Effect of Propolis Mouthwash on Growth and Sensitivity of Dual-Species Porphyromonas Gingivalis and Staphylococcus Aureus Biofilms (In vitro)

Sri Angky Soekanto[1]*, Alfi Zhafira[2], Eben Kalemben[2], Citra Fragrantia Theodorea[1], Endang Winiati Bachtiar[1], Boy Muchlis Bachtiar[1]

1. Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jalan Salemba Raya No. 4, Central Jakarta 10430, Indonesia.
2. Undergraduate Program, Faculty of Dentistry, Universitas Indonesia, Jalan Salemba Raya No. 4, Jakarta 10430, Indonesia.

Abstract

Propolis inhibits the growth of gram-negative and gram-positive bacteria, such as Porphyromonas gingivalis and Staphylococcus aureus, which interact antagonistically. Here, we investigated the effect of 5% propolis mouthwash on the growth, bacterial interactions, and sensitivity of the two bacteria in dual-species P. gingivalis and S. aureus biofilms.

Biofilms were formed in 96-well plates by incubating the bacteria for 24 h and assessed using a crystal violet test. The biofilms in 5% propolis mouthwash- and distilled water-treated (control) groups were observed using an inverted microscope after 0, 3, and 24 h of incubation. The proportions of P. gingivalis and S. aureus in the dual-species biofilms were assessed using real-time polymerase chain reaction.

The biofilm mass formed by P. gingivalis was higher than that by S. aureus. Propolis mouthwash reduced the formation of dual-species biofilms compared to the control. The proportion of P. gingivalis was higher than that of S. aureus accounting for 9.71% and 1.24% of all bacteria, respectively.

The findings reveal the effects of 5% propolis mouthwash on the growth of P. gingivalis and S. aureus and suggest the key roles of the active ingredients in 5% propolis mouthwash and differences in the cell structure of the two bacteria.

Experimental article (J Int Dent Med Res 2023; 16(3): 1010-1013)

Keywords: Propolis mouthwash, Biofilm dual-species, Porphyromonas gingivalis, Staphylococcus aureus.

Received date: 16 May 2023 **Accept date:** 25 July 2023

Introduction

Periodontitis is one of the most common periodontal tissue diseases caused by plaque accumulation in the oral cavity,[1] with a prevalence of 74.1% [Basic Health Research (RISKESDAS), 2018]—out of 10 Indonesian residents, 7 suffer from periodontitis.[2]Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola, collectively known as red complex bacteria, are the main etiological factors underlying the development of periodontal disease.[3] Among these, *P. gingivalis*, a gram-negative, obligate anaerobe, non-motile, and asaccharolytic,[4] is the primary triggering bacteria that causes an inflammatory response in periodontal disease. In the subgingival environment, P. gingivalis interacts antagonistically with Staphylococcus aureus, which secretes a bacteriocin-like substance that inhibits the growth of periodontopathogenic bacteria, including P. gingivalis.[5]

Natural products with antibacterial effects in inhibiting oral biofilm-related diseases, such as periodontitis, have gained increased research interest. Propolis, resinous natural product collected from various types of bees, including honey bees (Apis mellifera) and "stingless" bees (Trigona sp. and Tetragonula sp.),[6–8] comprises several active natural ingredients[7,9] and exhibits antimicrobial, antioxidant, anti-inflammatory, and antiproliferative activities.[10,11] Propolis eradicates periodontopathogenic bacteria and controls biofilm growth in the oral cavity by suppressing

Corresponding author:
Sri Angky Soekanto,
DDS., PhD., FISID., FIAOB., FICD., CMC.
Department of Oral Biology,
Faculty of Dentistry, Universitas Indonesia,
Jalan Salemba Raya No. 4, Central Jakarta 10430, Indonesia.
E-mail: sasoekanto@gmail.com

bacterial development via blocking cell mitosis and protein synthesis or by destroying the bacterial cytoplasm, cell membrane, and cell wall.[12]

However, no studies have analyzed the effect of 5% propolis mouthwash on dual-species P. gingivalis and S. aureus. Therefore, this study aimed to examine the effect of a 5% propolis mouthwash on the growth and sensitivity of dual-species P. gingivalis and S. aureus biofilms.

Materials and methods

Bacteria culture and biofilm formation

P. gingivalis (ATCC 33277) and S. aureus (ATCC 25923) were used. The bacteria were cultured in Brain Heart Infusion (BHI) medium and incubated at 37°C in a microaerophilic atmosphere for 24 h. The bacteria that grew on the agar were cultured in BHI broth. 5 g Propolis was used to prepare a mouthwash using powder extracts. The saliva coating procedure was performed on a 96-well plate to represent the oral cavity conditions. Approximately 80 µL 5% propolis mouthwash was added into the wells coated with saliva. Afterward, 10 µL S. aureus and P. gingivalis were added to each well, and the plates were incubated at 37°C in a microaerophilic atmosphere for 24 h. After incubation, observations were made using an inverted microscope (Zeiss Observer Z1, UK) to observe biofilm growth qualitatively.

Crystal violet test

Crystal violet dye (1%; 200 µL) was added to 96 wells for 2–3 min and washed with PBS for 1 min. Afterward, ethanol (96%) was added, and the biofilm mass was calculated using a Microplate Reader (Metertech, M965, Taipei, Taiwan ROC).

Real-Time Polymerase Chain Reaction (RT-PCR)

DNA concentration was measured using a Qubit® 1.0 Fluorometer (Invitrogen life technologies). All reactions were performed using a Step One Plus Real-Time PCR System (Applied Biosystems, USA) and a SensiFAST SYBR® Hi-ROX Kit (). The primers for P. gingivalis, S. aureus, and 16S rRNA are listed in Table 1. Real-time PCR was performed with a final volume of 10 µL, comprising 5 µL Sybr Green, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL nuclease-free water, and 3 µL DNA sample. The thermal profile comprised initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 ºC for 30 s. The cycle threshold (CT) values of each sample were obtained at the end of the RT-PCR and were analyzed using the relative quantification method.

Statistical analyses

All statistical analyzes were performed using SPSS ver. 25 (SPSS Inc., Chicago, Ill.) with a p value <0.05 which was considered significant. Absorbance values and $2^{-\Delta\Delta Ct}$ values for P. gingivalis and S. aureus were analyzed using the Mann Whitney test.

Bacteria	Primer	*Sequence 5'-3'*	Reference
Porphyromonas gingivalis	PG-F	TAC CCA TCG TCG CCT TGG T	17
	PG-R	CGG ACT AAA ACC GCA TAC ACT TG	
Staphylococcus aureus	Aureus_F	CAA GCA CAA GGC AGT GGT AT	18
	Aureus_R	GTG GCG TTG CAA TCT CCT TA	
Total bakteri (16S rRNA)	U16S 1020 - F	TTA AAC TCA AAG GAA TTG ACG G	19
	U16S 1190 - R	CTC ACG RCA CGA GCT GAC GAC	

Table 1. Primers used in Real-Time PCR.

Results

The average mass of the dual-species biofilms formed after 24 h of incubation is shown in Figure 1. The biofilm mass in samples treated with the propolis mouthwash was lower than that in the control. S. aureus showed the lowest biofilm mass after 24 h, whereas P. gingivalis showed the highest, indicating the highest inhibition of biofilm growth by S. aureus as shown in Figure 2. These results were directly proportional to the results of the RT-PCR. As shown in Figure 3, the proportion of P. gingivalis (9.71% of the total bacteria) was higher than that of S. aureus (1.24% of the total bacteria) (). The results showed P. gingivalis DNA was more detectable than S. aureus DNA in dual-species biofilms. Based on microscopic images, the density of biofilms in the control group was higher than that in the treatment group (Figure 4).

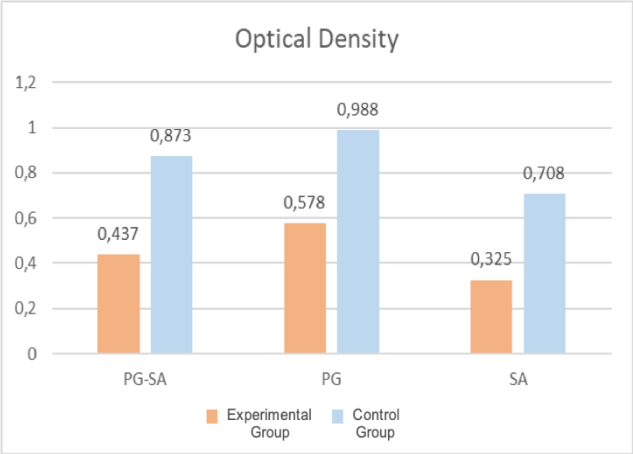

Figure 1. Dual-species and single-species biofilm mass test results for Porphyromonas gingivalis (PG) and Staphylococcus aureus (SA) using the crystal violet test observed at an optical density of 600 nm. PG-SA, dual culture with P. gingivalis and S. aureus.

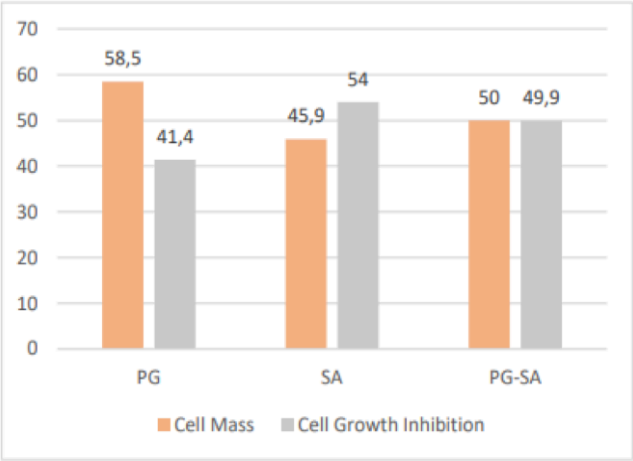

Figure 2. Percentage of biofilm mass and growth inhibition.

Discussion

The present study demonstrated the efficacy of 5% propolis mouthwash in inhibiting the growth of the biofilms formed by P. gingivalis and S. aureus. Between the mono-species and dual-species biofilms, the highest biofilm mass was observed in the mono-species P. gingivalis, followed by the dual-species, and the lowest in the mono-species S. aureus. Furthermore, P. gingivalis DNA in the dual-species biofilm was more detectable than S. aureus DNA. These results indicated the higher sensitivity of S. aureus to 5% propolis mouthwash than P. gingivalis.

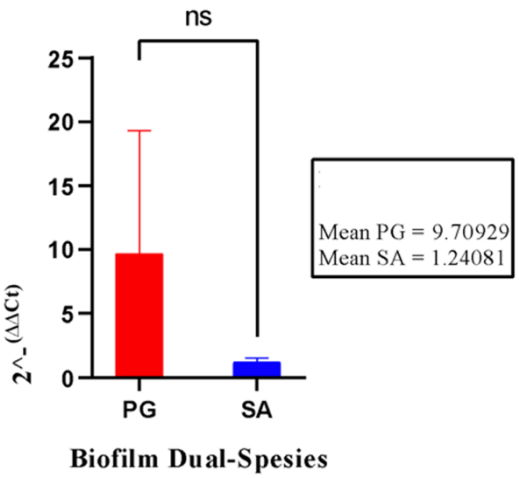

Figure 3. Differences in proportions of Porphyromonas gingivalis and Staphylococcus aureus.

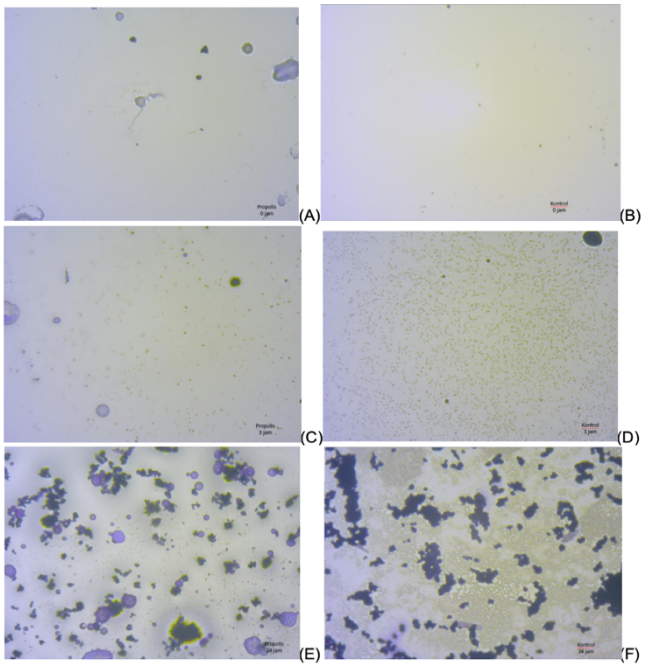

Figure 4. Microscopic observation of dual-species biofilm in propolis-treated and control groups using an inverted microscope. Biofilm formation (A, B) at 0 h of treatment with (A) 5% propolis mouthwash and (B) control; (C, D) at 3 h of treatment with (C) 5% propolis mouthwash and (D) control; (E, F) at 24 h of treatment with (E) 5% propolis mouthwash (F) control.

The differences in biofilm mass and proportion between P. gingivalis and S. aureus in this study could be due to differences in the cell wall structures of the two bacteria. S. aureus, a Gram-positive bacterium, has a thick peptidoglycan but no outer membrane. In contrast, P. gingivalis, a Gram-negative

bacterium, has a thin peptidoglycan and an outer membrane comprising lipopolysaccharide and proteins. The more complex cell wall structure in P. gingivalis could have inhibited the 5% propolis mouthwash from entering the cells.[13]

The findings revealed the antibacterial properties of propolis to inhibit bacterial growth in biofilms effectively. Previous studies have demonstrated that flavonoids inhibit biofilm growth by increasing the permeability of cell membranes, causing changes in the integrity of cell structures and cytoplasmic leakage, leading to cell death. In addition, flavonoids also inhibit energy metabolism in bacteria, inhibiting biofilm growth.[13,14] Based on these results, we speculate that the antibacterial properties of 5% propolis mouthwash could be attributed to flavonoids. Furthermore, the mass of dual-species biofilms treated with 5% propolis was lower than that of P. gingivalis mono-species biofilms. This result indicated an antagonistic interaction between P. gingivalis and S. aureus, which inhibited the growth of P. gingivalis in dual-species biofilms.

Qualitative observation using an inverted microscope showed increased biofilm density with increased incubation time. The higher biofilm density after 24 h of incubation than that after 3 h of incubation could be attributed to the formation of biofilms that had reached the maturation stage and an increasing number of Extracellular Polymeric Substances (EPS) matrices, thereby increasing the biofilm tolerance to external factors such as antimicrobial agents.[15] The matrix can biologically and actively retain water, nutrients, and enzymes within biofilms. The chemical mechanism of the matrix can also prevent the entry of other molecules, such as antimicrobial agents.[16]

Conclusions

The findings demonstrated that *S. aureus* was more sensitive to 5% propolis mouthwash than P. gingivalis. The 5% propolis mouthwash inhibited the growth of dual-species P. gingivalis and S. aureus biofilms and did not affect the antagonistic interactions between the two bacteria. The implication of this study was that the 5% propolis mouthwash eradicates periodontopathogenic bacteria and controls biofilm growth in the oral cavity.

Declaration of Interest

The authors report no conflict of interest.

Acknowledgement

This work is supported by Universitas Indonesia, PUTI Grant contract number BA1322/UN2.RST/PPM.00.03.01/2020

References

1. Lim G, Janu U, Chiou LL, Gandhi KK, Palomo L, John V. Periodontal Health and Systemic Conditions. Dent J (Basel). 2020;8(4):130.
2. Laporan Hasil Riset Kesehatan Dasar (Riskesdas) Available at: “https://www.litbang.kemkes.go.id/laporan-riset-kesehatan-dasar-riskesdas/” Accessed September 30, 2021.
3. Thurnheer T, Belibasakis GN, Bostanci N. Colonisation of gingival epithelia by subgingivabiofilms in vitro: role of "red complex" bacteria. Arch Oral Biol. 2014;59(9):977-986.
4. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence. 2011;2(5):435-444.
5. Grenier D. Antagonistic effect of oral bacteria towards Treponema denticola. J Clin Microbiol. 1996;34(5):1249-1252.
6. Saeed MA, Khabeer A, Faridi MA, Makhdoom G. Effectiveness of propolis in maintaining oral health: a scoping review. Can J Dent Hyg. 2021;55(3):167-176.
7. Pratami DK, Mun’im A, Yohda M, et al. Total phenolic content and antioxidant activity of spray-dried microcapsules propolis from Tetragonula species. AIP Conference Proceedings. 2019;2085(1):020040.
8. Parolia A, Thomas MS, Kundabala M, Mohan M. Propolis and its potential uses in oral health. International Journal of Medicine and Medical Sciences 2010;2(7): 210-215.
9. Amalina R, Soekanto SA, Gunawan HA, Sahlan M. Analysis of CPP-ACP Complex in Combination with Propolis to Remineralize Enamel. Journal of International Dental and Medical Research. 2017;10:814-819.
10. Soekanto SA, Marpaung LJ, Himmatushohwah, Djais A, Darwita RR. Efficacy of propolis fluoride and nano silver fluoride for inhibition of streptococcus mutans and enterococcus faecalis biofilm formation. International Journal of Applied Pharmaceutics. 2017;9(2):51-54.
11. Zulhendri F, Felitti R, Fearnley J, Ravalia M. The use of propolis in dentistry, oral health, and medicine: A review. J Oral Biosci. 2021;63(1):23–34.
12. Yuan J, Yuan W, Guo Y, Wu Q, Wang F, Xuan H. Anti-Biofilm Activities of Chinese Poplar Propolis Essential Oil against Streptococcus mutans. Nutrients. 2022;14(16):3290.
13. Przybyłek I, Karpiński TM. Antibacterial Properties of Propolis. Molecules. 2019;24(11):2047.
14. Valentina U, Oktiani BW, Panjaitan FUA. Inhibitory Test of Flavonid Propolis Kelulut Extracts (G. thorasica) on Porphyromonas gingivalis as an Etiologic Factor of Chronic Periodontitis. Dentino: Jurnal Kedokteran Gigi. 2019;4(2):140–144.
15. Singh S, Datta S, Narayanan KB, Rajnish KN. Bacterial exo-polysaccharides in biofilms: role in antimicrobial resistance and treatments. J Genet Eng Biotechnol. 2021;19(1):140.
16. Oral Microbiology - 6th Edition. Available at: “https://www.elsevier.com/books/oral-microbiology/marsh/978-0-7020-6106-6” Accessed May 12, 2023.
17. Dwiyanti S, Soeroso Y, Sunarto H, Radi B. Relationship between quantitative measurement of Porphyromonas gingivalis on dental plaque with periodontal status of patients with coronary heart disease. AIP Conference Proceedings. 2017;1817(1):030003.
18. Kim E, Yang SM, Won JE, Kim DY, Kim DS, Kim HY. Real-Time PCR Method for the Rapid Detection and Quantification of Pathogenic Staphylococcus Species Based on Novel Molecular Target Genes. Foods. 2021;10(11):2839.
19. Sedgley CM, Nagel AC, Shelburne CE, Clewell DB, Appelbe O, Molander A. Quantitative real-time PCR detection of oral Enterococcus faecalis in humans. Archives of Oral Biology. 2005;50(6):575–83.