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Effect of Brazilian propolis from the state of Bahia on oral bacteria

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Oral care from a young age is important for the prevention of dental caries and periodontal disease, as well as systemic diseases. Propolis, which has been used in traditional medicine for centuries, has value as a new oral care product because of its low toxicity, low allergenicity, and antimicrobial activity. However, the chemical composition of propolis extracts are extremely complex and influenced by region in the area where honey been live. In order to use propolis in oral care products, it is necessary to find out what ingredients it contains and how active they are. In this study, we evaluated the antimicrobial activity of a new Bahia propolis against oral pathogens. The results showed that the product has antimicrobial activity against the periodontopathogenic bacteria *Porphyromonas gingivalis* (*P. gingivalis*) and early colonizer *Actinomyces oris.* In particular, the effect on *P. gingivalis* was comparable to that of antimicrobials and disinfectants. Our results showed that the new Bahia propolis is a promising antimicrobial agent that may prevent oral infections. (J Osaka Dent Univ 2023; 57: 125-129)

Key words: Propolis; Bahia; Antibacterial activity; Oral bacteria; *Porphyromonas gingivalis*; *Actinomyces oris*

INTRODUCTION

With the rapidly growing aging population in Japan, urgent measures are needed to maintain a high quality of life for the elderly. One such measure is to maintain proper oral functions. Keeping good oral health is linked to prevention of frailty[1, 2] and aspiration pneumonia[3] in seniors, as well as prevention of systemic diseases[4] (such as endocarditis, atherosclerosis, cardiovascular disease, and gastric cancer) closely related to oral commensal bacteria[5] in middle-aged populations. For this reason, continuous oral care and control of oral conditions must be provided from a young age. In recent years, as home oral care has increased, many products, such as toothpastes and mouthwashes, have been developed that utilize naturally occurring substances (especially essential oils and propolis) that have antibacterial properties in biomedical applications.[6] The broad-spectrum antimicrobial activity, low toxicity, and limited allergic properties of propolis have long been utilized in traditional folk medicine throughout the world[7] as a substance that aids in treatment and prevention. Propolis has shown antibacterial, antifungal, antiviral, anticancer, anti-inflammatory and antioxidant activities.[8, 9]

Propolis is a resinous solid natural substance made from plant components, such as tree buds and sap, collected by honey bees from various plants, and mixed with bee saliva enzymes and beeswax components.[8, 10] Propolis originates from plants collected by honey bees, and its constituents are known to vary from region to region.[8] Brazilian propolis is categorized into 12 different types[11] according to regional and physiochemical properties; however the core bioactive substances common to various propolis are polyphenols, including flavonoids and phenolic carboxylic acids.[8] Due to its antimicrobial, antioxidant and anti-inflammatory activities, propolis may have new applications in dentistry. This study focuses on the antibacterial activity of a novel propolis extract against commensal oral bacteria for prevention of oral diseases.

MATERIALS AND METHODS

Preparation of ethanol-extracted propolis (EEP)

The Bahia propolis (Lot No.201223) used in this study was formulated by dissolving 50 mg of EEP powder obtained from Yamada Bee Company, Inc., Okayama, Japan, in 1 mL of 70% ethanol and stored the solution at −30°C until use.

Bacterial strains

The bacterial strains used in this study included *Escherichia coli* DH5*α* (*E. coli*), *Actinomyces oris* MG1 (*A. oris*), *Staphylococcus aureus* ATCC 12600 (*S. aureus*), *Pseudomonas aeruginosa* ATCC 10145 (*P. aeruginosa*), *Porphyromonas gingivalis* ATCC 33277 (*P. gingivalis*), and *Fusobacterium nucleatum* subsp. *polymorphum* JCM 12990 *(F. nucleatum).*

Growth conditions

E. coli, A. oris, S. aureus, and *P. aeruginosa* were cultured in heart infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) under aerobic conditions. *P. gingivalis* and *F. nucleatum* were cultured in modified Gifu anaerobic medium (Nissui, Tokyo, Japan) in an anaerobic chamber (Te-Her ANAEROBOX ANX-3; Hirasawa, Tokyo, Japan) at 37°C with 80% N_2, 10% H_2, and 10% CO_2.

Detection of bacterial growth via optical density

Overnight cultures of *E. coli, A. oris, S. aureus, P. aeruginosa, P. gingivalis,* and *F. nucleatum* were adjusted to $OD_{600}=0.1$ to be used as inoculum in the growth experiment. Five micro liters of bacterial suspensions were grown in a 10 mL tube in the presence of Bahia EEP (50 *μ*L) at a final concentration of 0, 50 or 100 *μ*g/mL. Growth was observed spectrophotometrically at 600 nm after 0, 3 and 24 h incubation at 37°C. Two bacterial species (*A. oris* and *P. gingivalis*) whose growth was affected by Bahia EEP were examined in detail. The bacteria were incubated at 37°C with Bahia EEP solution at a final concentration of 0, 50 and 100 *μ*g/mL. The turbidity was measured 0, 3, 6, 12 and 24 hrs later.

Antimicrobial activity

The minimum inhibitory concentration (MIC) of Bahia EEP against *A. oris* and *P. gingivalis* was determined using the microdilution method. Overnight cultures were adjusted to $OD_{600}=0.05$. Fifty mg/mL solution of Bahia EEP was subjected to a stepwise dilution (0～100 *μ*g/mL) using 70% ethanol. Then, 180 *μ*L of the adjusted bacterial suspension and 20 *μ*L of the Bahia EEP solution were added to a 96-well multi-microplate and incubated for 24 hrs. The turbidity was then measured at 600 nm using a M 50 S multi-microplate reader (Molecular Devices, Sunnyvale, CA, USA). The concentration at which no increase in turbidity was observed was defined as the MIC. Seven microliters of culture were collected from the wells where no increase in turbidity was observed, dropped onto a plane medium, and incubated for 2 days for *A. oris* and 5 days for *P. gingivalis* to examine growth. The concentration, at which no growth was observed, was defined as the minimum bacterial concentration (MBC). The MIC and MBC values of cetylpyridinium chloride (CPC) (Wako, Osaka, Japan), minocycline (MIN) (Wako), and erythromycin (EM) (Wako) were measured to estimate the potency of the EEP. The same experiment was performed three times independently.

RESULTS

The effect of Bahia EEP on the growth of *E. coli, A. oris, S. aureus, P. aeruginosa, P. gingivalis,* and *F. nucleatum* was analyzed using a visual spectrophotometer at 600 nm. The growth curves for *E. coli, S. aureus, P. aeruginosa* and *F. nucleatum* are shown (Figure 1). No significant effect of Bahia EEP was observed in *E. coli, S. aureus, P. aeruginosa* or *F. nucleatum.* Conversely, strong antibacterial activity was observed against *A. oris* and *P. gingivalis* (Figure 2). The MIC values for *A. oris* and *P. gingivalis* were then verified by using microdilution methos. The turbidity of *A. oris* was ob-

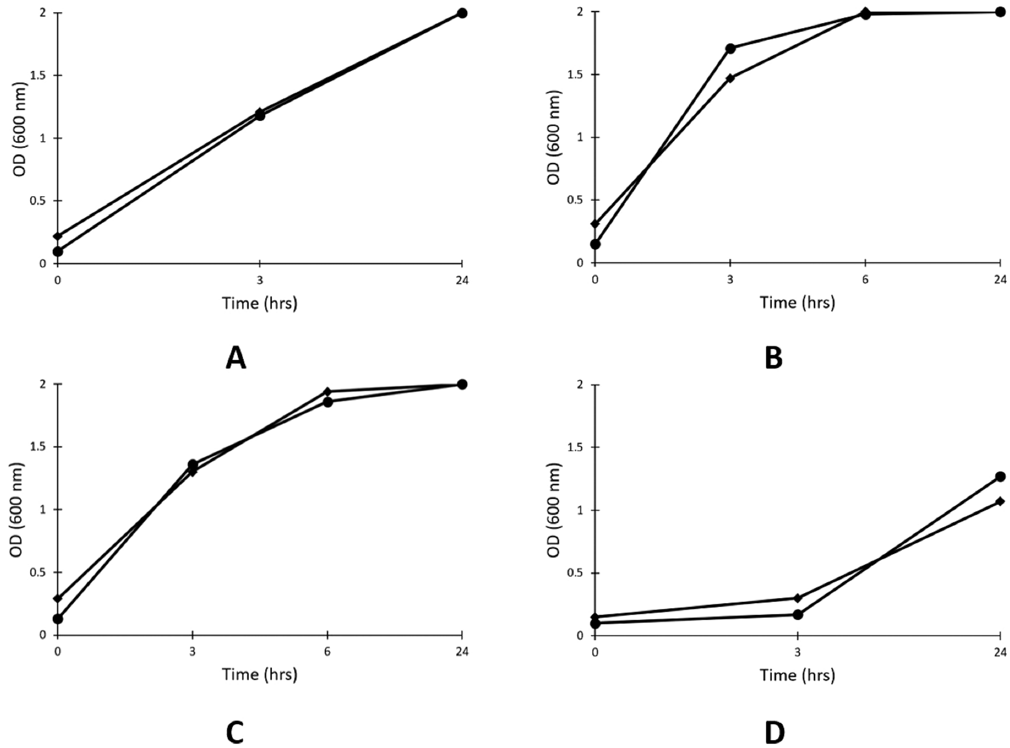

Fig. 1 Effect of EEP on the growth of (A) *E. coli,* (B) *S. aureus,* (C) *P. aeruginosa,* and (D) *F. nucleatum* (●0 μg/mL, ◆50 μg/mL).

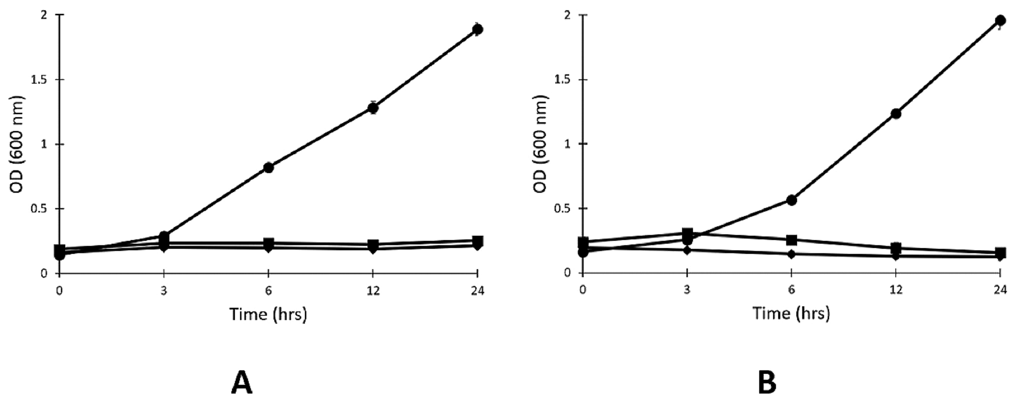

Fig. 2 Effect of EEP on the growth of (A) *A. oris* and (B) *P. gingivalis* (●0 μg/mL, ◆50 μg/mL, ■100 μg/mL).

Table 1 MIC and MBC of Bahia propolis against *A. oris* and *P. gingivalis*

Bacteria	Bahia EEP		CPC		MIN		EM	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
A. oris	16	＞64	0.5	0.5	2	4	0.015	0.0625
P. gingivalis	8	16	4	6.67	0.015	1	0.031	8

（μg/mL）

served at a concentration of 16 μg/mL, but not at 32 μg/mL. Similarly, *P. gingivalis* was found at 8 μg/mL, but not at 16 μg/mL. Seven microliters were removed from the wells where no turbidity was observed, and dropped onto new plane plates. No bactericidal effect was observed at 16, 32 or 64 μg/mL in *A. oris.* The bactericidal growth was precluded at 16 μg/mL in *P. gingivalis.* The MIC and MBC values of Bahia EEP were determined to be 16 and $>$64 μg/mL for *A. oris,* while it was 8 μg/mL and 16 μg/mL for *P. gingivalis.* The same experiment was performed three times independently (Table 1).

DISCUSSION

In this study, although the new Bahia EEP showed no effect on the growth of *E. coli, S. auresus, P. aeruginosa* or *F. nucleatum,* it had specific antibacterial activity against two species of oral bacteria (*P. gingivalis* and *A. oris*). The MIC and MBC values of Bahia EEP estimated for *P. gingivalis* were 8 and 16 mg/mL, respectively. The MIC and MBC values of CPC, a common oral care disinfectant, were 4 and 6.67 mg/mL, respectively, while the MIC and MBC values of EM and MIN, antimicrobial agents commonly used to treat periodontal disease, were 0.031 and 0.015 mg/mL and 0.015 and 1 mg/mL, respectively. The very low MIC value of Bahia EEP for *P. gingivalis* could be as effective as that of antimicrobials and disinfectants. The antimicrobial activities of various propolis EEPs, including Brazilian,[12, 13] Chinese,[14] and Hawaiian,[15] against *P. gingivalis* suggests that a substance common to all propolis extracts plays a central role in its antimicrobial activity. Yoshimasu *et al.* analyzed the constituents of Brazilian green propolis and reported that the main antimicrobial substances were artepillin C, baccharin, and ursolic acid.[16] Of these, they also mentioned that ursolic acid was the most effective antimicrobial agent against *P. gingivalis,* and that its mechanism of action involved cell membrane damage.

Actinomyces spp., along with *Streptococcus* spp., are known to act as the initial colonizing communities of the oral cavity.[17-19] *A. oris* has two types of fimbriae. Type 1 fimbriae mediate adhesion to pellicle, while the other is responsible for binding to oral *Streptococci* and host cells.[20] In this sense, *A. oris* is a key bacterium in dental plaque formation. In this study, the MIC and MBC values for *A. oris* were 16 and $>$64 mg/mL, respectively. The MIC and MBC values of CPC were 0.5 mg/mL, while the MIC and MBC values of EM were 2 and 4 mg/mL, and the MIC and MBC values of MIN were 0.015 and 0.0625 mg/mL, respectively. Few reports have revealed the antimicrobial activity of propolis against *A. oris,* only mentioning Hawaiian EEP which had MIC and MBC values of 10.6 and 21.3 mg/mL, respectively.[15] The MIC value of Brazilian green propolis was less than 100 mg/mL against *Actinomyces naeslundii,* the same genus as *A. oris.*[21] Although not as potent as the antimicrobials and disinfectants commonly used in oral care, Bahia EEP might be effective in inhibiting *A. oris* growth. This suggests that sustained EEP use may control plaque formation and maturation. Inhibition of the growth of early colonizers such as *A. oris* could control the maturation of subgingival and supragingival plaque, and might be an effective measure to prevent dental caries and periodontal diseaseies.

Although the Bahia EEP used in this study was an ethanol extract, we believe there is room to investigate antimicrobial components using other extraction methods. The Bahia EEP dissolved in ethanol is a complex collection of materials. Various solvents, such as water, ethanol, methanol, chloroform, dichloromethane, ether, and acetone, are used to extract propolis components. The eluted substances are different for each solvent. It is said that water or 70% ethanol extraction contains the most components with antimicrobial activities.[8] It is difficult to compare the numerous studies showing the antimicrobial activities of propolis from different sources and extraction methods. However, the very low MIC value of Bahia EEP against *P. gingivalis* is noteworthy. The Bahia EEP used in this study exhibited antimicrobial activities against oral pathogenic bacteria. Further component analysis is needed to identify the substance underlying the antimicrobial activities.

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REFERENCES

1. Ogawa T, Hirose Y, Honda-Ogawa M, Sugimoto M, Sasaki S, Kibi M, Kawabata S, Ikebe K, Maeda Y. Composition of salivary microbiota in elderly subjects. *Sci Rep* 2018: doi. 10.1038/s41598-017-18677-0 (9 pages)
2. Sawada N, Takeuchi N, Ekuni D, Morita M. Oral function, nutritional status and physical status in Japanese independent older adults. *Gerodontology* 2022; **39**: 359-365.
3. Ortega O, Parra C, Zarcero S, Nart J, Sakwinska O, Clavé P. Oral health in older patients with oropharyngeal dysphagia. *Age Ageing* 2014; **43**: 132-137.
4. Li X, Kolltveit KM, Tronstad L, Olsen I. Systemic Diseases Caused by Oral Infection. *Clin Microbiol Rev* 2000; **13**: 547-558.
5. Dörfer C, Benz C, Aida J, Campard G. The relationship of oral health with general health and NCDs: a brief review. *Int Dent J* 2017; **67**: 14-18.
6. Ghorbani A, Esmaeilizadeh M. Pharmacological properties of Salvia officinalis and its components. *J Tradit Complement Med* 2017; **7**: 433-440.
7. Toreti VC, Sato HH, Pastore GM, Park YK. Recent progress of propolis for its biological and chemical compositions and its botanical origin. *Evidence-based Complementary and Alternative Medicine* 2013: doi. 10.1155/2013/697390 (13 pages).
8. Machado CS, Mokochinski JB, de Lira TO, de Oliveira Fde C, Cardoso MV, Ferreira RG, Sawaya AC, Ferreira AG, Pessoa C, Cuesta-Rubio O, Monteiro MC, de Campos MS, Torres YR. Comparative Study of Chemical Composition and Biological Activity of Yellow, Green, Brown, and Red Brazilian Propolis. *Evidence-based Complementary and Alternative Medicine* 2016: doi. 10.1155/2016/6057650 (11 pages).
9. Bankova V, Christov R, Kujumgievb A, Marcuccic MC, Popov S. Chemical Composition and Antibacterial Activity of Brazilian Propolis. *Z Naturforsch CJ Biosci* 1995; **50**: 167-172.
10. Wagh VD. Propolis: A wonder bees product and its pharmacological potentials. *Adv Pharmacol Sci* 2013; **2013**: doi.10.1155/2013/308249 (11 pages).
11. Park YK, Alencar SM, Aguiar CL. Botanical origin and chemical composition of Brazilian propolis. *J Agric Food Chem* 2002; **50**: 2502-2506.
12. Namikawa D, Maruyama H, Masago A, Mashimo C, Nambu T, Okinaga T, Takahashi K. Species-specific growth inhibitory effect of propolis. *J Osaka Dent Univ* 2021; **55**: 83-89.
13. Nakao R, Senpuku H, Ohnishi M, Takai H, Ogata Y. Effect of topical administration of propolis in chronic periodontitis. *Odontology* 2020; **108**: 704-714.
14. Agarwal G, Vemanaradhya GG, Mehta DS. Evaluation of chemical composition and efficacy of Chinese propolis extract on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*: An in vitro study. *Contemp Clin Dent* 2012; **3**: 256-261.
15. Kang W, Maruyama H, Kawano A, Okinaga T. Antimicrobial property of Hawaiian propolis against oral pathogenic bacteria. *J Osaka Dent Univ* 2022; **56**: 161-165.
16. Yoshimasu Y, Ikeda T, Sakai N, Yagi A, Hirayama S, Morinaga Y, Nakao R. Rapid Bactericidal Action of Propolis against *Porphyromonas gingivalis. J Dent Res* 2018; **97**: 928-936.
17. Palmer RJ, Gordon SM, Cisar JO, Kolenbrander PE. Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. *J Bacteriol* 2003; **185**: 3400-3409.
18. Periasamy S, Kolenbrander PE. Central role of the early colonizer *Veillonella sp.* in establishing multispecies biofilm communities with initial, middle, and late colonizers of enamel. *J Bacteriol* 2010; **192**: 2965-2972.
19. Cavalcanti IMG, del Bel Cury AA, Jenkinson HF, Nobbs AH. Interactions between *Streptococcus oralis, Actinomyces oris,* and *Candida albicans* in the development of multispecies oral microbial biofilms on salivary pellicle. *Mol Oral Microbiol* 2017; **32**: 60-73.
20. Suzuki I, Shimizu T, Senpuku H. Short chain fatty acids induced the type 1 and type 2 fimbrillin-dependent and fimbrillin-independent initial attachment and colonization of *Actinomyces oris* monoculture but not coculture with streptococci. *BMC Microbiol* 2020: doi. 10.1186/s12866-020-01976-4 (14 pages)
21. Stähli A, Schröter H, Bullitta S, Serralutzu F, Dore A, Nietzsche S, Milia E, Sculean A, Eick S. *In vitro* activity of propolis on oral microorganisms and biofilms. *Antibiotics* 2021: doi. 10.3390/antibiotics10091045 (17 pages)