean colony count of 11.50 CFU/mL). The MBC was defined as the lowest concentration that achieved ≥99.9% bacterial killing, which was observed at 12.5%, where no bacterial growth occurred on MHA.[36]

The antibacterial activity of *P. pellucida* may be attributed to its phytochemical constituents, flavonoids, alkaloids, and tannins.[37,38] Flavonoids act by denaturing bacterial cell proteins, disrupting membrane permeability, and impairing cell function.[39] Alkaloids modify bacterial cell membranes, inhibit protein synthesis, and interfere with nucleic acid synthesis (DNA and RNA), thereby preventing replication and the expression of virulence genes, and exhibiting activity against both Gram-positive and Gram-negative bacteria.[40] Tannins bind to proteins via hydrogen bonds and hydrophobic interactions, disrupting bacterial metabolism. Their antibacterial effectiveness depends on pH, temperature, solvent type, and contact time.[25] Tannins can penetrate bacterial cell walls to the inner membrane, inhibit bacterial adhesion to surfaces, and ultimately cause cell death.[25,41]

CHX was used as the positive control due to its broad-spectrum antimicrobial properties and dual bacteriostatic and bactericidal effects. Its positively charged (cationic) molecules can penetrate bacterial membranes, altering permeability and leading to cell death.[22] Previous studies have reported that 0.2% CHX is a bis-biguanide antiseptic with rapid bactericidal activity, capable of reducing oral microbial counts by up to 80% with low toxicity.[42] This supports the present study's finding of no significant differences between CHX and *P. pellucida* extract concentrations of 12.5%, 25%, 50%, and 100%, as both may share similar antibacterial mechanisms involving membrane penetration and permeability disruption. This suggests that *P. pellucida* extract could serve as a potential alternative antibacterial agent, potentially reducing the adverse effects associated with long-term CHX use.[41,42] The anaerobic bacterial testing in this study followed WHO recommendations, employing (MHA) as the medium for evaluating antimicrobial activity across different test groups.[22,43]

This study reported no limitations that could affect the results or generalizability. However,

uncontrolled external factors may exist that could potentially influence outcomes, although none were observed in this research.

Based on the findings of this study, it is recommended that future research incorporate *time–kill assays* to evaluate the bactericidal properties of *Peperomia pellucida*. This method would allow assessment of bacterial killing dynamics over time, ideally presented as a kill–time curve or survival curve within 48 hours. Furthermore, the *time–kill assay* could be employed to investigate potential synergistic effects when combining plant-derived compounds.

CONCLUSION

The extract of *Peperomia pellucida* (L.) Kunth at concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100% demonstrated antibacterial activity against *Aggregatibacter actinomycetemcomitans*. There was a statistically significant difference in antibacterial effectiveness among these concentrations. The MIC of *P. pellucida* extract for inhibiting the growth of *A. actinomycetemcomitans* was determined to be 6.25%, while the MBC required to completely kill the bacteria was 12.5%.

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