ORIGINAL ARTICLE

The effectiveness of *Nicotiana tabacum* leaf extract as antibacterial agent against *Aggregatibacter actinomycetemcomitans*: an in vitro study

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Received: 07 Februari 2025
Revised: 12 June 2025
Accepted: 08 July 2025
Published: 31 July 2025
DOI: 10.24198/pjd.vol37no2.61637

p-ISSN 1979-0201
e-ISSN 2549-6212

Citation:
Vinsensius, TC; Widyastuti, W; The effectiveness of *Nicotiana tabacum leaf* extract as antibacterial agent against *Aggregatibacter actinomycetemcomitans*: an in vitro study. Padj J Dent, July. 2025; 37(2): 179-186.

ABSTRACT

Introduction: Dental caries remains a major public health issue in Indonesia and is often associated with bacterial pathogens such as *Aggregatibacter actinomycetemcomitans*, which lower the oral pH and contribute to tooth damage. Calcium hydroxide (Ca(OH)2) is commonly used in vital pulp therapy due to its antibacterial properties; however, it may cause adverse effects, including pulp necrosis. As natural alternative, tobacco leaf extract (*Nicotiana tabacum*) extract has shown potential due to its content of antibacterial compounds such as flavonoids and terpenoids. This study aims to analyse the antibacterial effect of *Nicotiana tabacum* extract against *A. actinomycetemomitans* as a potential alternative to Ca(OH)2. **Methods**: This study was an in-vitro experimental laboratory research with a post-test-only control group design. The antibacterial activity was tested using the plate count method on samples from seven different groups. The test was conducted using the microdilution method in 96-well plates, followed by the plate count method for bacterial enumeration. The test samples included *Nicotiana tabacum* leaf extract at concentrations of 3.125%, 6.25%, 12,5%, 25%, and 50%, with calcium hydroxide serving as the positive control and distilled water as the negative control. The data were analyzed using one-way ANOVA followed by Tukey' HSD post hoc test. **Results**: The study showed that the Minimum Inhibitory Concentration (MIC) of the extract against *Aggregatibacter actinomycetemcomitans* ranged from 12.5% to just below 25%, while a concentration of 25% represented the Minimum Bactericidal Concentration (MBC). The 25% concentration exhibited antibacterial activity comparable to that of calcium hydroxide. The Tukey HSD post hoc test revealed significant differences between the negative control and the *Nicotiana tabacum* leaf extract at concentrations of 6.25%, 12.5%, 25%, and 50%. However, no significant difference was found at the 3.125% concentration. **Conclusion**: *Nicotiana tabacum* leaf extract exhibits antibacterial activity against *Aggregatibacter actinomycetemcomitans*, with an MIC ranging from 12.5% to less than 25%, and an MBC of 25%.

KEYWORDS

Nicotiana tabacum leaf extract; antibacterial effects; *Aggregatibacter actinomycetemcomitans;* calcium hydroxide

INTRODUCTION

Dental caries is an infectious disease caused by bacterial interactions and is commonly found in humans.[1] In Indonesia, dental caries ranks as the most prevalent chronic disease among the population.[2] According to the 2023 SKI survey, 56.9% of Indonesians aged over three years experience dental and oral health issues, with a national caries prevalence reaching 88.80%.[3] One of the biofilm-forming microorganisms in the oral cavity is *Aggregatibacter actinomycetemcomitans*, a gram-negative bacterium that thrives under anaerobic

conditions.[4] The presence of *A. actinomycetemcomitans* can lower the pH of the oral cavity below 5.5, initiating the demineralization of hydroxyapatite crystals in enamel and proteolytic degradation of hard dental tissue structures.[5] Studies have shown that *A. actinomycetemcomitans* is found in 33.9% of deep carious lesions and significantly contributes to reversible pulpitis.[6]

Untreated tooth decay can lead to pain.[7] One way to maintain pulp vitality is by maintaining oral hygiene and treating teeth affected by deep caries.[8] A common treatment for caries involving the vital pulp is pulp capping using calcium hydroxide ($Ca(OH)_2$), which has long been considered as the gold standard for this therapy.[9] $Ca(OH)_2$ is a strong base with a pH of 12.5 and is known for its strong antibacterial properties as well as its ability to stimulate the formation of dentin bridges in areas of exposed pulp.[10] However, due to its high pH, $Ca(OH)_2$ can cause superficial necrosis of the pulp surface, which may lead to re-opening of the dentin bridge.[11] In addition, the application of $Ca(OH)_2$ at certain concentrations may also result in fibroblast cell death, leading to pulp tissue damage.[12]

Currently, natural materials are widely used as alternative treatments to reduce the side effects of chemical substances in healthcare.[13] One natural material commonly used in Indonesia is the tobacco plant (*Nicotiana tabacum*). Tobacco leaves have long been used by the kretek cigarette industry as the main ingredient, especially to create the distinctive aroma of kretek cigarettes.[14] Research has shown that in addition to their role in the tobacco industry, *Nicotiana tabacum* leaf extracts contain various active compounds with potential antibacterial and antifungal properties, which may help overcome some limitations of $Ca(OH)_2$, as the gold standard in vital pulp treatment. However, it is important to note that *Nicotiana tabacum* also contains toxic compounds, such as nicotine and other alkaloids, which may pose cytotoxic effects; therefore, its safety and dosage must be carefully evaluated prior to clinical application.[15]

Previous studies have tested the antibacterial activity of *Nicotiana tabacum* leaf extract against *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Candida albicans* using the disc diffusion method. The results showed that a 60% concentration of *Nicotiana tabacum* leaf extract effectively inhibited these oral pathogens, with inhibition zones increased proportionally with higher extract concentrations.[16] This study aims to further examine the antibacterial activity of *Nicotiana tabacum* leaf extract against *A. actinomycetemcomitans*, and to evaluate its potential as an alternative agent for pulp capping treatment. The novelty of this research lies in the application of *Nicotiana tabacum* for a bacterial target that has not been previously studied in this context, using a methodological approach distinct from previous studies.

METHODS

This study was an in-vitro experimental laboratory research with a post-test-only control group design. The research was conducted at the MiCORE Laboratory, Faculty of Dentistry, Universitas Trisakti, from September to November 2024. The procedure began with the preparation of leaf extract samples. Virsginia tobacco (*Nicotiana tabacum*) leaves aged 2 to 3 months characterized by healthy, green, unblemished appearance, were selected and processed into powder form. The powdered leaves were then macerated and dissolved using 96% ethanol at a ratio of 1:5 (tobacco leaf powder to ethanol). The resulting mixture was filtered using flannel cloth, and the ethanol solvent was removed using a rotary evaporator.

The extract was diluted into five concentrations (50%, 25%, 12.5%, 6.25%, and 3.125%) using sterile distilled water. In this study, a 100% concentration of *Nicotiana tabacum* leaf extract was not tested, as previous research had already proven that a 60% concentration is effective in eliminating oral cavity microbes, including *S. mutans*, *P. gingivalis*, and *Candida albicans*. The dilution process was calculated using the formula **V1 × M1 = V2 × M2,** where V1 represents the

volume of the pure solution (in microliters), M1 is the concentration of the stock solution (in percent), V2 is the total volume of the final solution (in microliters), and M2 is the desired concentration of the final solution (in percent).[17]

Bacterial media preparation is conducted by weighing 7.4 grams of Brain Heart Infusion Broth (BHI-B) powder and 3 grams of bacteriological agar powder using a digital scale. These were then dissolved in 200 mL of sterile distilled water in an Erlenmeyer flask. The mixture was stirred with a glass rod until homogeneous, forming Brain Heart Infusion Agar (BHI-A). The flask was covered with aluminum foil and sterilized in an autoclave at 121°C and 1 atm pressure for 15 minutes. After autoclaving, the BHI-A medium was poured into seven sterile Petri dishes to a thickness of approximately 4 mm and left to solidify.[17]

The bacterial sample used was *A. actinomycetemcomitans* ATCC 29522. The bacteriawere suspended in 10 mL of BHI-B, vortexed, and incubated. A microplate reader was used to measure the turbidity and determine the bacterial colonies, with the turbidity standard following McFarland 0.5 or 1.5 x 10^8 CFU/mL. The antibacterial activity test was conducted using the microdilution method in 96-well plates, followed by the plate count method for bacterial enumeration.[17]

The preparation of the positive control began by mixing the base and catalyst components of calcium hydroxide ($Ca(OH)_2$) from Dentsply using a cement spatula on a paper pad until evenly blended. Then, 0.28 grams of the $Ca(OH)_2$ mixture was placed into a microcentrifuge tube, followed by the addition of 0.7 mL of distilled water, and vortexed for 15 seconds. A micropipette was used to dispense the resulting solution into 96-well plates with four replicates, and the mixture was then combined with the bacterial suspension.[17]

The antibacterial test against *A. actinomycetemcomitans* was performed using a dilution method involving seven test groups: *Nicotiana tabacum* leaf extract at concentrations of 3.125%, 6.25%, 12.5%, 25%, and 50%, distilled water as a negative control, and $Ca(OH)_2$ as a positive control. A micropipette is used to dispense 100 µL of *A. actinomycetemcomitans* bacterial suspension and dispense it into each well of the 96-well plates, followed by the addition of 100 µL of each test solution. The mixture was then incubated for 24 hours at 37°C under anaerobic conditions.

After incubation, each mixture of bacterial suspension and test solution was diluted 1000 times using a micropipette. Then, 5 µL of the diluted solution was streaked onto petri dishes containing BHI-A medium and incubated for 24 hours at 37°C under anaerobic conditions. Bacterial colony counts were conducted to determine the Minimum Inhibitory Concentration (MIC). The BHI-A media was further incubated for another 24 hours to determine the Minimum Bactericidal Concentration (MBC). Bacterial colonies were counted four times on each Petri dish using the plate count method.[17]

The data were then analyzed by applying the Shapiro-Wilk test to evaluate the normality. If the data were normally distributed ($p > 0.05$), a parametric test using one-way Analysis of Variance (ANOVA) was performed. When significant differences were detected ($p < 0.05$), the Tukey HSD (Honestly Significant Difference) post hoc test was used to identify specific groups differences.

RESULTS

The results of antibacterial test for all groups treated with *Nicotiana tabacum* leaf extract are presented in the following figure.

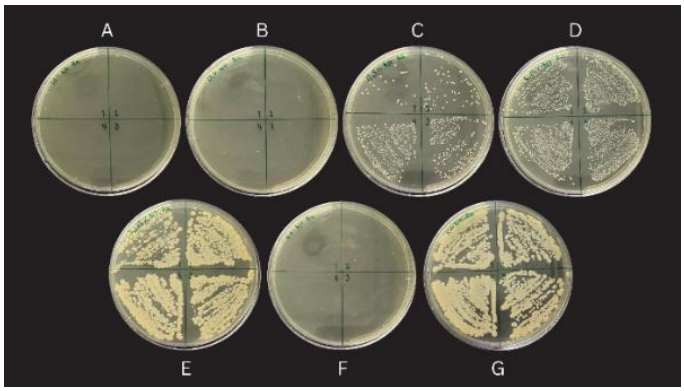

Figure 1. Antibacterial test results of *Nicotiana tabacum* leaf extract against *A. actinomycetemcomitans* using the plate count method. (A) *Nicotiana tabacum* leaf extract 50%; (B) *Nicotiana tabacum* leaf extract 25%; (C) *Nicotiana tabacum* leaf extract 12.5%; (D) *Nicotiana tabacum* leaf extract 6.25%; (E) *Nicotiana tabacum* leaf extract 3.125%; (F) Ca(OH)2 (positive control); (G) Aquadest (negative control).

On petri dishes containing *A. actinomycetemcomitans*, no bacterial colonies were observed in the groups treated with *Nicotiana tabacum* leaf extract at concentrations of 50%, 25%, as wll as in the $Ca(OH)_2$ positive control group,. Treatment with *Nicotiana tabacum* leaf extract at a concentration of 12.5% resulted in an average colony count of (239 ± 215) x 10^5 CFU/mL. At a 6.25%, the average colony count was (423 ± 66.42) x 10^5 CFU/mL, and at a 3.125%, the average count increased to (642 ± 132.12) x 10^5 CFU/mL. The negative control group (distilled water) exhibited the highest average colony count, at (716 ± 43.99) x 10^5 CFU/mL (Table 1). *Nicotiana tabacum* leaf extracts contain active compounds with antibacterial effects against *A. actinomycetemcomitans* as presented in the Table 1 below.

Table 1. Average Colony Count of *Aggregatibacter actinomycetemcomitans*

	Concentration Group	Average number of colonies ± SD (CFU/mL)
A	*Nicotiana tabacum* leaf extract 50%	(0,00 ± 0) x 10^5
B	*Nicotiana tabacum* leaf extract 25%	(0,00 ± 0) x 10^5
C	*Nicotiana tabacum* leaf extract 12,5%	(239 ± 215) x 10^5
D	*Nicotiana tabacum* leaf extract 6,25%	(423 ± 66,42) x 10^5
E	*Nicotiana tabacum* leaf extract 3,125%	(642 ± 132,12) x 10^5
F	Ca(OH)2	(0,00 ± 0) x 10^5
G	Aquadest	(716 ± 43,99) x 10^5

The normality test showed that the data were normally distributed ($p>0.05$), allowing for a one-way ANOVA test (Table 2).

Table 2. Tests of normality results using Shapiro-Wilk

	K-	3,125%	6,25%	12,5%	25%	50%	K+
Sig.	.220	.057	.533	.505	.	.	.

The one-way ANOVA revealed a significant difference among the test groups ($p<0.05$). Therefore, the analysis was followed by the Tukey HSD post hoc test. The results indicated significant differences between the negative control and the *Nicotiana tabacum* leaf extract at concentrations of 6.25%, 12.5%, 25%, and 50%. However, no significant difference was observed at a concentration of 3.125% (Table 3).

Table 3. Antibacterial test results of *Nicotiana tabacum* leaf extract compared to negative and positive controls using Tukey HSD post hoc test

	3,125%	6,25%	12,5%	25%	50%
K (-)	0,967	0,025*	0,000*	0,000*	0,000*
K (+)	0,000*	0,000*	0,095	1	1

* = significant difference with a p-value < 0.05

Based on these results, the Minimum Inhibitory Concentration (MIC) of *Nicotiana tabacum* leaf extract against *Aggregatibacter actinomycetemcomitans* lies between 12.5% and less than 25%, as bacterial growth was still observed at 12.5% but not at 25%. The Minimum Bactericidal Concentration (MBC) was determined to be 25%, since no bacterial colonies were observed at this concentration and higher.

DISCUSSION

In this study, *Nicotiana tabacum* leaves were macerated with 96% ethanol to obtain the extract. The maceration technique is a preferred method, as it minimizes the risk of damaging the active compounds present *in Nicotiana tabacum* leaves during extraction process.[18] The use of 96% ethanol as a solvent is considered effective due its low toxicity and high solubility.[19] According to the literature, ethanol-based solvents can optimize the extraction of flavonoids and phenolic compounds found in plants.[20]

The antibacterial test was conducted on *A. actinomycetemcomitans,* one of the bacteria commonly associated with deep caries. Along with other deep pathogens, *A. actinomycetemcomitans* can contributes to pulp irritation.[6] Calcium hydroxide ($Ca(OH)_2$) was used as the positive control in this study due its potent antibacterial properties , making it known as the gold standard for vital pulp treatment materials. In vital pulp treatment, particularly pulp capping, $Ca(OH)_2$ helps protect the pulp complex and maintain pulp vitality.[9] Distilled water, which has neutral properties and does not promote bacterial growth, was used as the negative control.[21] Antibacterial testing performed to determine the MIC and MBC values was conducted using the microdilution method, followed by the plate count method. The plate count method for bacterial colony enumeration is relatively simple and does not require complex equipment, making it accessible for laboratory use.[22]

The findings of this study showed that *Nicotiana tabacum* leaf extract has antibacterial activity against *A. actinomycetemcomitans*. At a concentration of 12.5%, bacterial growth was still observed with an average colony count of (239 ± 215) x 10^5 CFU/mL. In contrast, no bacterial growth was detected at concentrations of 25% and 50% concentrations. The MIC value was defined as the lowest concentration of *Nicotiana tabacum* leaf extract that inhibited visible bacterial growth, while the MBC reffered to the lowest concentration that completely eliminated bacterial colonies.[23] Based on these criteria, the 12.5% concentration was identified as the MIC, and the 25% concentration was determined to be as the MBC.

Nicotiana tabacum leaf extract at a 12.5% concentration significantly inhibited the growth of *A. actinomycetemcomitans*, possibly because *A. actinomycetemcomitans* is a gram-negative bacterium with a cell wall containing high lipid content. In this study, it is suggested that the lipid compounds in the cell wall of *A. actinomycetemcomitans* were disrupted upon contact with the extract, causing bacterial cell walls to form pores that facilitated the penetration of active compounds from the *Nicotiana tabacum* leaf extract.[24] The 25% concentration demonstrated antibacterial effectiveness similar to that of $Ca(OH)_2$, used as the positive control. Overall, the findings indicate that the antibacterial

activity of *Nicotiana tabacum* against *A. actinomycetemcomitans* increases with higher extract concentrations.

These results are in line with previous studies that evaluated the antibacterial effects of *Nicotiana tabacum* leaf extract against *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Candida albicans*. Those studies, which used the well diffusion method, showed that the antibacterial activity became stronger with higher concentration -similar to the findings observed in this study.[25] However, other research examining *N. tabacum* extract against *Staphylococcus aureus* reported different results. In that study, the antibacterial effect did not consistently increase with higher concentrations. This inconsistency may be attributed to the formation of larger extract particles at higher concentrations, which could hinder their ability to penetrate the bacterial cell wall. In this case of *S. aureus,* this may be due to limited transport through membrane proteins.[26]

The antibacterial effects of *Nicotiana tabacum* leaf extract against *A. actinomycetemcomitans* may be attributed to the presence of active compounds such as flavonoids, saponins, steroids, and terpenoids. Each of these compounds has its own unique mechanism for inhibiting bacterial growth. Flavonoids, a type of polyphenol, inhibit bacterial proliferation by disrupting energy metabolism, damaging the cell membrane, and reducing nucleic acid synthesis.[25] Saponins and steroids have been reported to possess anti-genotoxic, anti-mutagenic, and anti-inflammatory properties. The antibacterial mechanism of terpenoids in *Nicotiana tabacum* is believed to involve the inhibition of the bacterial enzymes responsible for cell wall synthesis.[26]

The average total colony count of *A. actinomycetemcomitans* showed a relatively high standard deviation, which may be attributed to several limitations in the study. First, the experimental procedures were highly sensitive to technical variations, such as inconsistencies in bacterial streaking, pipetting errors, or potential cross-contamination, all of which could affect the accuracy and reproducibility of the results. Additionally, environmental factors such as pH, temperature, and media composition in the test conditions may not fully replicate cliical or from real-world settings, potentially affecting the observed antibacterial activity of the tested compounds.

These results indicate that *Nicotiana tabacum* leaf extract has the potential to serve as a promising and innovative alternative for vital pulp therapy. Its antibacterial activity against *A. actinomycetemcomitans* was similar to that of calcium hydroxide ($Ca(OH)_2$) . With further refinement and evaluation of its safety profile, *Nicotiana tabacum* may offer a natural, plat-based option for use in vital pulp treatment

To further explore the potential use of *Nicotiana tabacum* leaf extract as an alternative for vital pulp therapy, additional studies are recommended. These should include the use of different extraction methods to obtain a more optimal extract and its efficacy, in vitro toxicity and solubility assessments and investigations into its potential synergistic effects when combined with other biomaterials.

One limitation of this study is the use of crude extracts without isolating specific active compounds, which may result in unpredictable antibacterial effects. To address this, future studies should explore alternative extraction methods to obtain a more optimal (refined and standardized) *Nicotiana tabacum* leaf extract. Additionallym further testing is recommended to within the concentration range of 12.5% to 25% to determine a more accurate Minimum Inhibitory Concentration (MIC) value.

CONCLUSION

Nicotiana tabacum leaf extract exhibits antibacterial effects against *A. actinomycetemcomitans*, with a Minimum Inhibitory Concentration (MIC) ranging

from 12.5% to less than 25%, and a Minimum Bactericidal Concentration (MBC) of 25%. The 25% concentration as the MBC. The 25% *Nicotiana tabacum* leaf extract demonstrated antibacterial effects equivalent to those of Ca(OH)2. The implications of this research support the potential for further investigation into plant-based antibacterial agents as alternatives to chemical-based such as Ca(OH)2 for use in pulp therapy.

Acknowledgement: The author would like to thank the professionals who contributed to the research and preparation of this manuscript.
Author Contributions: Conceptualization, TCV and WW; methodology, TCV; software, TCV; validation, TCV and WW; formal analysis, TCV; investigation, TCV; resources, TCV; data curation, TCV; writing original draft preparation, TCV; writing review and editing, TCV; visualization, TCV; supervision, TCV; project administration, TCV; funding acquisition, WW. All authors have read and agreed to the published version of the manuscript.", please turn to the CRediT taxonomy for the term explanation.
Funding: This research received no funding.
Institutional Review Board Statement: Ethical review and approval were waived for this study, as it did not involve human participants or animal subjects.
Informed Consent Statements: Not applicable. This study did not involve human participants.
Data Availability Statement: Data supporting the findings of this study are available from the corresponding author upon reasonable request.
Conflicts of Interest: The authors declare no conflict of interest or potential commercial background related to this research.

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