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Antimicrobial property of Hawaiian propolis against oral pathogenic bacteria

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Intestinal microflora plays a significant role in systemic health and immunity. However, oral microflora is still poorly understood and research is ongoing to identify substances that improve it. Propolis, a resinous substance found in beehives, is produced by honeybees by mixing plant shoots and resin with saliva and other enzymatic secretions. The natural antimicrobial properties of propolis from several regions such as Brazil, Mexico, Taiwan, and Okinawa have been previously reported. However, the anti-microbial property of Hawaiian propolis, which is from the same botanical source in subtropical regions such as Taiwan and Okinawa remains to be characterized. In this study, we investigated the antibacterial activities of Hawaiian propolis against *Actinomyces oris* (*A. oris*), early adherents of biofilm formation on the tooth surface, and against *Porphyromonas gingivalis* (*P. gingivalis*), a periodontopathic bacterium. We measured the inhibitory effect of ethanol-extracted propolis on the growth of *A. oris* and *P. gingivalis* and determined the minimum inhibitory concentration and minimum bactericidal concentration of ethanol-extracted propolis against these oral pathogen bacteria. Our results suggest that Hawaiian propolis is an effective antimicrobial against *A. oris* and *P. gingivalis* with implications for usage in aiding dental health. (J Osaka Dent Univ 2022; 56: 161-165)

Key words: Propolis; Antibacterial activity; Hawaiian; Growth-inhibition; *Actinomyces oris*; *Porphyromonas gingivalis*

INTRODUCTION

Propolis is a sticky, resinous substance produced by bees by mixing various plant products, such as bud exudates, flowers, and leaves with bee secretions and waxes.[1, 2] Owing to its antiseptic and antimicrobial properties, propolis is used by bees to build their hives and to protect colonies from diseases.[3] Analogously, propolis is known for its antibacterial,[4] antifungal,[5, 6] antiviral,[7] anti-inflammatory,[8] anti-tumor,[9] and immunomodulatory activities.

The composition of propolis varies based on several factors, including plant species growing around the hive, altitude, illumination, and seasonal variation.[10] However, the main components in propolis have been characterized to be flavonoids, phenolics, and mixtures of aromatic substances.[11-13] Propolis is found worldwide, with Brazil being a popular place of origin with reports of 13 types of propolis. They can be distinguished based on their color, texture, botanical origin, and chemical profile.[14-16] Among these, green and red propolis have been well-studied. The antimicrobial activity of their hydro-alcoholic extracts against multidrug-resistant bacteria has been reported.[14] Okinawa propolis and Taiwanese propolis are known varieties that originate from *Macaranga tanarius* (*M. tanarius*), also known as *Macaranga*-type propolis. Inui *et al.* reported that the botanical origin of Hawaiian propolis is also *M. tanarius.*[15] Although, Okinawa propolis has been reported to have a wide range of pharmacological benefits, including antioxidant, anti-inflammatory, antimicrobial, anticancer, antidiabetic, anti-Alzheimer's, anti-melanogenic, and longevity-extend-

ing effects,[16] these properties have not been characterized in Hawaiian propolis.

We investigated the antimicrobial properties of Hawaiian propolis against an *in vitro* culture of oral pathogenic bacteria using growth inhibition and susceptibility studies. We believe the implications of our study extend to improving dental healthcare using the naturally occurring Hawaiian propolis.

MATERIALS AND METHODS

Preparation of ethanol extracted propolis (EEP)

Hawaiian EEP was obtained from the Yamada Bee Company, Inc., Okayama, Japan. To prepare the solution, we dissolved 50 mg of EEP powder in 1 mL of 70% ethanol.

Strain and growth conditions

Actinomyces oris (*A. oris,* strain MG1), *Porphyromonas gingivalis* (*P. gingivalis,* strain ATCC 33277), and *Escherichia coli* (*E. coli,* strain DH5α) were used in this study. *A. oris* and *E. coli* were cultured aerobically in Bacto™ Heart Infusion Broth and LB medium, respectively (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), whereas *P. gingivalis* was cultured anaerobically in modified Gifu anaerobic medium (GAM broth) (Nissui, Tokyo, Japan) using an anaerobic chamber (Hirasawa, Tokyo, Japan) at 37°C.

Measurement of the growth curve

The growth characteristics of *A. oris, P. gingivalis,* and *E. coli* were measured in a visible photometer (Biochrom, Cambridge, UK) using absorbance at a wavelength of 600 nm. Each strain was pre-cultured in a liquid medium, and the turbidity of each bacterial solution was adjusted to $OD_{600}=0.1$. EEP at a final concentration of 0, 50 or 100 μg/mL was added to the adjusted solutions. *A. oris* was incubated under aerobic conditions, whereas *P. gingivalis* was incubated under anaerobic conditions, both at 37°C with shaking. The turbidity of the culture was measured after 3-24 h.

Calculation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

We determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Hawaiian EEP against *A. oris* and *P. gingivalis.* To measure the MIC, we employed a microdilution method where the pre-cultured bacterial solution was adjusted to a turbidity of $OD_{600}=0.05$ using a visible photometer, after which EEP was added at a concentration of 0-64 μg/mL. After 24 hours of incubation, we measured its turbidity using a multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). The lowest concentration of EEP that inhibited the growth in the culture, while maintaining it at the initial levels, was taken to be the MIC. All experiments were performed in triplicate. To determine MBC, 7 μL of the solution with no visible growth was plated onto the solid medium and incubated for 48 hours. The lowest concentration that revealed no visible bacterial growth after sub-culturing was taken to be the MBC.

RESULTS

We quantitatively evaluated the antibacterial activity of Hawaiian EEP by measuring the OD 600 of bacteria using a visible photometer. Figures 1-3 show the growth curve of *A. oris* (Fig. 1), *P. gingivalis* (Fig. 2), and *E. coli* (Fig. 3) treated with increasing concentrations of Hawaiian EEP. Remarkably, Ha-

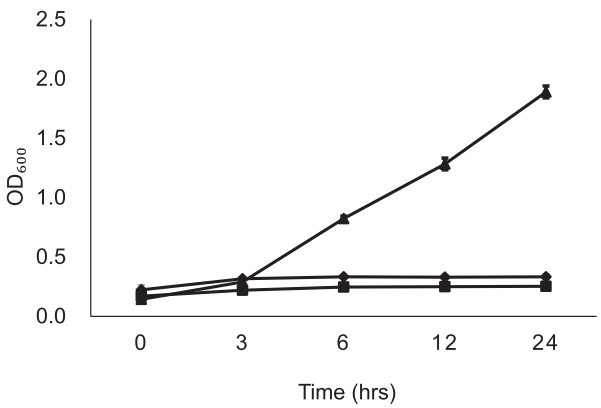

Fig. 1 Effect of EEP on the proliferation of the early colonizer, *Actinomyces oris* at concentrations of ◆100 μg/ml, ■ 50 μg/ml, and ▲0 μg/ml calculated as the mean from triplicate assays.

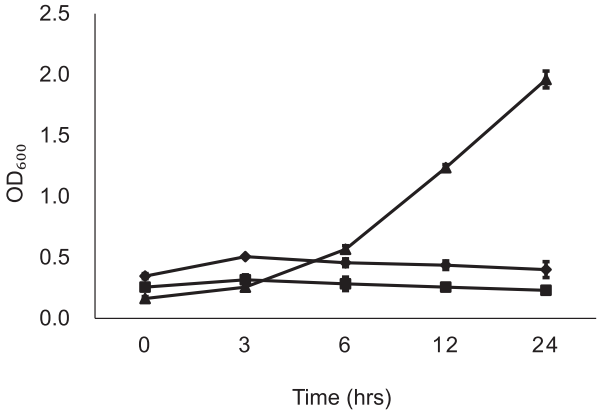

Fig. 2 Effect of EEP on the proliferation of periodontopathic bacteria, *Porphyromonas gingivalis* at concentrations of ◆ 100 μg/ml, ■50 μg/ml, and ▲0 μg/ml calculated as the mean from triplicate assays.

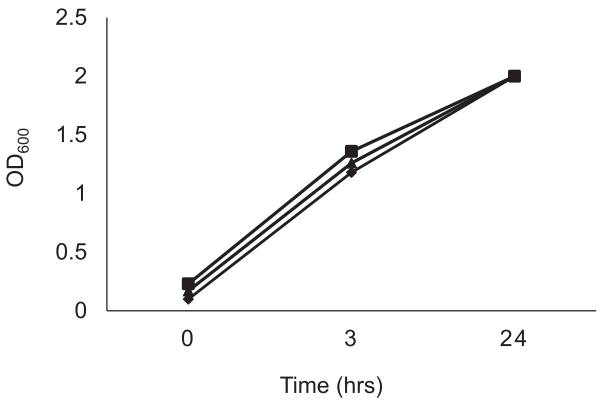

Fig. 3 Effect of EEP on the proliferation of *Escherichia coli* at concentrations of ◆100 μg/ml, ■50 μg/ml, and ▲0 μg/ml.

waiian EEP did not inhibit the growth of *E. coli.* However, 50 μg/mL Hawaiian EEP showed strong antibacterial activity against *A. oris* and *P. gingivalis.* This revealed the significant antibacterial capacity of Hawaiian propolis against both gram-positive and gram-negative oral pathogenic bacteria.

Next, we measured the susceptibility of the pathogens using broth microdilution as well as agar dilution techniques. Following MIC determination of Hawaiian EEP, aliquots from all the tubes that showed no visible bacterial growth were seeded on agar plates and incubated. The lowest concentration of Hawaiian EEP that killed 99.9% of the bacterial population was taken to be the MBC (Table). After incubation for 24 h at 4 μg/mL and 8 μg/mL of EEP, we saw turbidity in the tubes containing *A. oris,* whereas at 8 μg/mL and 16 μg/mL no turbidity was seen, indicating complete clearance. Similarly, we noticed turbidity in the tubes containing *P. gingivalis* cells at 4 μg/mL. However, at a concentration of 8 μg/mL of EEP, tubes containing *P. gingivalis* exhibited growth inhibition. The suspension from the tubes at concentrations of 2, 4, 8, 16, 32 and 64 μg/mL was inoculated in agar plates and incubated for 24 h. No growth of *A. oris* was observed at 16 and 32 μg/mL and no growth of *P gingivalis* was observed at 8 and 16 μg/mL. We determined the MIC and MBC of Hawaiian EEP to be 10.6 μg/mL and 21.3 μg/mL for *A. oris,* and 8 μg/mL and 9.6 μg/mL for *P. gingivalis,* respectively (average of triplicate experiments).

Table 1 MIC and MBC of Hawaiian propolis against *Actinomyces oris* and *Porphyromonas gingivalis.*

Hawaiian propolis		
Bacteria	MIC (μg/ml)	MBC (μg/mL)
Actinomyces oris	10.6	21.3
Porphyromonas gingivalis	8.0	9.6

DISCUSSION

Although the antibacterial activity of propolis from Brazil, Mexico, Taiwan, and Okinawa have been reported, little is known about the antibacterial properties of Hawaiian propolis.[10, 16-18] To the best of our knowledge, our study is the first to report the activity of Hawaiian propolis against oral pathogenic bacteria where we used *A. oris* and *P. gingivalis* as model organisms. *A. oris* is among the most abundant microorganisms present in supra- and subgingival dental plaques. It is a well-known early colonizer that interacts with other oral bacteria, such as *Streptococcus gordonii* and *Streptococcus sanguinis,* on the surface of the tooth during the progression of dental caries.[19] *P. gingivalis* is a key pathogen associated with periodontitis. We found that Hawaiian propolis inhibited the growth of both *A. oris* (gram-positive) and *P. gingivalis* (gram-

negative).

Previous studies have reported the use of different solvents, such as methanol and water, to perform *in vitro* experiments with propolis extracts. This led to inconsistencies in propolis standardization as different solvents extract different compounds, influencing its activity.[20] In our study, we used ethanol as the solvent because it has been widely used to obtain low wax propolis extracts, rich in biologically active compounds. Additionally, the most active ingredients of propolis seemed to be soluble in ethanol.

Remarkably, the nine prenylflavonoids (propolin A, propolin B, prokinawan, propolin E, nymphaeol-B (NB), isinymphaeol-B (INB), nymphaeol-A (NA), 3'-geranylnaringenin, and nymphaeol-C (NC)) that have been isolated from Okinawa propolis[21] have been identified in Hawaiian propolis using HPLC, HRESIMS data, and UV spectra in ethanolic extracts.[15] NA from Taiwanese propolis exhibits the highest antibacterial activity against gram-positive bacterial strains, such as *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis,* and also shows strong antibacterial activity when used in combination with NB and NC,[22] whereas NB from Egyptian propolis exhibits antibacterial activity against the gram-positive strains *Bacillus cereus* and *S. aureus,* and the gram-negative strains *Serratia* sp. and *Pseudomonas* sp.[23] Additionally, NA, NB, NC and INB (specifically NB) from *M. tanarius,* have been found to be potential flavonoids with significant antimicrobial effects.[24] Bryan *et al.* reported that the flavonoids of Okinawa propolis and Taiwanese propolis can interact with the bacterial cell wall, leading to cell lysis and death.[25] However, the mechanism of action of flavonoids on the cell wall of gram-positive and gram-negative bacteria is still unknown. Analogously, the interaction of prenylated flavonoids in Hawaiian propolis with other functional compounds in antibacterial activity remains to be investigated.

The antibacterial activity of propolis is two-pronged, direct action on bacteria and stimulation of the immune system leading to the activation of natural defense against bacteria.[26, 27] Regarding the direct action on bacteria, Yoshimasu *et al.* reported that EEP derived from Brazilian propolis triggered the development of aberrant membrane blebs on the surface of *P. gingivalis.*[28] In their study, they isolated artepillin C, baccharin, and ursolic acid as antibacterial compounds against *P. gingivalis.* Additionally, they reported the MIC of Brazil propolis EEP to be 64 μg/mL against *P. gingivalis.* In comparison, EEP derived from Hawaiian propolis had a MIC of 8 and 10.6 μg/mL against *A. oris* and *P. gingivalis,* respectively, indicating greater antibacterial activity against these oral pathogen bacteria. The constituents of Hawaiian propolis that give rise to its anti-microbial property need to be analyzed in future studies. Nonetheless, our conclusions prove the potential of Hawaiian propolis as a natural substance to improve oral health.

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Conflicts of interest

The authors declare they have no conflicts of interest.

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