Interdental Jurnal Kedokteran Gigi
Website: https://e-journal.unmas.ac.id/index.php/interdental
ISSN 1979-9144 (print), ISSN 2685-5208 (online)

Research Article

Antibacterial Effects Of Tobacco Leaf Extract (Nicotiana tabacum) On Fusobacterium Nucleatum (In Vitro Study)

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Received date: March 21, 2024 **Accepted date: April 5, 2025** **Published date: April 22, 2025**

KEYWORDS

Fusobacterium nucleatum, Nicotiana tabacum leaf extract, antibacterial effects, MIC, MBC

ABSTRACT

Introduction: Deep caries can involve the pulp tissue, necessitating pulp capping treatment. Fusobacterium nucleatum is frequently found in deep caries. One of the essential requirements for pulp capping materials is antibacterial properties. Mineral Trioxide Aggregate (MTA) is a commonly used pulp capping material due to its favorable characteristics, such as antibacterial properties, biocompatibility, and more. However, MTA has several drawbacks, including a long setting time, difficult manipulation, risk of staining and discoloration, and high cost. Nicotiana tabacum leaf extract has the potential to serve as an herbal alternative for pulp capping materials, as it is known to possess antibacterial properties derived from its secondary metabolites. This study aimed to determine the antibacterial effects of Nicotiana tabacum leaf extract on Fusobacterium nucleatum.
Materials and Methods: An in vitro experimental laboratory study with a post-test only control group design was conducted. The antibacterial test used the 96-well microdilution and plate count methods to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). There were nine test groups, consisting of Nicotiana tabacum leaf extract at 3.125%, 6.25%, 12.5%, 25%, and 50% of 1000 µg/µL (31.25 µg/µL, 62.5 µg/µL, 125 µg/µL, 250 µg/µL, and 500 µg/µL), sterile aquadest (negative control), MTA (positive control), and internal controls (Nicotiana tabacum leaf extract and sterile aquadest).
Results: The number of bacterial colonies significantly decreased as the extract concentration increased. Nicotiana tabacum leaf extract showed MIC values at 12.5% (125 µg/µL) and MBC values at 25% (250 µg/µL). The antibacterial activity of Nicotiana tabacum leaf extract is attributed to its secondary metabolites, including flavonoids, saponins, steroids, and triterpenoids, which disrupt bacterial structures and functions.
Conclusion: Nicotiana tabacum leaf extract has antibacterial effects (bacteriostatic and bactericidal) on Fusobacterium nucleatum.

DOI : 10.46862/interdental.v21i1.11388

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How to cite this article: Wilsan M, Widyastuti W, Wedagama DM. (2025). Antibacterial Effects of Tobacco Leaf Extract (Nicotiana tabacum) on Fusobacterium nucleatum (in vitro Study). Interdental Jurnal Kedokteran Gigi 21(1), 64-71 DOI: 10.46862/interdental.v21i1.11388

INTRODUCTION

Teeth play a vital role in chewing, speaking, and aesthetics,[1] making proper maintenance essential for optimal function. One of the most prevalent oral diseases is dental caries,[2] a progressive condition characterized by enamel demineralization and subsequent destruction of deeper tooth structures, potentially reaching the pulp.[3] Caries develops through the interaction of four main factors: the host (tooth structure), substrate (carbohydrates), microorganisms (bacteria), and time.[4] Bacteria within the biofilm metabolize carbohydrates and produce acids, which lower the pH and lead to tooth demineralization.[5] If not treated, this can lead to pulp inflammation, which can evolve into necrotic tissue. Therefore, pulp treatment is crucial for maintaining the vitality of carious teeth.[6]

Many bacterial species contribute to the development of deep caries, including *Fusobacterium nucleatum*,[7] a gram-negative obligate anaerobe.[8] Studies have shown that *Fusobacterium nucleatum* is frequently found in the deep layers of carious lesions, with a prevalence of 74%. This bacterium acts as a co-aggregator with other bacteria, promoting an acidic environment that accelerates demineralization and contributes to further caries progression through inflammation.[9,10]

Treatment of exposed pulp often requires root canal therapy (RCT) or even tooth extraction. RCT involves multiple visits and significant costs. Pulp capping has emerged as an alternative method to preserve pulp vitality and avoid more extensive treatments. In pulp capping procedures, biomaterials can be placed directly on the exposed pulp (direct) or over the remaining thin layer of dentin (indirect) to maintain pulp vitality.[11] Mineral Trioxide Aggregate (MTA) is a preferred material due to its physical and bioactive properties, including antibacterial effects, biocompatibility, high pH, and the ability to stimulate dentin bridge formation. However, the high cost of MTA, long setting time, manipulation difficulties, and potential staining and discoloration of teeth are its limitations.[12]

In countries with rich biodiversity, such as Indonesia, the use of herbal-based medicine is common.[13] Its use is preferred due to its affordability, availability, and perceived safety compared to conventional medicine.[14] As the sixth-largest producer of *Nicotiana tabacum* (tobacco) in the world, Indonesia contributes approximately 1.91% of the global supply,[15] with tobacco leaves and cigarettes being the primary products. Tobacco leaves contain various secondary metabolites such as flavonoids, saponins, steroids, terpenoids, and alkaloids, which exhibit significant antibacterial activity.[16] These secondary metabolites act by inhibiting cell wall synthesis, altering membrane permeability, and inhibiting protein and nucleic acid metabolism.[17]

One important characteristic of pulp capping materials is their antibacterial effect. In addition to this, pulp capping materials must contain calcium, which plays a crucial role in reparative dentin formation and is considered a primary mediator of the mineralization process.[11] *Nicotiana tabacum* does not naturally contain calcium. This study aims to evaluate the antibacterial effects of *Nicotiana tabacum* leaf extract against *Fusobacterium nucleatum* as a potential material for pulp capping (as preliminary research). While previous studies have investigated the antibacterial effects of *Nicotiana tabacum* leaf extract on *Staphylococcus aureus*,[18] *Streptococcus mutans*, and *Porphyromonas gingivalis*,[19] its effect on *Fusobacterium nucleatum* has not been explored.

MATERIAL AND METHOD

This study received ethical exemption from the Ethics Committee of the Faculty of Dentistry, Universitas Trisakti (Approval No. 776/S1/KEPK/FKG/6/2024) prior to its commencement. This *in vitro* laboratory experimental research used a *post-test only control group design* and was conducted at the Microbiology Center of Research and Education (MiCORE), Faculty of Dentistry, Universitas Trisakti. The sample size was calculated using the unpaired numerical analytic formula by Lemeshow. Based on this calculation, the minimum required sample size for each group was one. However, to enhance validity, obtain more accurate results, and minimize potential data

errors, this study was conducted with four repetitions for each group.

The extraction of *Nicotiana tabacum* leaves was carried out at Balai Pengujian Standar Instrumen Tanaman Rempah, Obat dan Aromatik (BPSI-TROA). Healthy and mature *Nicotiana tabacum* leaves were selected. A total of 1500 grams of leaves were dried in an oven at 40–50°C for one week, then ground into a fine powder. The maceration process was carried out using 96% ethanol (1:5 ratio), shaken for 2–3 hours, and left at room temperature for 24 hours. The macerate was filtered, and the solvent was evaporated using a rotary evaporator to obtain a thick extract. Subsequently, qualitative phytochemical screening was conducted at BPSI-TROA using standard reagents to identify secondary metabolites in *Nicotiana tabacum* leaf extract.

The antibacterial test solutions were prepared using *Nicotiana tabacum* leaf extract at an initial concentration of 1000 µg/µL. Serial dilutions were performed with sterile aquadest to obtain final concentrations of 500 µg/µL (50%), 250 µg/µL (25%), 125 µg/µL (12.5%), 62.5 µg/µL (6.25%), and 31.25 µg/µL (3.125%). The MTA test solution was prepared by weighing 0.28 g of MTA powder and mixing it with 0.7 mL of sterile aquadest at a 4:10 ratio, which is equivalent to 0.4 g/mL.[20] The mixture was then homogenized using a vortex mixer to achieve a concentration of 400 mg/mL.

Bacterial growth media were prepared by weighing 2.7 g of bacteriological agar and 6.66 g of Brain Heart Infusion Broth (BHI-B) using a digital balance. The components were mixed with 180 mL of sterile aquadest in an Erlenmeyer flask and stirred until homogeneous. The flask was covered with aluminum foil and sterilized in an autoclave at 121°C for 15 minutes. After sterilization, the media were aseptically poured into nine sterile Petri dishes and left to solidify at room temperature. The Petri dishes were then incubated at 37°C for 24 hours to confirm the absence of contamination before use.

A bacterial culture of *Fusobacterium nucleatum* ATCC 25586 was prepared by retrieving 10 µL of bacteria from cryopreserved stock stored at -80°C using a micropipette. The bacteria were transferred into a 1.5 mL microcentrifuge tube containing 1 mL of sterile BHI-B and homogenized with a vortex mixer. The tube was then incubated anaerobically at 37°C for 24 hours. Once turbidity was observed, bacterial density was measured using a microplate reader at 600 nm. If the optical density (OD) ranged from 0.08 to 0.1, it was considered equivalent to McFarland standard 0.5 or approximately 1.5×10^8 CFU/mL. The bacterial suspension was then diluted to achieve a final concentration of 1.5×10^5 CFU/mL.

The study included nine test groups: *Nicotiana tabacum* leaf extract at concentrations of 3.125%, 6.25%, 12.5%, 25%, and 50%; sterile aquadest (negative control); MTA (positive control); and internal controls (containing only *Nicotiana tabacum* leaf extract or sterile aquadest) to detect potential contamination. A 100 µL bacterial suspension was pipetted into each well of a 96-well plate, followed by the addition of 100 µL of each test solution, except for the internal control wells, which received 200 µL of either *Nicotiana tabacum* leaf extract or sterile aquadest only. The plates were incubated anaerobically at 37°C for 48 hours. After incubation, serial dilutions were performed to achieve a final dilution of 1:1000. A 5 µL aliquot from each diluted solution was streaked onto BHI-A agar in the Petri dishes using an inoculating loop, with each Petri dish divided into four sections. The nine Petri dishes were then incubated at 37°C for 48 hours, and colony counts were performed to determine the Minimum Inhibitory Concentration (MIC). The dishes were then reincubated for an additional 24 hours to determine the Minimum Bactericidal Concentration (MBC).

The data were then processed and analyzed using the Statistical Product and Service Solutions (SPSS) software. Data normality was assessed using the Shapiro-Wilk test. The results indicated that the data were not normally distributed ($p < 0.05$). Therefore, the Kruskal-Wallis test was applied. Significant differences were observed ($p < 0.05$), leading to further analysis using the Mann-Whitney test to identify the groups with significant differences.

RESULT AND DISCUSSION

The qualitative phytochemical analysis of *Nicotiana tabacum* leaf extract revealed the presence of

several secondary metabolites. The results were positive for flavonoids, saponins, steroids, and triterpenoids. This indicates the presence of bioactive compounds responsible for the extract's antibacterial activity.

The results indicate that *Nicotiana tabacum* leaf extract exhibits antibacterial effects, including the ability to inhibit and kill *Fusobacterium nucleatum* at specific concentrations. The average *Fusobacterium nucleatum* colony count in the negative control (sterile aquadest) was significantly high (exceeding the normal range established by the FDA, which is 25–250), while the average colony counts for *Nicotiana tabacum* leaf extract were 18 ± 8.92 CFU at 3.125%, 5 ± 1.71 CFU at 6.25%, 4 ± 3.69 CFU at 12.5%, 1 ± 0.58 CFU at 25%, and 0 ± 0.00 CFU at 50%. The internal control for *Nicotiana tabacum* leaf extract showed an average of 0 ± 0.50 CFU, while the sterile aquadest internal control had 0 ± 0.00 CFU. The positive control (MTA) also had an average of 0 ± 0.00 CFU (Table 1, Figure 1). The variations in standard deviation at all tested concentrations suggest a slight pipetting error at the 12.5% concentration.

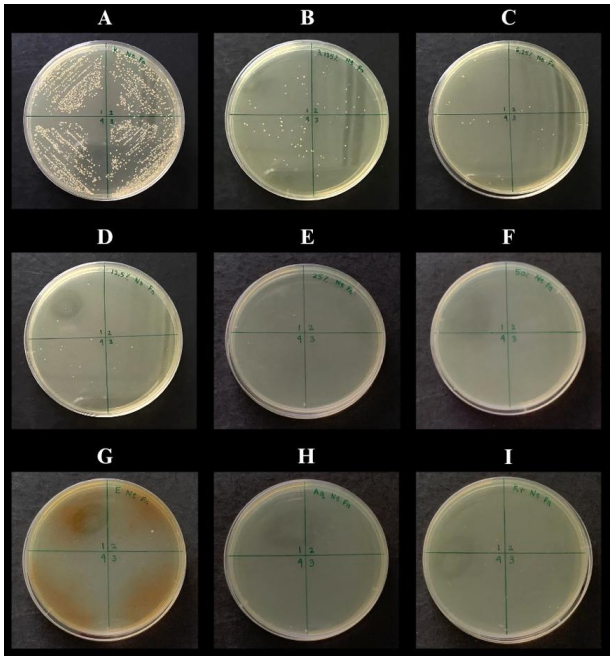

Figure 1. Antibacterial test results of *Nicotiana tabacum* leaf extract against *Fusobacterium nucleatum* using the Plate Count method. (A) Negative control (sterile aquadest); (B) *Nicotiana tabacum* leaf extract 3.125%; (C) *Nicotiana tabacum* leaf extract 6.25%; (D) *Nicotiana tabacum* leaf extract 12.5%; (E) *Nicotiana tabacum* leaf extract 25%; (F) *Nicotiana tabacum* leaf extract 50%; (G) Internal control (*Nicotiana tabacum* leaf extract); (H) Internal control (sterile aquadest); (I) Positive control (MTA).

Table 1. Average total colony count of *Fusobacterium nucleatum*

No.	Test Group	Mean ± SD (CFU)
A	Negative control (sterile aquadest)	> 250
B	*Nicotiana tabacum* leaf extract 3.125%	18 ± 8.92
C	*Nicotiana tabacum* leaf extract 6.25%	5 ± 1.71
D	*Nicotiana tabacum* leaf extract 12.5%	4 ± 3.69
E	*Nicotiana tabacum* leaf extract 25%	1 ± 0.58
F	*Nicotiana tabacum* leaf extract 50%	0 ± 0.00
G	Internal control (*Nicotiana tabacum* leaf extract)	0 ± 0.50
H	Internal control (sterile aquadest)	0 ± 0.00
I	Positive control (MTA)	0 ± 0.00

The results of data processing and analysis using the SPSS software indicated that the data were not normally distributed, as assessed by the Shapiro-Wilk test ($p < 0.05$). Consequently, the Kruskal-Wallis test was applied, revealing significant differences between the test groups ($p < 0.05$). Further analysis using the Mann-Whitney test confirmed significant differences ($p < 0.05$) between the negative control and all concentrations of *Nicotiana tabacum* leaf extract.

Ethanol 96% was chosen as the solvent due to its universal polarity, non-toxicity, ease of evaporation, and selective ability to extract compounds efficiently. Its high concentration allows better penetration into the cell wall, resulting in a more concentrated extract.[21,22] MTA was used as a positive control because of its known antibacterial properties as a pulp capping material, while sterile aquadest was used as a negative control because of its neutral composition, ensuring no effect on bacterial growth.[23]

The maceration method was chosen for its effectiveness in isolating natural compounds without requiring heat, thereby minimizing the risk of degradation of active compounds. It is also cost-effective and easy to perform.[24,25] The 96-well microdilution method was chosen for its quantitative results, minimal sample requirement, high sensitivity, and short testing time. The plate count method was chosen for its high sensitivity, as it measures only viable cells and has a simple procedure.[26]

The concentrations of *Nicotiana tabacum* leaf extract used in this study (3.125%, 6.25%, 12.5%, 25%, and 50%) were selected based on previous studies showing the antibacterial efficacy of *Nicotiana tabacum* leaf extract at higher concentrations. A study by Riria Hendarto Putri

et al. reported that the extract effectively inhibited *Streptococcus mutans* at 80% concentration and *Porphyromonas gingivalis* at 100%.[19] Other research indicated that the extract demonstrated optimal antibacterial activity against *Staphylococcus aureus* at 60% concentration.[18]

The findings of this study revealed that *Nicotiana tabacum* leaf extract exhibited significant antibacterial activity against *Fusobacterium nucleatum*. This activity was observed through a reduction in bacterial colony count as extract concentration increase. At 3.125%, bacterial inhibition began to appear, although colony numbers remained relatively high. The MIC was determined at 12.5%, as bacterial colony growth was significantly reduced, indicating strong inhibitory activity. Meanwhile, at 25%, only two bacterial colonies remained, which were considered insignificant, fulfilling the criteria for MBC and indicating bactericidal activity. This aligns with the study by Mogana *et al.*, which stated that concentrations resulting in fewer than ten colonies could be classified as MBC.[27] At the highest concentration (50%), the extract demonstrated maximum antibacterial effectiveness, with no bacterial growth observed.

The antibacterial activity of *Nicotiana tabacum* leaf extract is due to its secondary metabolites. Steroids disrupt bacterial lipid membranes, compromising membrane stability and inhibiting essential nutrient uptake required for bacterial growth.[28,29] Triterpenoids interfere with transmembrane protein structures, reducing cell wall permeability and impairing bacterial nutrient absorption.[30] Flavonoids inhibit nucleic acid synthesis, membrane function, and energy metabolism, as well as disrupt bacterial permeability.[28] Saponins increase membrane permeability, causing leakage of intracellular bacterial components.[31] The synergistic combination of these compounds in *Nicotiana tabacum* leaf extract provides significant antibacterial activity.

The positive control (MTA) showed no bacterial growth, confirming its effectiveness as an antibacterial agent. The negative control (sterile aquadest) showed extensive bacterial colony growth, indicating that *Fusobacterium nucleatum* proliferated optimally without intervention. The internal control for *Nicotiana tabacum* leaf extract showed very low bacterial colony counts, with only one colony in one of four samples, considered insignificant.[27] Meanwhile, the internal control for sterile aquadest exhibited no bacterial colonies, confirming that the test media were free from contamination. These internal control results verify that there was no significant contaminant influence on antibacterial testing, ensuring the validity of the data obtained.

In addition to the presence of antibacterial agents, bacterial morphology also plays a crucial role in the mechanism of antibacterial action. The ability of each bacterium to resist antibacterial agents varies depending on the composition and thickness of its cell wall. Active antibacterial compounds diffuse through the bacterial cell wall to exert their bactericidal effects.[32]

A comparison with the study conducted by Anis Uswatun Khasanah *et al.* on the antibacterial activity of *Nicotiana tabacum* ethanolic extract against *Staphylococcus aureus* reveals differences in the effective concentrations required for inhibition. In their study, the optimal concentration against *Staphylococcus aureus* was 60%, producing an inhibition zone of 12 mm.[18] Meanwhile, in this study, the MIC against *Fusobacterium nucleatum* was determined to be only 12.5%. This difference may be attributed to the distinct cell wall structures of the two bacteria. *Fusobacterium nucleatum* is a gram-negative bacterium with a thin peptidoglycan layer located within the periplasmic space, situated between the inner and outer membranes composed of lipopolysaccharides (LPS) and phospholipids. This structure is relatively more permeable to active antibacterial compounds. Conversely, *Staphylococcus aureus* is a gram-positive bacterium with a cell wall primarily composed of a thick peptidoglycan layer, where the peptidoglycan chains are interconnected by peptide bonds. Teichoic acids further stabilize these layers, creating a dense and rigid cell wall. This structure provides additional protection to the bacterium and makes the diffusion of active compounds into the cell more challenging.[33–35] As a result, a higher concentration of *Nicotiana tabacum* leaf extract is required to achieve antibacterial efficacy against *Staphylococcus aureus* compared to *Fusobacterium nucleatum*.

CONCLUSSION

Based on the findings of this study, it can be concluded that Nicotiana tabacum leaf extract exhibits antibacterial activity against Fusobacterium nucleatum. This is evidenced by a significant reduction in bacterial colony counts with increasing extract concentrations. The MIC required to inhibit Fusobacterium nucleatum growth is 12.5% (125 µg/µL), while the MBC needed to eliminate Fusobacterium nucleatum is 25% (250 µg/µL). At 25%, the extract shows antibacterial effects comparable to MTA.

Further research is recommended to evaluate the effectiveness of Nicotiana tabacum leaf extract against other bacteria associated with deep caries, characterize its active compounds, and explore its combination with calcium to enhance its potential as a pulp capping material. Additionally, cytotoxicity and biocompatibility tests, along with developing a more stable formulation, should be conducted before clinical application.

ACKNOWLEDGEMENT

We would like to express our gratitude to Mario Richi for his assistance in the laboratory and support during this research. We also extend our thanks to the faculty members for their guidance and support in completing this study.

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