Effectiveness of Pineapple (Ananas Comosus) Hump Extract in Inhibiting Periodontal Pathogens Biofilm Growth and Adhesion

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Abstract

Pineapple (Ananas comosus) hump extract is a waste product of the pineapple plant. The extract contains alkaloids, flavonoids, saponins, and bromelain enzymes, all of which can inhibit the growth of bacteria that cause periodontal disease.

Aim: To determine the effectiveness of pineapple hump extract against Treponema denticola, Aggregatibacter actinomycetemcomitans, and Fusobacterium nucleatum biofilm growth and adhesion. The bacteria were inoculated into nutrient broth medium and then incubated for 24 hours at 37°C. The antibacterial and antibiofilm effectiveness of a pineapple hump extract were evaluated using diffusion inhibition test and crystal violet biofilm assay. Chlorhexidine (0.2%) served as a positive control, and distilled water served as a negative control. Each bacterium (200 µL) was plated in 96 well-plates, followed by incubation for 24 hours at 37°C to form biofilm. Subsequently, pineapple hump extract was added into biofilm well and incubated for 1,6 and 24h. After staining with crystal violet, the biofilm density was calculated using a microplate reader.

The Shapiro–Wilk test was used to determine whether the data had a normal distribution. For data with a normal distribution, a one-way analysis of variance (ANOVA) test was conducted. In all tests, a value of $p<0.05$ was considered statistically significant. Showed that pineapple hump extract were effective in inhibit the bacterial growth. A one-way analysis of variance (ANOVA) test and post hoc test revealed a significant difference ($p < 0.05$) between the ability of the different concentrations of pineapple hump extract to inhibit A. actinomycetemcomitans, T. denticola, and F. nucleatum biofilm growth. The pineapple hump extract has been proven to inhibit bacterial growth and eradicate biofilms attachment of periodontal pathogens.

The pineapple hump extract has potential as antibacterial and antibiofilm agent to prevent periodontal diseases. However, further studies are still needed to explore this result.

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Introduction

Periodontal disease begins with inflammation of the gingiva and progresses to a breakdown of hard and soft tissues and leads to tooth loss.[1] The prevalence of periodontitis in Indonesia is relatively high. According to Basic Health Research (RIKESDAS) in 2018, the prevalence of periodontitis in the Indonesian population was 74.1%.[2] Periodontal disease occurs due to an accumulation of Gram-negative bacteria, such as *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*, normally found in the human oral cavity, resulting in the formation of biofilms on the surfaces of teeth.[3–5] Biofilms on the tooth surface can cause dental caries, whereas biofilms on the gingival sulcus can cause periodontal disease.[6] Chemicals, such as chlorhexidine, in antibacterial mouthwashes, can inhibit biofilm formation on tooth surfaces. However, long-term use of mouthwashes that contain alcohol can have detrimental effects, such as a burning sensation in the oral cavity and discoloration of teeth, and are also linked with a risk of oral cancer.[7,8]

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A. actinomycetemcomitans and *T. denticola* are found in plaque which causes gingivitis and periodontitis. These bacteria can cause localized aggressive periodontitis, which is common in adolescents.[3] Aggressive periodontitis is associated with attachment loss and gingival recession. It occurs four times faster than chronic periodontitis.[9] *F. nucleatum*, an obligate anaerobic bacterium, is one of the most common species found in the oral cavity. Increased numbers of these bacteria are found in the saliva of patients with gingivitis and periodontitis.[10]

Antimicrobial targeted therapy can reduce periodontitis by inhibiting the causative pathogens of periodontitis. Antibiotics such as tetracyclines and doxycycline could inhibit *A. actinomycetemcomitans*.[11] However, antimicrobial therapy has disadvantages, including the possible development of antimicrobial resistance[12] and an imbalance in the antibiotic concentration at the targeted site, which can cause adverse reactions.[11] As a result of the aforementioned problems with chemical-based mouthwashes and antimicrobial therapy, researchers are focusing on alternative herbal medications, with possibly fewer adverse effects. Several studies have documented the possibility of using herbal medications to inhibit biofilm growth.[13–19] According to one study, pineapple fruit extract has antibacterial effects against oral pathogens.[20]

Pineapple (*Ananas comosus*) is a tropical plant that has long been used as a traditional medicine.[21] Due to Indonesia's tropical climate, pineapples are grown in almost all regions in Indonesia. Indonesia can produce enough pineapples for the Southeast Asian region, and it is the third largest pineapple producer with pineapple production accounting for 23% of GDP.[22] The pineapple plant contains bromelain, which belongs to a group of protein-digesting enzymes, alongside papain from papaya.[23] The concentration of bromelain in pineapple hump (pineapple plant waste) is higher than in pineapple flesh. Previous studies reported that bromelain exerted antibiotic activity against causative pathogenic agents of periodontal disease.[24,25]

Previous research reported that pineapple hump extract contains alkaloids, flavonoids, and saponins, which have antibacterial properties that could inhibit the growth of *Streptococcus mutans, Enterococcus faecalis* and *Porphyromonas gingivalis* bacteria.[26] However, the antimicrobial and antibacterial potential of pineapple hump extract in terms of combatting biofilm growth and adhesion toward *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* as periodontal pathogens has not been studied. Therefore, the aim of this research is to determine the effectiveness of pineapple hump extract against *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* biofilm growth and adhesion.

Materials and methods

Pineapple hump extraction

The pineapple hump extract process was performed at the Indonesian Herbal and Medicinal Plants Research Institute. The pineapple humps were first separated from the pulp and then dried and placed in a grinder to obtain a fine powder of pineapple humps. The pineapple hump powder was extracted by the maceration method using 96% ethanol. In total, 500 g of pineapple hump were extracted. Subsequently, the filtered solution was placed in a rotary evaporator to evaporate the remaining ethanol solution and produce a viscous extract free of solvents. The extract was then diluted with distilled water to obtain concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.12%.

Phytochemical analysis of pineapple hump extract

Several phytochemical tests were conducted to qualitatively identify the content of the pineapple hump extract. These tests were as follows: 1) Flavonoid test: The pineapple hump extract (2 ml) was added into a test tube, along with magnesium powder and concentrated hydrochloric acid (HCl). The formation of a pink to the red color indicated the presence of flavonoid compounds.; 2) Phenol test: The sample (2 ml) was added, together with a few drops of hot water and one or two drops of 1% ferric chloride ($FeCl_3$). A phenolic compound was confirmed by the formation of a blue/purple color; 3) Saponin test: The sample (2 ml) was added, along with 10 ml of distilled water and shaken vigorously. If foam formed and was maintained for at least 5 minutes, a saponin compound was confirmed; 4) Terpenoid test: Chloroform (2 ml)

was added to a test tube, along with 0.5 ml of the extract. This was followed by the addition of 3 ml of concentrated sulfuric acid. A reddish-brown color confirmed the presence of terpenoids; 5) Alkaloid test: The extract (2 ml) was added, along with 0.2 ml of diluted HCl, followed by the addition of 1 ml of Mayer's reagent. The formation of a yellowish color indicated the presence of alkaloids; 6) Tannin test: Pineapple hump extract (5 ml) was added, together with 2 ml of 5% $FeCl_3$ solution. The formation of a greenish-black precipitate indicated the presence of tannin in the extract.

Bacterial cultures

Treponema denticola ATCC 35405, *Aggregatibacter actinomycetemcomitans* ATCC 29522, and *Fusobacterium nucleatum* ATCC 25586 bacterial suspension were inoculated into nutrient (Oxoid, Hampshire) broth and then incubated for 24 hours at 37°C in anaerobic jars (Oxoid, Hampshire) to maintain anaerobic conditions. After incubation, each bacterium was standardized to the equivalent of 0.5 McFarland standard (1.5 x 10^8 CFU/ml) prior to the upcoming tests below.

Diffusion inhibition test

A bacterial inhibition test was performed using the agar well diffusion method with Petri-dishes containing Brain Heart Infusion Agar (Oxoid, Hampshire) (BHI-A). In total, 20 µl of *T. denticola*, *A. actinomycetemcomitans*, and *F. nucleatum* suspensions were inoculated unto BHI-A medium and were spread on the surface of the agar using a spreader. Five wells were made in each petri dish, and 50 µl of the different concentrations of pineapple hump extract (100%, 50%, 25%, 12.5%, 6.25%, and 3.12%) were added. Each concentration of the pineapple hump extract was tested in triplicate. Chlorhexidine served as a positive control, and distilled water served as a negative control. Subsequently, the inoculated plates were incubated at 37°C. After 24 hours, the zone of inhibition that formed around each well was measured.

Biofilm assay

The bacterial cultures in nutrient broth medium were dispensed into a 96-well plate and then incubated for 48 hours at 37°C under anaerobic conditions. The leftover medium and the unattached cells were then removed from each well. The attached biofilm cells were rinsed with sterile phosphate-buffered saline. Subsequently, different concentrations of pineapple hump extract (100%, 50%, 25%, 12.5%, 6.25%, and 3.12%) were dispensed into the well plate and incubated for 1 hour, 6 hours, and 24 hours at 37°C. After incubation, each well plate extract was removed and was then rinsed using phosphate-buffered saline to clean leftover extract. The already treated biofilm was fixed by passing the wells quickly over an open fire. Furthermore, crystal violet (0.5% w/v) was added to each well for 15 minutes to stain the treated-biofilm, followed by rinsing with phosphate-buffered saline to remove the leftover stain.

Finally, the treated-biofilm was measured from the remaining purple crystal extraction on the well plate after the addition of 200 µl of 96% ethanol. The biofilm density was calculated using a microplate reader (SAFAS MP96, SAFAS, Monaco) with an optical density of 490 nm. The positive control used was 0.2% chlorhexidine, and the negative control used was sterile distilled water.

Statistical analysis

The Shapiro–Wilk test was used for normally distributed data analysis. Furthermore, a one-way analysis of variance (ANOVA) test was conducted to analyze the significant difference on each group in this study. In all the tests, a value of $p < 0.05$ was considered statistically significant. Statistical significance was confirmed by a post hoc least significant difference (LSD) test. Statistical analysis was performed using SPSS Statistics for Windows software v. 26 (IBM, Armonk, NY).

Results

Phytochemical and Diffusion inhibition test result

As shown by the results of the phytochemical tests, active substances (alkaloids, flavonoids, and saponins) were detected in the pineapple hump extract (Table 1). Based on the results of the bacterial zone of inhibition test, pineapple hump extract with a concentration of 100% had similar efficacy as 0.2% chlorhexidine, which both inhibited *A. actinomycetemcomitans*, *T. denticola,* and *F. nucleatum* (Figs. 1 – 2). The pineapple hump

extract also inhibited F. *nucleatum* at the concentration of 50% (Figure 1).

Phytochemical tests	Qualitative result
- Alkaloid	+
- Flavonoid	+
- Tannin	-
- Saponin	+
- Steroid	-
- Triterpenoid	-

Table 1. Phytochemical analysis result.

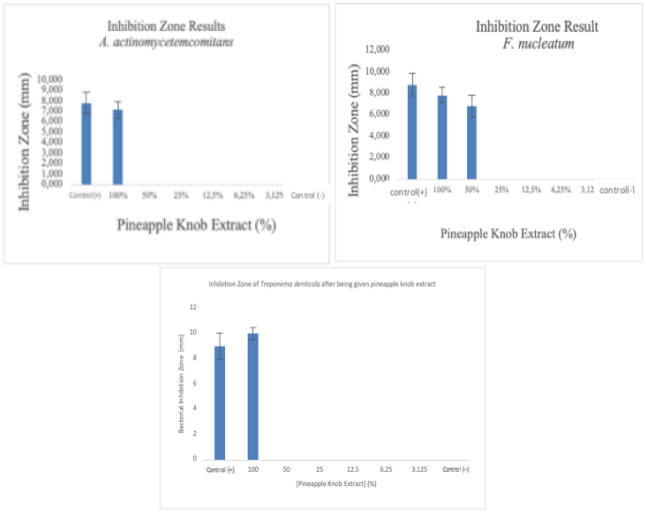

Figure 1. Graph (A) showing the average diameter of the zone of inhibition for pineapple hump extract in different concentration against *A. actinomycetemcomitans* and picture (B) showing the average diameter of the zone of inhibition for pineapple hump extract in different concentration against *F. nucleatum* and picture (C) showing the average diameter of the zone of inhibition for pineapple hump extract in different concentration against *T. denticola*.

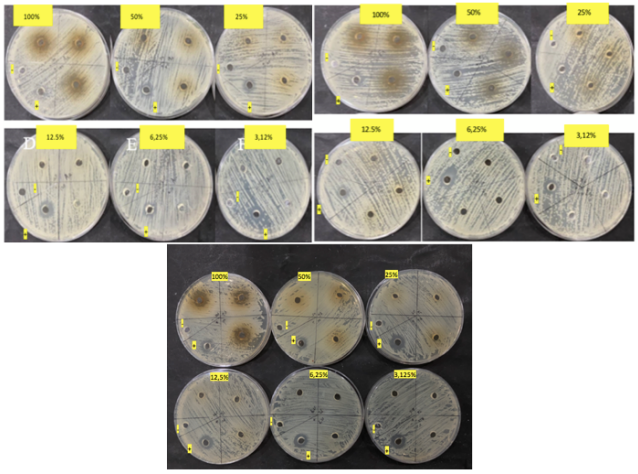

Figure 2. Graph (A) showing the diameter of the zone of inhibition for pineapple hump extract different concentration against *A. actinomycetemcomitans* in brain heart infusion agar plate. Chlorhexidine (0.2%) served as a positive control, and distilled water served as a negative control. Graph (B) showing the diameter of the zone of inhibition for pineapple hump extract different concentration against *F. nucleatum* in brain heart infusion agar plate. Chlorhexidine (0.2%) served as a positive control, and distilled water served as a negative control. Graph (C) showing the diameter of the zone of inhibition for pineapple hump extract different concentration against *T. denticola* in brain heart infusion agar plate. Chlorhexidine (0.2%) served as a positive control, and distilled water served as a negative control.

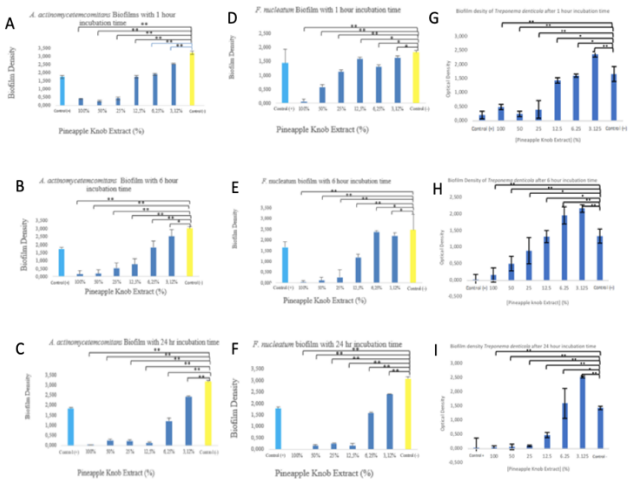

Figure 3. Average biofilm optical density of the *A. actinomycetemcomitans* after the pineapple

hump extract treatment in different concentration (100%, 50%, 25%, 12.5%, 6.25%, and 3.12%.) with incubation for 1 hour (A), 6-hour (B), and 24-hour (C). Average biofilm optical density of the *F. nucleatum* after the pineapple hump extract treatment in different concentration (100%, 50%, 25%, 12.5%, 6.25%, and 3.12%.) with incubation for 1 hour (D), 6-hour (E), and 24-hour (F). Average biofilm optical density of the *T. denticola* after the pineapple hump extract treatment in different concentration (100%, 50%, 25%, 12.5%, 6.25%, and 3.12%.) with incubation for 1 hour (G), 6-hour (H), and 24-hour (I). Chlorhexidine (0.2%) served as a positive control, and distilled water served as a negative control. *p<0.05, **p<0.01.

Biofilm assay result

According to the results of the biofilm assay, treatment with the pineapple hump extract generally reduced *A. actinomycetemcomitans, F. nucleatum,* and *T. denticola*, biofilm mass. Using 100% down to 25% of pineapple hump extract had a significant reduction to *A. actinomycetemcomitans* biofilm mass after 1-hour and 6-hour exposure time (Figure 3A, 3B), whilst after 24-hour exposure time, the 12.5% pineapple hump extract also provided similar results to 100%, 50%, and 25% (Figure 3C). On the other hand, only 100% pineapple hump extract had a significant reduction of *F. nucleatum* biofilm after 1-hour exposure (Figure 3D), but as the exposure time increases, *F. nucleatum* biofilm mass was also reduced with even lower concentrations. At 6-hour exposure time, 100% down to 25% concentrations had similar effects (Figure 3E), while at 24-hour exposure time, 100% down to 12.5% concentrations also gave similar effects (Figure 3F). In *T. denticola* biofilm assay, the 100% down to 25% concentration of pineapple hump extract was capable to reduce *T. denticola* biofilm mass in all 1-hour (Figure 3G), 6-hour (Figure 3H), and 24-hour (Figure 3I) exposure times, respectively.

Discussion

As shown by the result of the zone inhibition assay, pineapple hump extract with a concentration of 100% impeded the growth of *A. actinomycetemcomitans* and *T. denticola*. In terms of *F. nucleatum*, an inhibition zone began to form at a concentration of 50% and increased at a concentration of 100%. Based on the results of the present study, pineapple hump extract has the potential to inhibit the growth of bacteria, such as *A. actinomycetemcomitans, F. nucleatum*, and *T. denticola* that cause periodontal disease. Among mouthwashes, chlorhexidine is considered the gold standard for periodontal treatment due to its strong antibacterial properties.[27] The present study, as indicated by the difference in the inhibition zone that formed in the pineapple hump extract treatments and chlorhexidine (positive control) treatments, showed that the pineapple hump extract has an antibacterial effect on all bacteria, even though chlorhexidine as gold standard has a bigger inhibition zone. Pineapple hump extract is a natural ingredient, thus unlike chlorhexidine which is a chemical substance. As a chemical, chlorhexidine causes discoloration of teeth, facilitating the formation of supragingival calculus, and changes in taste sensation.[7]

This study showed that pineapple hump extract has an anti-bacterial effect. The results of this study were supported by Praveen *et al*. study[1] who tested the inhibitory activity of pineapple hump extract against *S. mutans* using the agar well technique diffusion method. Praveen *et al*. study showed that pineapple hump extract with a concentration of 2mg/ml has an inhibitory effect against *S. mutans*. The difference in the bacterial inhibition effect of the pineapple hump extract in this study as compared to the study by Praveen *et al*. may be due to structural differences in bacterial cell walls. Gram-positive bacteria possess a thick (20–80 nm) cell wall as outer shell of the cell. In contrast Gram-negative bacteria have a relatively thin (<10 nm) layer of cell wall, but harbor an additional outer membrane with several pores and appendices. These differences in the cell envelope confer different properties to the cell, in particular responses to external stresses, including heat, UV radiation and antibiotics.[1] Praveen *et al.* demonstrated antibacterial effects of pineapple hump extract against *Porphyromonas gingivalis*, with bacterial

inhibition zones beginning to form in their study at a concentration of 4.15 mg/ml.[1] Umarudin *et al*. demonstrated an antibacterial effect of pineapple hump extract against *Staphylococcus aureus* growth.[28] In their study, based on an LSD test, the optimum concentration of pineapple hump extract with antibacterial activity against *S. aureus* was 70%.

The mechanism underlying the antibacterial activity of alkaloids involves interfering with peptidoglycan formation and therefore cell wall formation in bacterial cells.[29] Flavonoids act as an antibacterial by inhibiting cell membrane function, nucleic acid synthesis, and energy metabolism.[30] The antibacterial activity of saponins is attributed to their ability to reduce the surface tension of bacterial cell walls and the stability of the bacterial cell membrane, which results in the release of intracellular compounds on which bacterial survival depends.[31]

Conclusions

The present study showed that pineapple hump extract has the ability to inhibit bacterial growth and eradicate *T. denticola*, *A. actinomycetemcomitans*, and *F. nucleatum* biofilms. Therefore, we can conclude that the pineapple hump extract has the potential as antibacterial and antibiofilm agent to prevent periodontal diseases. However, further studies such as toxicity and in vivo study are still needed to confirm this result.

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Declaration of Interest

The authors report no conflict of interest.

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