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Inhibition of the growth of cariogenic bacteria in vitro by plant flavanones

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Abstract. Phytoalexins, defensive compounds produced by plants against microbial infections, were purified from *Sophora exigua* (Leguminosae) and their growth inhibitory effects on oral cariogenic bacteria were determined in vitro. Among three isolated compounds, 5,7,2′,4′-tetrahydroxy-8-lavandulylflavanone completely inhibited the growth of oral bacteria including primary cariogenic mutans streptococci, other oral streptococci, actinomycetes, and lactobacilli, at concentrations of 1.56 to 6.25 μg/ml.

Key words. Antibacterial activity; caries prevention; cariogenic bacteria; flavonoid; growth inhibition; phytoalexin.

Dental caries is an infectious disease caused by cariogenic bacteria, like streptococci of the mutans group[1,2]. Strategies to prevent caries include elimination of the cariogenic bacteria from the oral cavity, inhibition of bacterial plaque formation and increasing the resistance of the teeth to demineralization. Antibacterial substances that could eliminate the causative agents of caries have been actively studied[3–5]. Among them, plant-derived compounds (phytochemicals) have recently been attracting much interest as an alternative to synthetic chemical substances for caries prevention[6,7]. However, for most phytochemicals described up to now, the antibacterial activity against cariogenic bacteria is equivocal[8–11].

Phytoalexins are synthesized by plants in response to microbial infections, and function as the plant's self-defense system against pathogenic microorganisms[12]. Plants which synthesize these compounds include various food plants such as soybean, carrot, tomato, potato, eggplant and rice. Phytoalexins possess broad antimicrobial spectra[12,13]. This reliable antibacterial effect, and their origin from food plants, make them a promising source of substances with low toxicity for caries prevention.

As the first step in developing a plant-derived preventive agent for dental caries, we purified phytoalexins from *Sophora exigua* (Leguminosae), evaluated their growth inhibitory effects on cariogenic bacteria, and identified their chemical structures.

Materials and methods

Fractionation of phytochemicals. The dried roots (120 g) of *S. exigua*, collected in Thailand, were pulverized and extracted twice with chloroform (500 ml). The combined extracts were fractionated by two-step silica gel chromatography based on the method of Ruangrungsi et al.[14], and the antibacterial activity of each fraction was determined. The active fractions were purified by reversed-phase HPLC using a 6.0 × 150 mm Shim-pack CLC-ODS column (5 μm particle size; Shimadzu, Kyoto, Japan); eluent: acetonitrile-water (65:35, v:v); elution rate: 1.2 ml/min; column temperature: 25 °C; UV detection: 290 nm. Three substances, compounds **1, 2** and **3**, were finally isolated, and their minimum inhibitory concentrations (MICs) were measured.

Antibacterial assay. Bacterial strains (see table) were laboratory stock cultures or were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Bacterial cells (10^8 cfu/ml, based on the OD values) were suspended in BHI broth (Difco, Detroit, Michigan, USA) and used for inoculation.

All fractions were dissolved in triethanolamine-water (30:70, v:v) and added to BHI agar medium (125 μg/ml for the 1st screening and 6.25–25.0 μg/ml for the 2nd screening). BHI agar was used for all assays because the tested strains grew poorly on Mueller-Hinton agar[7,9]. The inoculated plates were incubated anaerobically at 37 °C for 48 h, and then aerobically for 24 h. A fraction was defined as having antibacterial activity when no colony was observed after both incubations.

Compounds **1, 2** and **3** were dissolved in ethanol and their MICs were determined by the two-fold serial agar dilution method under the above-mentioned conditions. The lowest concentration in which no colony was observed after both incubations was defined as the MIC value. The MICs of commercially available chlorhexidine gluconate, chrysine, quercetine and shikonine were similarly determined. Using Scheffe's test, the confidence intervals of the MICs of compound **2**, compound **3** and chlorhexidine, and the statistical comparison between them, were analyzed by Dr. H. Todoriki (School of Medicine, University of the Ryukyus, Okinawa, Japan).

Antibacterial action. An ethanolic solution of compound **3** was added to the cell suspension (1.8×10^7 cfu/ml) of *S. mutans* (OMZ-175) in 0.1 M phosphate-buffered saline (pH 7.0) to give the concentration of 6.25 µg/ml (the MIC value). The mixture was incubated at 37 °C and the change in the number of viable cells was followed with time using Mitis Salivarius agar plates (Difco)[15].

Identification of isolated compounds. Compounds **1, 2** and **3** were subjected to elemental analysis, determination of the melting point (mp) and spectral measurements including EI-MS, HR-MS, ^{1}H-NMR, ^{13}C-NMR, UV/Vis and IR, using standard techniques[14].

Results and discussion

Antibacterial activity was finally found in compounds **1, 2** and **3** after serial fractionation and measurement of the activity in both 1st and 2nd screenings. Compound **1** showed MICs of 25.0 µg/ml or more. Compounds **2** and **3** were more active against cariogenic bacteria than compound **1** (table). The antibacterial activity of compound **3** was intensive, with MIC values for primary cariogenic mutans streptococci, actinomycetes and lactobacilli ranging from 1.56 to 6.25 µg/ml. Compound **3** also inhibited the growth of the other streptococci related to plaque formation at 3.13 to 6.25 µg/ml. The MIC values

Minimum inhibitory concentrations to cariogenic bacteria.

Bacterial strains	Minimum inhibitory concentration (µg/ml)		
	Compound-**2**	Compound-**3**	Chlorhexidine
Mutans streptococci:			
S. mutans (ATCC 25175)	12.5	6.25	1.56
S. mutans (LM-7)	12.5	6.25	6.25
S. mutans (OMZ-175)	12.5	6.25	1.56
S. mutans (GS-5)	6.25	3.13	1.56
S. sobrinus (ATCC 33478)	–	6.25	0.78
S. sobrinus (6715)	–	3.13	0.78
S. sobrinus (OMZ-176)	–	6.25	1.56
S. rattus (BHT)	12.5	3.13	1.56
S. rattus (FA-1)	6.25	3.13	0.78
S. rattus (HS-6)	12.5	6.25	3.13
S. cricetus (E-49)	12.5	6.25	3.13
mean	10.49*	5.12*	2.06*
confidence interval	8.82–13.06	3.31–6.92	0.25–3.87
Actinomyces:			
A. viscosus (ATCC 15987)	–	1.56	<0.39
A. viscosus (ATCC 15988)	–	1.56	<0.39
A. viscosus (ATCC 19246)	–	1.56	0.78
A. naeslundii (ATCC 12104)	–	1.56	<0.39
A. israelii (ATCC 10048)	–	1.56	0.78
A. israelii (ATCC 12102)	–	1.56	<0.39
mean		1.56*	0.52*
confidence interval		1.39–1.73	0.35–0.69
Lactobacillus:			
L. casei (ATCC 7469)	–	3.13	3.13
other oral streptococci:			
S. salivarius (ATCC 7073)	6.25	3.13	6.25
S. salivarius (ATCC 25975)	12.5	3.13	1.56
S. salivarius (1911)	12.5	6.25	1.56
S. salivarius (2613)	12.5	6.25	1.56
S. sanguis (ATCC 10556)	6.25	3.13	6.25
S. sanguis (YST-002)	12.5	6.25	3.13
S. sanguis (YST-074)	12.5	6.25	3.13
S. sanguis (0501)	12.5	3.13	1.56
S. sanguis (1033)	6.25	3.13	1.56
S. gordonii (ATCC 10558)	12.5	3.13	3.13
S. oralis (ATCC 10557)	6.25	3.13	3.13
S. oralis (ATCC 35037)	12.5	3.13	1.56
S. oralis (1903)	12.5	6.25	1.56
S. mitis (ATCC 33399)	6.25	3.13	3.13
S. mitis (ATCC 9811)	6.25	3.13	6.25
S. mitis (ATCC 903)	6.25	3.13	6.25
mean	9.58*	3.96*	3.23*
confidence interval	7.82–11.35	2.20–5.72	1.47–4.99

–, not determined. *, $p < 0.01$.

Minimum inhibitory concentrations of compound **2**, compound **3** and chlorhexidine for oral bacteria. The differences between the values for compound **2**, compound **3** and chlorhexidine were statistically significant ($p < 0.01$ in mutans streptococci, actinomyces and other oral streptococci).

of compound **3** were significantly smaller with all tested bacteria than those of compound **2** ($p < 0.01$).

The MICs of chlorhexidine for the same strains varied in the range from <0.39 to 6.25 µg/ml. These values were smaller for both primary cariogenic and plaque-forming bacteria than those of compound **3** ($p < 0.01$). The antibacterial activity of compound **3** was, however, much superior to that of potentially anti-cariogenic phytochemicals reported previously, such as chrysine, quercetine and shikonine[16], which showed MICs of 50 µg/ml or more, against all strains.

The decrease in viability of *S. mutans* (OMZ-175) was determined by incubating the cells in the presence of compound **3**. At the concentration corresponding to the MIC value (6.25 µg/ml), the cell numbers decreased from 1.8×10^7 to 5.3×10^5 cfu/ml in the first 30 min, but after that the viability was only gradually reduced further (1.2×10^4 cfu/ml after 3 h). The antibacterial effect of compound **3** is considered to be bacteriostatic rather than bactericidal, like that of chlorhexidine[17].

Compound **2**, mp 179 °C (benzene), was obtained as off-white crystals. From spectral data, it was identified as 5,7,2′,6′-tetrahydroxy-8-lavandulylflavanone, exiguaflavanone A[14] (see figure). Compound **3**, mp 174–175 °C (benzene), was obtained as colorless needles. Its spectral data agreed with those of 5,7,2′,4′-tetrahydroxy-8-lavandulylflavanone, sophoraflavanone G[14] (see figure).

More than 200 phytoalexins have been characterized from over 20 plant families, and nearly half of them are flavonoids[18]. Various natural flavonoids possess sufficient antimicrobial potential to be medicinally important as preventive and therapeutic agents[16,19,20]. Considering the widespread occurrence of flavonoids in edible plants and beverages, it is likely that among the flavonoid phytoalexins there are substances which could be used in the future as caries preventive agents of low toxicity[21]. Various antibiotics reduce the numbers of cariogenic bacteria and inhibit plaque formation[22]. There is, however, general agreement that antibiotics should not be routinely used in caries prevention because of the risk that the oral microflora will be altered by their use, and other pathogenic microorganisms will multiply rapidly[23,24]. To overcome this problem, substances which could offer an alternative to antibiotics have been searched for. More attention has recently been paid to the use of antimicrobial phytochemicals in preventive and therapeutic strategies for infectious diseases, including dental caries[8–10,23,25–27]. Phytochemicals, in contrast to antibiotics, appear scarcely to induce resistance in oral microorganisms[28,29].

Since many interrelated factors are responsible for the initiation and progression of dental caries[1,2], multiple prevention against different factors is the most promising approach. Although the antibacterial activity of the tea components studied previously is not as intensive as that of some other agents, they reduce plaque formation by inhibiting glucosyltransferase and bacterial adherence[9,26,27]. The two flavanone phytoalexines isolated in this study are chemically analogous to the anti-cariogenic polyhydroxyl flavonoids from tea leaves[10,27], so these flavanones may also show an inhibitory effect on plaque formation.

When caries preventive agents are administered, they will initially be present at relatively high concentrations (possibly more than the MICs) in oral environments, and thereafter be desorbed so that the concentrations will fall[5]. In addition to reliable antibacterial activity, the practical use of preventive agents requires effective delivery to and adequate retention in the oral cavity. Certain phytochemicals active against cariogenic bacteria meet this requirement[30,31].

For the compounds described here, the effectiveness in vivo remains to be studied, together with their retention in the oral cavity, other biological activities and toxicity.

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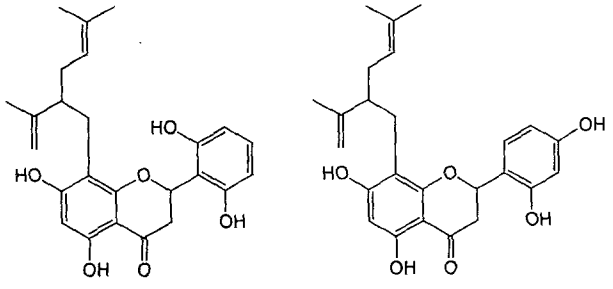

Compound-2 Compound-3

Chemical structures of compound **2** (5,7,2′,6′-tetrahydroxy-8-lavandulylflavanone) and compound **3** (5,7,2′,4′-tetrahydroxy-8-lavandulylflavanone).

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