World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

Check for updates

Investigation of the antibacterial effects of hinokitiol on *Fusobacterium nucleatum*

Norimasa Tsuji [1,*], Hideki Yoshimatsu [2], Ryuhei Kanda [3], Masataka Hashimoto [1], and Hiroshi Maeda [1]

[1] *Department of Endodontics, Osaka Dental University 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan.*
[2] *Department of Preventive and Community Dentistry 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan.*
[3] *Division of Creative and Integrated Medicine, Advanced Medicine Research Center, Translational Research Institute for Medical Innovation (TRIMI), Osaka Dental University 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan.*

World Journal of Advanced Research and Reviews, 2025, 25(03), 103-108

Publication history: Received on 13 January 2025; revised on 24 February 2025; accepted on 27 February 2025

Article DOI: https://doi.org/10.30574/wjarr.2025.25.3.0607

Abstract

Hinokitiol (HNK), which is known to have stress-relieving properties in living organisms and to show antimicrobial activity against both bacteria and fungi, has been used as a form of aromatherapy in oral care products, such as bath additives, toothpaste, and mouthwash. This study was performed to assess the antibacterial effects of HNK on *Fusobacterium nucleatum* isolates from patients with periodontitis. *F. nucleatum* was cultured on Gifu Anaerobic Medium (GAM) for 24 h. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of HNK against *F. nucleatum* were determined using the liquid dilution method. Furthermore, the bactericidal effects of HNK on *F. nucleatum* were evaluated using adenosine triphosphate (ATP) bioluminescence assay and flow cytometry.

The MIC and MBC of HNK against *F. nucleatum* were 50 μg/mL and 200 μg/mL, respectively. When HNK solution was added to *F. nucleatum* cultures adjusted to 106 CFU/mL at the MIC, the ATP levels within the *F. nucleatum* cells decreased significantly in the experimental group compared with the controls after 24 h. Moreover, staining with propidium iodide (PI) and fluorescence intensity measurements using flow cytometry revealed that the addition of HNK increased the number of dead cells. In summary, HNK demonstrated significant antibacterial effects against *F. nucleatum*, suggesting its potential as a plant-derived agent for prevention of periodontitis. This study highlighted the potential of HNK as a component of dental care for prevention of periodontitis.

Keywords: Hinokitiol; *Fusobacterium Nucleatum*; Antibacterial Activity; Periodontitis

1. Introduction

Hinokitiol (HNK), a tropolone-related compound also known as β-thujaplicin, present in essential oils extracted from Japanese cypress [1], possesses various biological properties, including antimicrobial, antifungal, antitumor, antiinflammatory, and antioxidant activities [2-6]. Notably, HNK exhibits significant antibacterial effects against oral pathogens, such as *Streptococcus mutans* (the primary etiological agent of dental caries), *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*, which have been implicated in periodontal disease [7-9]. Due to these properties, HNK has been incorporated into oral care products, such as toothpaste and gels, aimed at preventing dental caries, periodontal disease, and halitosis [10,11].

Fusobacterium nucleatum is a gram-negative anaerobic rod-shaped bacterium commensal to the human oral cavity, which is associated with not only oral diseases, such as gingivitis and periodontitis, but also with digestive disorders, including colon cancer and inflammatory bowel disease [12-13]. The pathogenic role of *F. nucleatum* in periodontitis makes it an important target for antimicrobial therapies aimed at preventing and treating oral diseases [14-16].

* Corresponding author: Norimasa Tsuji

This study was performed to evaluate the antibacterial effects of HNK on *F. nucleatum* using a variety of methods, including time-kill assay, adenosine triphosphate (ATP) bioluminescence assay, MTT assay, and flow cytometry.

2. Material and methods

2.1. Bacterial strain and cultivation

F. nucleatum (ATCC 10953) was cultured in Gifu Anaerobic Medium (GAM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) under anaerobic conditions. Cultures were initiated during the logarithmic growth phase.

2.2. Hinokitiol

HNK was purchased from Tokyo Chemical Industries (Tokyo, Japan) and prepared as a stock solution at 100 mg/mL in dimethyl sulfoxide (DMSO). The final concentration of hinokitiol (HNK) in the culture medium, with a solvent concentration of 0.1%, was achieved by adding the stock solution to the working medium at a 1/1000 ratio.

2.3. Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) of HNK against *F. nucleatum* was determined following the Clinical and Laboratory Standards Institute method [17]. *F. nucleatum* was precultured in GAM, and the bacterial suspension was adjusted to an optical density at 600 nm (OD_{600}) of 0.1. The HNK-containing GAM liquid medium was serially diluted in 96-well plates, and 10 μL of the bacterial suspension was added. The MIC was determined after 24 h of anaerobic culture. The minimum bactericidal concentration (MBC) was determined by culturing in the medium that showed growth inhibition, and the lowest concentration without observable colony formation was considered the MBC.

2.4. Effects of hinokitiol on the *F. nucleatum* growth curve

The turbidity of cultures was determined by measuring the OD_{600} to confirm the inhibition of *F. nucleatum* growth. GAM liquid medium was prepared with or without 12.5 and 25 μg/mL HNK, and *F. nucleatum* preculture was added. The growth of *F. nucleatum* was monitored by measuring the OD_{600} at 6-h intervals over 24 h at 37°C under anaerobic conditions.

2.5. Membrane integrity assay

The effects of HNK on *F. nucleatum* cell membrane integrity were examined by propidium iodide (PI) staining as described previously [18]. Bacterial suspensions treated with HNK at different concentrations were incubated at 37°C for 24 h and then fixed with PI to a final concentration of 10 μg/mL for 20 min at 4°C in the dark. Subsequently, cells were collected by centrifugation at 6000 × *g* for 10 min, washed with 0.85% NaCl solution, and resuspended in the same solution to adjust their concentration to 10^6 CFU/mL before analysis. PI staining of the cells was performed using a FACSVerse (BD Bioscience, San Jose, CA, USA).

2.6. BacTiter-Glo microbial cell viability assay

To investigate the viability of *F. nucleatum* cells, ATP efflux was measured by BacTiter-Glo microbial cell viability assay (Promega, Madison, WI, USA) in accordance with the manufacturer's protocol. Briefly, approximately 100 μL of 10^6 CFU/mL *F. nucleatum* was cultured in white opaque 96-well plates, together with HNK at 0, 0.25, and 0.5 MIC. After 24 h, aliquots of 100 μL of the culture were taken from each well and mixed with 100 μL of the BacTiter-Glo reagent. Following a 5-min incubation period at room temperature, the bioluminescence response was determined in relative light units (RLUs) using a microplate reader. The results are presented as the means of three independent experiments.

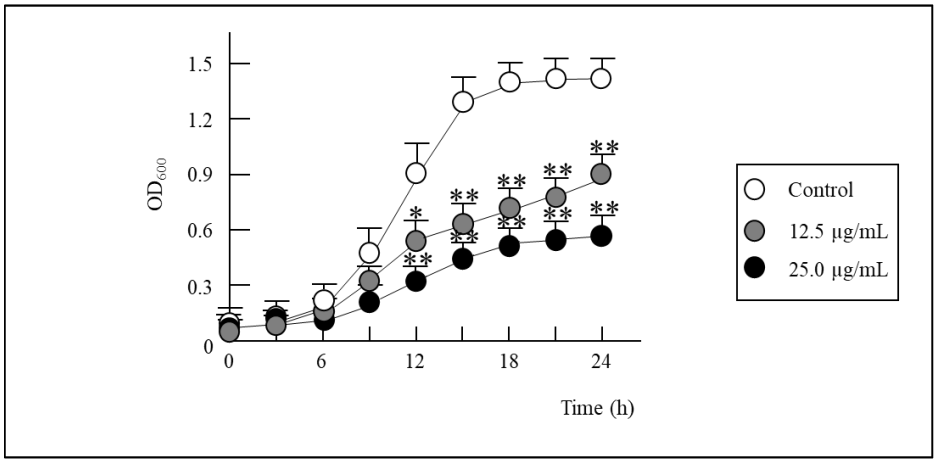
2.7. Statistical analysis

All experimental data are expressed as the mean and standard deviation of three separate observations. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test in SPSS 19.0 (IBM, Armonk, NY, USA). Differences between the control and experimental groups were considered statistically significant at $p < 0.01$ and $p < 0.05$.

3. Results

3.1. Antibacterial activity of hinokitiol against *F. nucleatum*

The MIC and MBC of HNK against *F. nucleatum* were established as 50 μg/mL and 100 μg/mL, respectively. *F. nucleatum* growth was inhibited for up to 24 h in culture in GAM supplemented with HNK at 12.5 μg/mL and 25 μg/mL (Fig. 1).

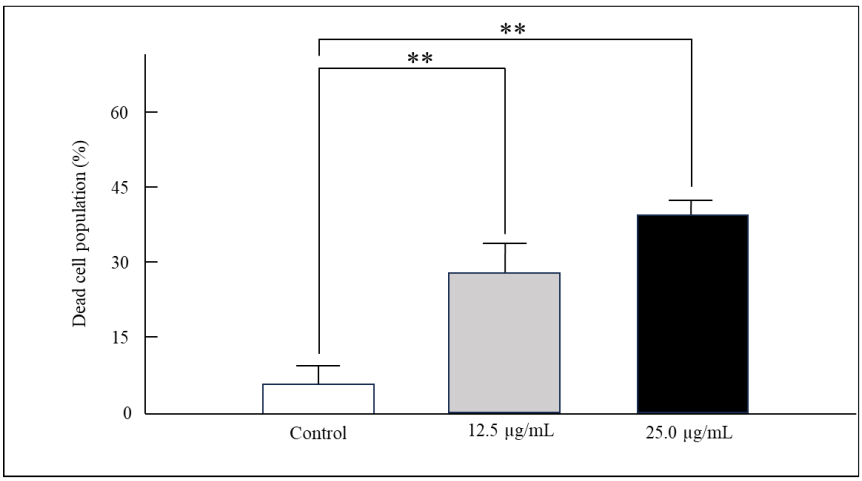


Bacterial cultures were grown in GAM liquid medium supplemented with various concentrations of HNK. Optical density at 600 nm (OD600) was measured using a spectrophotometer at 0, 3, 6, 9, 12, 15, 18, 21, and 24 hours. The graph represents the mean ± SD of three independent experiments. (*p<0.05, **p<0.01).

Figure 1 Effects of HNK on growth of *F. nucleatum*

3.2. Effects of hinokitiol on the bacterial membrane and intracellular disruptions

Representative results of flow cytometric analysis to determine the antifungal effects of HNK on *F. nucleatum* (Fig. 2). The responses of the bacterial membrane and the intracellular status of HNK are shown separately for *F. nucleatum* exposed to 0.25 and 0.5 MIC. The response patterns of the tested bacteria differed after 24 h of treatment. The dead cell populations of *F. nucleatum* cultures incubated for 24 h with 0, 0.25, and 0.5 MIC were 7.5% ± 2.6%, 25.2% ± 5.2%, 23.5% ± 3.2%, and 35.9% ± 5.5%, respectively. The dead cell counts increased continuously in a dose-dependent manner relative to the untreated control.

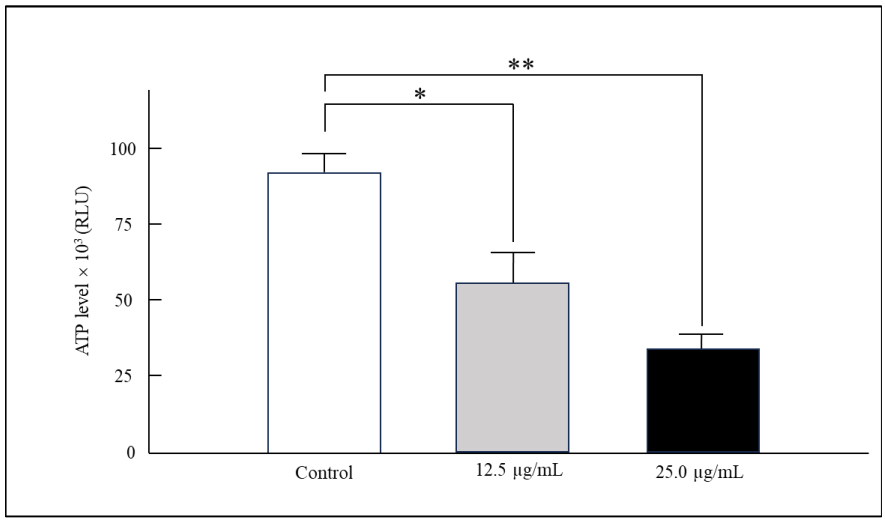


F. nucleatum were treated with 0.25 and 0.5 MIC or untreated control for 24h. After incubation, *F. nucleatum* were collected and were stained with propidium iodide (PI). The resulting cells were subjected to flow cytometry analysis by FACSVerse®. The values are presented as the means ± SD. (n = 3, *p < 0.05 compared with the control group by Tukey's test).

Figure 2 Dead cells in *F. nucleatum* treated with/without HNK, as assessed by flow cytometry assay

3.3. ATP leakage assay

To verify the effects of HNK on energy metabolism in *F. nucleatum*, we measured the intracellular ATP levels expressed in RLUs. As shown in Fig. 3, after treatment with 0.25 and 0.5 MIC of HNK, the intracellular ATP RLUs in *F. nucleatum* decreased significantly in the experimental group compared with the untreated control group.



ATP efflux due to the addition of HNK at various concentrations was measured using BacTiter-Glo microbial cell viability assay. Three independent experiments were performed, and the mean and standard deviation were calculated (n = 3, *p < 0.05, **p < 0.01 compared with the control group by Tukey's test).

Figure 3 Effects of HNK on the viability of *F. nucleatum* cells

4. Discussion

The MIC and MBC of HNK against *F. nucleatum* were 50.0 μg/mL and 100 μg/mL, respectively. The growth of *F. nucleatum* cultured in GAM liquid culture medium supplemented with 12.5 μg/mL and 25.0 μg/mL HNK was suppressed up to 24 h after the start of culture (Fig. 1).

As the MIC and MBC of HNK against *F. nucleatum* were similar, the antibacterial action of HNK against *F. nucleatum* appeared to be bactericidal in nature. The influx of PI indicated that HNK damaged the membrane, decreased its integrity, and increased its permeability (Fig. 2). Cell membranes have been studied extensively as potential targets of antibacterial agents. When the cell membrane structure is damaged, nucleic acids and proteins leak from the cell, causing irreversible damage and cell death. This study demonstrated that HNK damages the integrity of the cell wall of *F. nucleatum* and increases cell membrane permeability.

Our results demonstrated that the level of ATP in the bacteria, which plays a key role in cellular energy metabolism, decreased significantly after treatment with HNK (Fig. 3). Therefore, HNK may interfere with energy metabolism by affecting carbohydrate metabolism in *F. nucleatum*, further affecting the use of its catabolic products for cellular repair and thereby inhibiting bacterial growth. The precise mechanism underlying the antibacterial effect of HNK is not fully understood. However, previous reports suggested that HNK may act on bacterial cell membranes, causing metabolic disturbances [19]. HNK affected the cell membrane and disrupted membrane permeability, leading to leakage of intracellular components in the *F. nucleatum* strain used in this study. However, a detailed analysis of the antimicrobial mechanism of HNK was not conducted in this study, and further investigations are required.

5. Conclusion

The findings of the present study revealed the antibacterial efficacy of HNK against *F. nucleatum*. Developing a safe HNK-based method for the prevention of periodontitis is important because the prevalence of this condition is anticipated to increase in the future.

Compliance with ethical standards

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (C) (Grant No. JP23K09209) from the Japan Society for the Promotion of Science (JSPS), funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Disclosure of conflict of interest

The authors have no conflicts of interest to declare.

References

[1] Erdtman H, Gripenberg J. Antibiotic substances from the heart wood of Thuja plicata Don. Nature 1948; 13:719-19.

[2] Yamato M, Hashigaki K, Yasumoto Y, Sakai J, Luduena RF, Banerjee A, Tsukagoshi S, Tashiro T, Tsuruo T. Synthesis and antitumor activity of tropolone derivatives. J Med Chem. 1987; 30:1245-48.

[3] Lee JH, Moon JH, Lee YJ, Park SY. SIRT1, a class III histone deacetylase, regulates LPS-induced inflammation in human keratinocytes and medicated the anti-inflammatory effects of hinokitiol. J Invest Dermatol. 2017; 137:1257-66.

[4] Xu Y, Wang S, Miao Q, Jin K, Lou L, Ye X, Xi Y, Ye J. Protective role of hinokitiol against H2O2-induced injury in human corneal epithelium. Curr Eye Res. 2017; 42:47-53.

[5] Trust TJ, Coombs RW. Antibacterial activity of β-thujaplicin. Can J Microbiol. 1973; 19:1341-46.

[6] van der Kamp BJ. Effects of heartwood inhabiting fungi on thujaplicin content and decay resistance of Western redcedar (Thuja plicata Donn.). Wood Fiber Sci. 1986; 18:421-27.

[7] Wang TH, Hsia SM, Wu CH, Ko SY, Chen MY, Shih YH, Shieh TM, Chuang LC, Wu CY. Evaluation of the antibacterial potential of liquid and vapor phase phenolic essential oil compounds against oral microorganisms. PLoS One. 2016; 11:e0163147.

[8] Shih YH, Chang KW, Hsia SM, Yu CC, Fuh LJ, Chi TY, Shieh TM. In vitro antimicrobial and anticancer potential of hinokitiol against oral pathogens and oral cancer cell lines. Microbial Res. 2013; 168:254-62.

[9] Osawa K, Matsumoto T, Maruyama T, Takiguchi T, Okuda K, Takazoe I. Studies of the antibacterial activity of plant extracts and their constituents against periodontopathic bacteria. Bull Tokyo Dent Coll. 1990; 31:17-21.

[10] Nagao Y, Sata M. Effect of oral care gel on the quality of life for oral lichen planus in patients with chronic HCV infection. Virol J. 2011; 8:348.

[11] Iha K, Suzuki N, Yoneda M, Takeshita T, Hirofuji T. Effect of mouth cleaning with hinokitiol-containing gel on oral malodor: a randomized, open-label pilot study. Oral Surg Oral Med Oral Pathol Oral Radiol. 2003; 149:433-39.

[12] Strauss J, Kaplan GG, Beck PL, Rioux K, Panaccione R, Devinney R, Lynch T, Allen-Vercoe E. Invasive potential of gut mucosa-derived fusobacterium nucleatum positively correlates with IBD status of the host. Inflamm Bowel Dis. 2011; 17:1971-1978.

[13] Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa associated microbiota in patients with colorectal cancer. PLoS One. 2012; 7:e39743.

[14] Loozen G, Ozcelik O, Boon N, Mol DA, Schoen C, Quirynen M, Teughels W. Inter-bacterial correlations in subgingival biofilms: a large-scale survey. J Clin Periodontol. 2014; 41:1-10.

[15] Feng X, Zhang L, Xu L, Meng H, Lu R, Chen Z, Shi D, Wang X. Detection of eight periodontal microorganisms and distribution of Porphyromonas gingivalis fimA genotypes in Chinese patients with aggressive periodontitis. J Periodontol. 2014; 85:150-59.

[16] Ogawa Y, Kobayashi R, Kono T, Toda M, Okada H, Kurita-Ochiai T, Komiya M. Involvement of Fusobacterium nucleatum in bone resorption and periodontal tissue inflammation. Int J Oral-Med Sci. 2020; 18:296-302.

[17] CLSI (2007) Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Information Supplement. CLSI Document M100-S17 (M2-A7 and M7-A7) 27(1) Clinical and Laboratory Standards Institute, Wayne, PA.

[18] Musimum C, Chuysongmuang M, Permpoonpattana P, Chumkaew P, Sontikul Y, Ummarat N, Srisawat T. FACS analysis of bacterial responses to extracts of Vatica diospyroides fruit show dose and time dependent induction patterns. Walailak J Sci Technol. 2017; 14:883-91.

[19] Yee R, Holmgren C, Mulder J, Lama D, Walker D, van Palenstein Helderman W. Efficacy of silver diamine fluoride for arresting caries treatment. J Dent Res. 2009; 88:644-647.