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Screening of aqueous extracts of medicinal herbs for antimicrobial activity against oral bacteria

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ABSTRACT

Background: Dental caries is considered to be a preventable disease, and various antimicrobial agents have been developed for the prevention of dental diseases; however, many bacteria show resistance to existing agents. In this study, 14 medicinal herbs were evaluated for antimicrobial activity against five common oral bacteria as a screen for potential candidates for the development of natural antibiotics.

Methods: Aqueous extracts of medicinal herbs were tested for activity against *Enterococcus faecalis*, *Actinomyces viscosus*, *Streptococcus salivarius*, *Streptococcus mutans*, and *Streptococcus sanguis* grown in brain heart infusion (BHI) broth. A broth microdilution assay was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). A disk diffusion assay was performed by inoculating bacterial cultures on BHI agar plates with paper disks soaked in each of the medicinal herb extracts. Inhibition of the synthesis of water-insoluble glucans by *S. mutans* was also investigated.

Results: The aqueous extracts of many of the 14 medicinal herbs demonstrated antimicrobial activity against the five types of pathogenic oral bacteria. The extracts of Sappan Lignum, Coptidis Rhizoma, and Psoraleae Semen effectively inhibited the growth of oral bacteria and showed distinct bactericidal activity. The extracts of Notoginseng Radix, Perillae Herba, and Psoraleae Semen decreased the synthesis of water-insoluble glucans by the *S. mutans* enzyme glucosyltransferase (GTase). The present study is the first to confirm the antimicrobial activity of the extract of Sappan Lignum against all five species of oral bacteria strains.

Conclusion: These results suggest that certain herbal medicines with proven antimicrobial effects, such as Sappan Lignum and Psoraleae Semen, may be useful for the treatment of dental diseases.

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1. Introduction

Two major dental diseases in the world are dental caries and periodontal disease, both of which are caused by various bacteria in the oral cavity.¹ Dental caries is a common oral disease that usually develops secondary to the formation of plaque biofilms on the tooth surfaces; the causative agents are Gram-positive bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus* spp., and some non-mutans streptococci.^{2,3} Specific bacterial species such as *Actinomyces* spp. and *Enterococcus faecalis* contribute to tooth root caries and periodontal infections.^{4,5} Although dental disease is only slowly progressive, oral bacteria can also cause infections of the head and neck, such as periapical abscesses and infections of the jaw bones and fascia.⁶ Therefore, the control of oral bacteria is key to the prevention and treatment of these oral diseases. Various antibiotics, including ampicillin, chlorhexidine, erythromycin, spiramycin, and vancomycin, have been very effective at preventing dental caries, but these agents can cause unexpected side effects such as microorganism resistance, vomiting, and diarrhea.^{7,8} Furthermore, the use of antibiotics can promote the development of multidrug-resistant (MDR) strains of bacteria.⁹ These problems have led to a search for new antibacterial substances that are safe for humans and specific for oral pathogens. Various sources such as microorganisms, animals, and plants,¹⁰ have been examined for components with these properties.

In Asian countries, including Korea, China, and Japan, traditional herbal medicines have been used to treat infectious diseases since ancient times.¹¹ Most oral diseases are due to bacterial infections, and medicinal plants are well known to exert considerable antimicrobial activity against many microorganisms, including the bacteria responsible for dental caries.¹² Furthermore, the natural phytochemicals isolated from herbal medicines could offer effective alternatives to antibiotics and represent a promising approach to the prevention and treatment of dental caries and other oral infections.¹³ Screening for herbal medicines effective against oral bacteria is the required first step in the identification of natural phytochemicals that could be used as antimicrobial substances.¹¹ Therefore, the present study was performed to show that extracts of medicinal herbs inhibit the growth of oral bacteria as well as the synthesis of water-insoluble glucans by *S. mutans*.

2. Methods

2.1. Identification and preparation of medicinal herbs extracts

Aqueous extracts of medicinal herbs used in this study were purchased from the Korea Medicine Herbs Association (Yeongcheon, Korea). Identification of all herbal medicines was verified by Professor Bae of the College of Pharmacy, Chungnam National University, and the specimens of these herbs were deposited at Korean Institute of Oriental Medicine (KIOM). Each herbal medicine was extracted by heating in water of 8 to 10 times of the herb weight for 3 hours at 115 °C (Gyeongseo Extractor Cosmos-600, Incheon, Korea).

After boiling, the extract was filtered using standard testing sieves (150 µm) (Retsch, Haan, Germany) and freeze-dried to a powder. A 50 mg sample of each powdered herbal medicine was dissolved in 1 mL of distilled water and stored at -20 °C before use.

2.2. Microorganisms and growth conditions

Enterococcus faecalis (KTCT3206), *Actinomyces viscosus* (KTCT9146), *Streptococcus salivarius* (KTCT5091) and *Streptococcus mutans* (KCTC3065) were purchased from the Biological Resource Center (BRC) in Korea Research Institute of Bioscience and Biotechnology (KRIBB). *Streptococcus sanguis* (NCTC 9811) was purchased from the School of Dentistry Seoul National University. Five types of strains were incubated in brain heart infusion (BHI; Difco, BD, USA) broth and BHI agar (Difco, BD, USA) at 37 °C in the presence of 5% CO₂.

2.3. Determination of minimum inhibitory concentration and minimum bactericidal concentration of herbal medicine extracts on oral bacteria

Plate dilution method was used for determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of herbal medicine extracts on five kinds of oral bacterial strains. For MIC and MBC, the bacteria was incubated for 20 hours at 37 °C in 5% CO₂. To determine MIC, the extract concentration was diluted two-fold from 5000 µg/mL to 78 µg/mL and the inoculum of 1 × 10⁴ CFU/mL was used. The lowest concentration of the herbal medicine extracts that inhibited the growth of the organism, corresponding to an inhibition of 99% of the inoculum, was considered as the MIC. To determine MBC, we used a variant on the agar dilution method. The inoculation spots with no visible growth were cut and top down 3 µL of bacterial culture broth on BHI agar plate. The lowest concentration of herbal medicine extract that yielded no growth on the agar (99.9% kill) was defined as MBC.

2.4. Inhibition of water-insoluble glucan synthesis

The assay and preparation of crude GTase were based on the method previously described by Koo et al.¹⁴ The cell-free enzymes were precipitated from culture supernatant of *S. mutans*. After filtering the culture supernatant of *S. mutans* using a 0.2 µm membrane filter, the cell-free enzymes were extracted and precipitated by Amicon ultra centrifugal filter (MWCO 30 kDa, Millipore, USA). The crude enzymes were restored at -80 °C and used for synthesis of water-insoluble glucan. A reaction mixture consisting of 20 µL of crude enzyme, 180 µL of the diluted herbal medicine extract in 800 µL of 62.5 mM potassium phosphate buffer (pH 6.5) containing 12.5 g of sucrose and 0.25 g of sodium azide were incubated at 37 °C for 30 hours. After incubation, the fluid was removed, and the contents that stick to tube wall were washed with sterile water and dispersed by a sonicator (JAC-2010P, Kodo, Korea). The total amount of water-insoluble glucan was measured the absorbance by UV/VIS spectrophotometer (U-2900, Hitachi, Japan).

Table 1 – MIC and MBC of herbal medicines against oral bacteria.

	Herbal medicines	<i>E. faecalis</i>		<i>A. viscosus</i>		<i>S. salivarius</i>		<i>S. mutans</i>		<i>S. sanguis</i>	
		MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
1	Rhei Rhizoma	>5,000	>5,000	>5,000	>5,000	625	5,000	>5,000	>5,000	>5,000	>5,000
2	Sinomeni Caulis	2,500	>5,000	>5,000	>5,000	>5,000	>5,000	>5,000	>5,000	2,500	2,500
3	Saururi Radix	>5,000	>5,000	>5,000	>5,000	625	625	1,250	1,250	>5,000	>5,000
4	Notoginseng Radix	>5,000	>5,000	>5,000	>5,000	2,500	2,500	>5,000	>5,000	>5,000	>5,000
5	Sappan Lignum	156	1,250	156	625	<78	156	156	156	313	625
6	Perillae Herba	>5,000	>5,000	>5,000	>5,000	625	625	5,000	5,000	>5,000	>5,000
7	Alpiniae Rhizoma	>5,000	>5,000	>5,000	>5,000	>5,000	>5,000	2,500	5,000	>5,000	>5,000
8	Coptidis Rhizoma	2,500	2,500	2,500	2,500	78	156	313	313	625	625
9	Mori Cortex	2,500	>5,000	2,500	>5,000	313	625	125	2,500	1,250	1,250
10	Phellinus Linteus	2,500	>5,000	>5,000	>5,000	1,250	2,500	2,500	2,500	>5,000	>5,000
11	Sophorae Radix	>5,000	>5,000	>5,000	>5,000	625	2,500	1,250	5,000	>5,000	>5,000
12	Psoraleae Semen	625	625	313	313	<78	<78	156	156	313	625
13	Galla Rhois	1,250	2,500	5,000	5,000	156	2,500	625	2,500	313	313
14	Acanthopanax Cortex	5,000	>5,000	5,000	>5,000	>5,000	>5,000	>5,000	>5,000	>5,000	>5,000
A	Erythromycin	1.56	1.56	<0.78	<0.78	<0.78	<0.78	<0.78	<0.78	>50	>50
B	Spiramycin	6.25	12.5	6.25	6.25	<0.78	<0.78	<0.78	<0.78	50	50

Two kinds of antibiotics, A and B, were used as the positive control. MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.

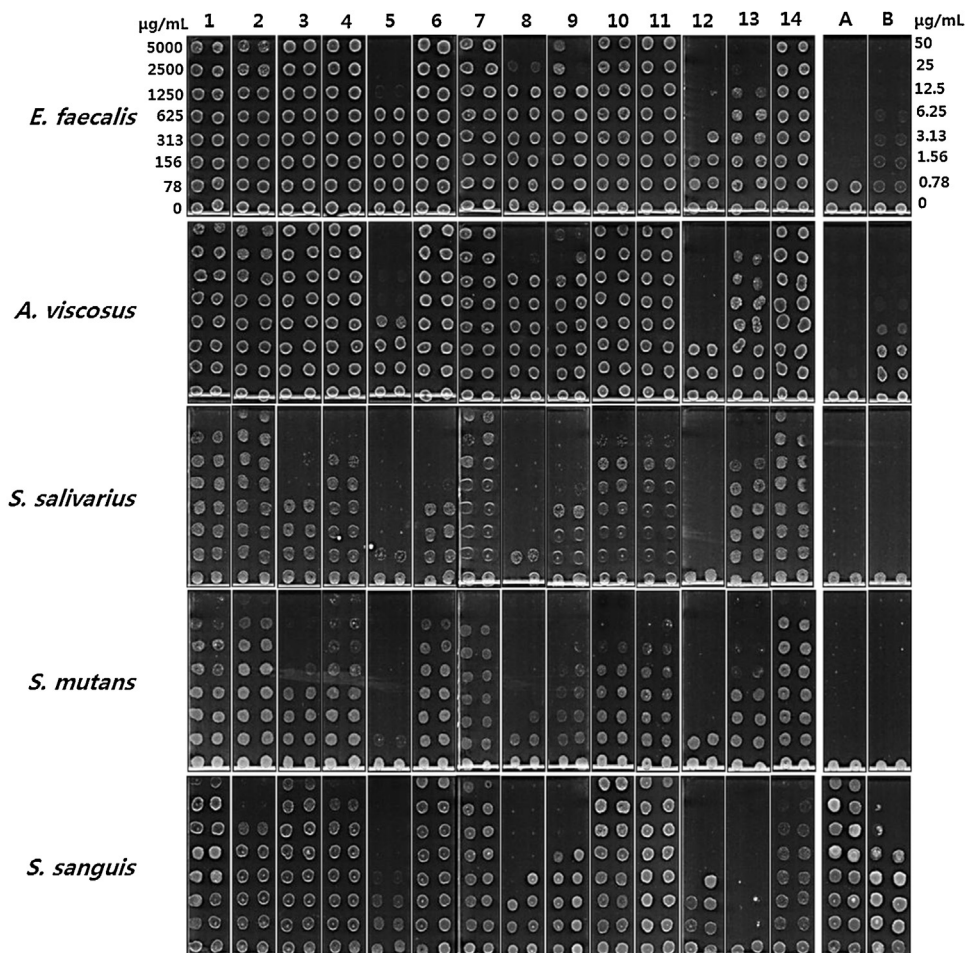


Fig. 1 – Comparison of MBC for extracts of herbal medicine against oral bacteria. Each extract was tested as a dilution series: 0, 78, 156, 313, 625, 1250, 2500, and 5000 $\mu\text{g/mL}$. Two kinds of antibiotics, erythromycin (A) and spiramycin (B), were respectively tested as a standard agents and these concentration were serially diluted: 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 $\mu\text{g/mL}$. 1, Rhei Rhizoma; 2, Sinomeni Caulis; 3, Saururi Radix; 4, Notoginseng Radix; 5, Sappan Lignum; 6, Perillae Herba; 7, Alpiniae Rhizoma; 8, Coptidis Rhizoma; 9, Mori Cortex; 10, Phellinus Linteus; 11, Sophorae Radix; 12, Psoraleae Semen; 13, Galla Rhois; and 14, Acanthopanax Cortex.

2.5. Agar diffusion assay

The antibiotic sensitivity profile of five kinds of oral bacteria was determined on assay plate including an inoculum of 1×10^4 CFU/mL on the top layer of the BHI agar plate. A sterile paper disk (8 mm) was soaked with extracts of herbal medicines, so that each disk was impregnated with 625–5000 μg of herbal medicine extract per disk. The plates were then incubated for 20 hours at 37 °C in 5% CO_2 . The antibacterial activity was evaluated by measuring the diameter (mm) of the inhibition zone.

2.6. Statistical analysis

The data are presented as the mean \pm SD. The Student t-test was used to assess the statistical significance of difference between control and the samples treated with each extract. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Herbal medicines have antimicrobial activity against oral bacteria.

The efficacies of 14 medicinal herbs against oral bacteria were investigated in terms of the MIC and MBC using a broth microdilution assay. A dot-blot assay was used to compare the MIC values of replicate samples from the multiwell broth assay with those of some commercially available antibacterial agents, including erythromycin and spiramycin. As shown in Table 1 and Fig. 1, Sappan Lignum (5), Coptidis Rhizoma (8), and Psoraleae Semen (12) showed significant antimicrobial activity against oral bacteria. The MIC values of Sappan Lignum (5) were 313 $\mu\text{g/mL}$ for *S. sanguis*, 156 $\mu\text{g/mL}$ each for *E. faecalis*, *A. viscosus*, and *S. mutans*, and <78 $\mu\text{g/mL}$ for *S. salivarius*. The MBC values for Sappan Lignum (5) were 1250 $\mu\text{g/mL}$, 625 $\mu\text{g/mL}$, 156 $\mu\text{g/mL}$, 156 $\mu\text{g/mL}$, and 625 $\mu\text{g/mL}$ against *E. faecalis*, *A. viscosus*, *S. salivarius*, *S. mutans*, and *S. sanguis*,

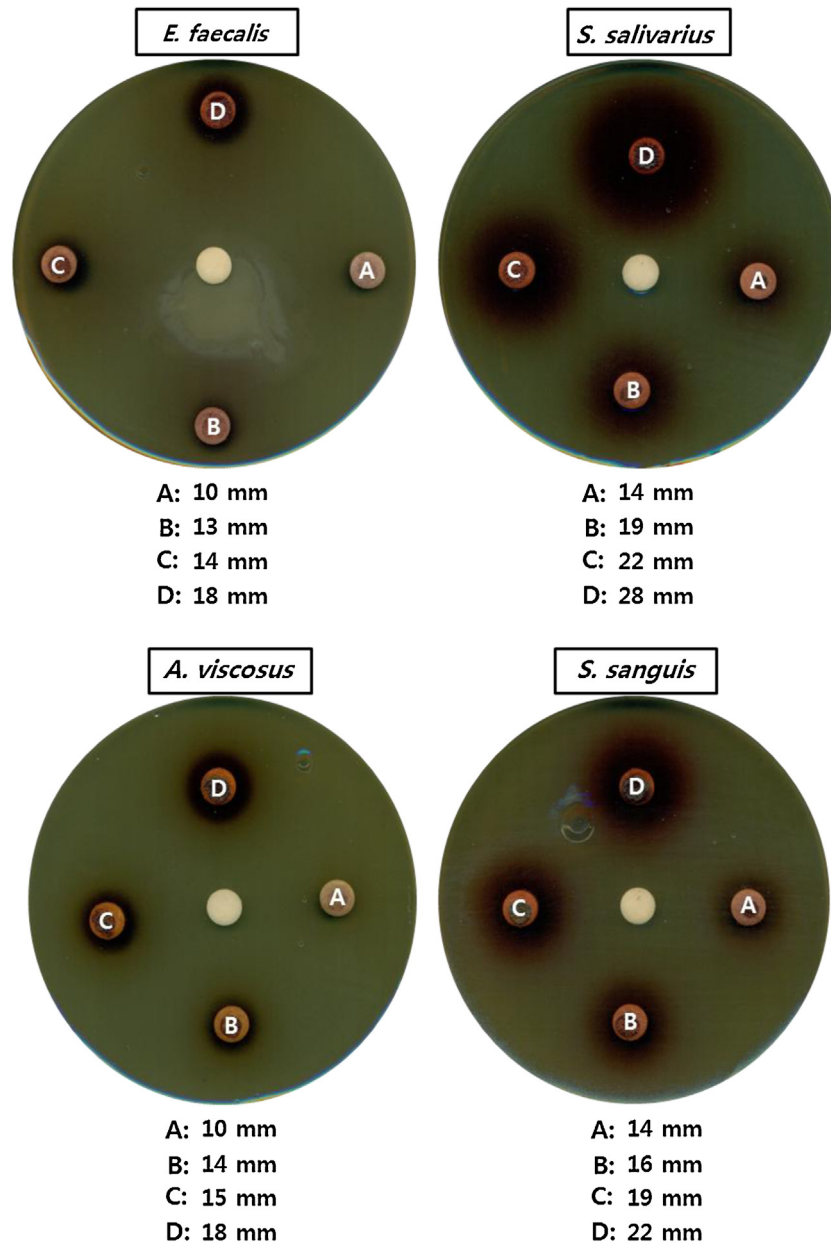


Fig. 2 – Disk diffusion assay of Sappan Lignum extract against oral bacteria. Inhibition zone formed according to the concentration of the Sappan Lignum extract was indicated in mm. A, 625 µg/mL; B, 1250 µg/mL; C, 2500 µg/mL; and D, 5000 µg/mL.

respectively. Of the five types of oral bacteria examined, *S. salivarius* and *S. mutans* were the most susceptible to Sappan Lignum (5). Psoraleae Semen (12) also showed low MIC values for oral bacteria, with specific results of 625 µg/mL, 313 µg/mL, 156 µg/mL, 313 µg/mL, and <78 µg/mL for *E. faecalis*, *A. viscosus*, *S. mutans*, *S. sanguis*, and *S. salivarius*, respectively. The MBC values for Psoraleae Semen (12) were 625 µg/mL, 313 µg/mL, 156 µg/mL, and 625 µg/mL for *E. faecalis*, *A. viscosus*, *S. mutans*, and *S. sanguis*, respectively. The MBC value of Psoraleae Semen (12) against *S. salivarius* was lower than 78 µg/mL; this was the strongest bactericidal effect observed among all of the tested herbal medicines. Coptidis Rhizoma (8) showed good antimicrobial activity against the three strains

of viridans streptococci, *S. salivarius*, *S. mutans*, and *S. sanguis*, but did not strongly affect *E. faecalis* or *A. viscosus*. Of the oral bacteria examined in the present study, *S. salivarius* was the most sensitive to treatment with all of the herbal medicines, except for Sinomeni Caulis (2), Alpiniae Rhizoma (7), and Acanthopanax Cortex (14).

3.2. Herbal medicines inhibit the synthesis of water-insoluble glucans by *S. mutans*.

We examined the inhibitory effects of the extracts of medicinal herbs on the synthesis of water-insoluble glucans by crude glucosyltransferase (GTase) from *S. mutans* (Table 2). Crude

Table 2 – Inhibitory activity of herbal medicines against water-insoluble glucan synthesis caused by GTase from *S. mutans*.

No.	Name of herbal medicine	Inhibition ratio (%)
1	Rhei Rhizoma	α
2	Sinomeni Caulis	α
3	Saururi Radix	n.e.
4	Notoginseng Radix	83.44 ± 2.05
5	Sappan Lignum	n.e.
6	Perillae Herba	44.16 ± 0.95
7	Alpiniae Rhizoma	n.e.
8	Coptidis Rhizoma	n.e.
9	Mori Cortex	α
10	Phellinus Linteus	n.e.
11	Sophorae Radix	α
12	Psoraleae Semen	56.67 ± 0.53
13	Galla Rhois	n.e.
14	Acanthopanax Cortex	n.e.

Data are represented as mean ± standard deviation.
GTase, glucosyltransferase; α, no inhibition; n.e., not evaluable.

GTase was treated with extracts of each medicinal herb at concentrations of 5 mg/mL, and Notoginseng Radix (4), Perillae Herba (6), and Psoraleae Semen (12) reduced the glucan formation by 83.44%, 44.16%, and 56.67%, respectively. The extracts of Rhei Rhizoma (1), Sinomeni Caulis (2), Mori Cortex (9), and Sophorae Radix (11) did not inhibit glucan synthesis. The extracts of the seven remaining herbs, including Saururi Radix (3), Sappan Lignum (5), Alpiniae Rhizoma (7), Coptidis Rhizoma (8), Phellinus Linteus (10), Galla Rhois (13), and Acanthopanax Cortex (14), were not evaluated for their effects on glucan formation because the colors of these extracts interfered with the assay used in the present study.

3.3. Sappan Lignum has strong activity against oral bacteria

Of the 14 herbal medicines tested, Sappan Lignum (5) demonstrated the most consistent antimicrobial activity, inhibiting the growth of all oral bacteria examined (Fig. 2). Agar plates spread with oral bacteria were treated with a series of dilutions, including 625, 1250, 2500, and 5000 µg/mL of Sappan Lignum. Most concentrations of Sappan Lignum produced inhibition zones in all tested oral bacteria, with the highest concentration, 5000 µg/mL, of Sappan Lignum producing wide inhibition zones of 18, 28, 18, and 22 mm for *E. faecalis*, *S. salivarius*, *A. viscosus*, and *S. sanguis*, respectively.

4. Discussion

Dental disease is one of the most prevalent public health concerns. The problems caused by dental caries affect all age groups, and treatment is both expensive and labor-intensive.¹ Dental caries and periodontal diseases are infectious diseases caused by common oral bacteria; therefore, controlling or even reducing the levels of these causative pathogens, such as *S. mutans* and *E. faecalis*, is a key step in the prevention and treatment of these diseases.² Dental caries is a common oral disease caused by many cariogenic microbes, including

Lactobacillus spp., *Streptococcus* spp., and *Actinomyces* spp., which usually form plaque biofilms on the tooth surfaces.¹ Dental plaque is initially synthesized by the GTase from *S. mutans*, and oral microorganisms then colonize and accumulate in this water-insoluble glucan layer. The viridans streptococci *S. salivarius*, *S. sanguis*, and *S. mutans* were the most representative human cariogenic bacteria included in the present study; however, these species are also moderately resistant to antibiotics.¹³ *Actinomyces* spp. are involved in early plaque development on tooth surfaces and contribute to root caries and periodontal infections.⁵ *E. faecalis* is an opportunistic pathogen that is frequently isolated from asymptomatic and persistent endodontic infections, especially from the failed root canals undergoing retreatment.¹⁵ *E. faecalis* is a better survivor than other root canal microbes, being able to resist various harsh conditions such as bile salts and starvation as well as many antibacterial agents.¹⁶ The characteristics of these oral bacteria determine the antimicrobial agents that can be used for the prevention and treatment of dental diseases. In our study, aqueous extracts of 14 medicinal herbs were prepared and evaluated for their antimicrobial activities against five species of oral bacteria.

Sappan Lignum has traditionally been used as a red dyestuff and also as a herbal medicine to treat inflammation or improve blood circulation.^{17,18} The antimicrobial activity of Sappan Lignum against *S. mutans* has been reported by You et al.¹⁹ However, we widely confirmed the antimicrobial activity of Sappan Lignum about periodontal diseases as well as dental caries. In our study, the aqueous extract of Sappan Lignum showed strong antimicrobial activity against all five species of oral bacteria. The MIC values of Sappan Lignum against *E. faecalis* and *A. viscosus* were especially low relative to those of the other herbal medicines. Further, Sappan Lignum extract had strong activity against oral bacteria in the agar diffusion assay; this activity was more pronounced for *Streptococcus* spp. than for other species such as *E. faecalis* and *A. viscosus*. Therefore, we have provided primary data showing that Sappan Lignum extract could be a potential treatment for both dental caries and periodontal diseases.

Psoraleae Semen is the fruit of *Psoralea corylifolia* L., and its active component, bakuchiol, has been reported to have various biological properties, including antimicrobial and anticancer activities.^{20,21} Bakuchiol isolated from Psoraleae Semen has been confirmed to have antimicrobial activity against several oral bacteria, including *S. mutans*, *S. sanguis*, *S. salivarius*, *S. sobrinus*, *E. faecalis*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *A. viscosus*, and *Porphyromonas gingivalis*.²² On the basis of previous reports, we suggest that the antimicrobial activity of Psoraleae Semen in the present study may have been due mainly to the bakuchiol contained in Psoraleae Semen. Therefore, we are in the process of confirming the antimicrobial activity of bakuchiol and its derivatives isolated from Psoraleae Semen.

S. mutans produces extracellular polysaccharides such as the water-insoluble glucans synthesized by GTase, which promote plaque formation.¹⁴ The synthesis of water-insoluble glucans by *S. mutans* is one of the most important contributors to the maturation of dental plaque. In the present study, several herbal medicines, including Notoginseng Radix, Perillae

Rhizoma, and Psoraleae Semen, at least partially inhibited the synthesis of water-insoluble glucans, although Notoginseng Radix and Perillae Rhizoma did not inhibit the growth of oral bacteria. These results suggest that Notoginseng Radix and Perillae Rhizoma may reduce the synthesis of water-insoluble glucans by inhibiting the enzymatic activity of the *S. mutans* GTase rather than by inhibiting bacterial growth. Furthermore, this is the first report that Notoginseng Radix or Perillae Rhizoma suppresses the synthesis of water-insoluble glucans.

In conclusion, we evaluated the antimicrobial activities of aqueous extracts of herbal medicines against oral bacteria. Of the tested herbal medicines, Sappan Lignum and Psoraleae Semen showed the strongest antimicrobial activity against all of the bacteria tested; notably, this is the first report of the inhibitory effect of Sappan Lignum on oral bacteria. Therefore, these results suggest that herbal medicines with proven antimicrobial effects, such as Sappan Lignum and Psoraleae Semen, may be useful for the treatment of dental diseases.

Conflict of interests

All authors have no conflict of interests.

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