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Inhibitory Effects of Flavonoids from *Spatholobus suberectus* on Sortase A and Sortase A-Mediated Aggregation of *Streptococcus mutans*

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Seven flavonoids were isolated from *Spatholobus suberectus* via repetitive column chromatography and high-performance liquid chromatography. The chemical structures of these compounds were identified by spectroscopic analysis and comparison with values reported in the literature. Among the flavonoids tested, 7-hydroxy-6-methoxyflavanone (**1**) and formononetin (**4**) exhibited strong inhibitory activity against *Streptococcus mutans* SrtA, with IC_{50} values of 46.1 and 41.8 μM, respectively, but did not affect cell viability. The onset and magnitude of inhibition of saliva-induced aggregation in *S. mutans* treated with compounds **1** and **4** were comparable to the behavior of a *srtA*-deletion mutant without treatment.

Keywords: *Spatholobus suberectus*, flavonoids, *Streptococcus mutans*, sortase A, cell aggregation

Sortase A (SrtA) is a membrane-associated transpeptidase responsible for the anchoring of surface-exposed proteins to the cell wall envelope of gram-positive bacteria [1]. Numerous genetic knockout experiments have shown that SrtA plays a critical role in the pathogenesis of gram-positive bacteria by modulating the ability of the bacterium to adhere to host tissue [2]. Because of the great interest in SrtA as a target for anti-infective therapies, many studies have been undertaken to identify a potent inhibitor of this enzyme [3]. *Streptococcus mutans* is the major pathogen responsible for the formation of dental caries [4]. SrtA is responsible for sorting and anchoring surface proteins to the cell wall of *S. mutans* [5]. A mutant strain of *S. mutans* lacking *srtA* exhibited a decreased ability to colonize the oral mucosa and teeth and consequently reduced caries formation [6]. Therefore, this enzyme could provide a future target for treatment to prevent dental caries. In our search for bioactive compounds employed in Korean folk medicine, we found that crude organic extracts from the stem of *Spatholobus suberectus* Dunn (Leguminosae) inhibited SrtA from *Staphylococcus aureus*. From the polar chromatographic fraction of *S. suberectus* extract, we recently identified 20 flavonoids as *S. aureus* SrtA inhibitors [7]. However, biological activity tests revealed that some of these compounds showed strong inhibitory activity against saliva-induced cell aggregation in *S. mutans* strain OMZ65, isolated from the human oral cavity. Here, we report the potential of seven flavonoids for inhibition of SrtA and saliva-induced cell aggregation in *S. mutans*.

The isolation of flavonoids from the stem of *S. suberectus* was carried out according to a previously published procedure [7]. The crude extracts (105.1 g) were partitioned between H_2O (78.2 g) and *n*-BuOH (25.4 g); then, the latter fraction was repartitioned between H_2O-MeOH (15:85) (22.6 g) and *n*-hexane (2.1 g). An aliquot (10.4 g) of the aqueous MeOH layer was separated by C_{18} reverse-phase vacuum flash chromatography using gradient mixtures of H_2O and MeOH as eluents (six fractions in a gradient from 50:50 H_2O-MeOH to 0:100), followed by acetone and then EtOAc. The H_2O-MeOH (30:70, 0.4 g) and H_2O-MeOH

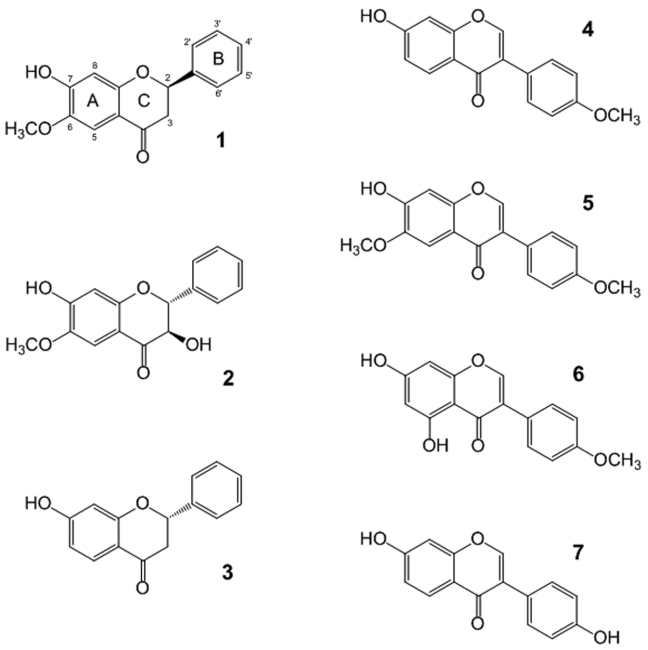

Fig. 1. The structures of compounds **1–7** isolated from the stem of *S. suberectus* Dunn.

7-Hydroxy-6-methoxyflavanone (**1**), (*2S,3R*)-3,7-dihydroxy-6-methoxyflavanone (**2**), 7-hydroxyflavanone (**3**), formononetin (**4**), afromosin (**5**), 5,7-dihydroxy-4′-methoxyisoflavone (**6**), and daidzein (**7**).

(20:80, 0.45 g) fractions were separated by reverse-phase HPLC (YMC-ODS column, 10 mm × 250 mm; H_2O-MeOH, 85:15 and 35:65, respectively). Individual compounds were purified using reverse-phase HPLC (H_2O-MeCN, 63:37). Purified metabolites were isolated in the following amounts: 4.0, 8.9, 3.7, 11.5, 7.7, 5.0, and 4.8 mg of compounds **1–7**, respectively. The spectroscopic data for these compounds were in accordance with literature values for 7-hydroxy-6-methoxyflavanone (**1**) [8], (*2S,3R*)-3,7-dihydroxy-6-methoxyflavanone (**2**) [9], 7-hydroxyflavanone (**3**) [10], formononetin (**4**) [11], afromosin (**5**) [12], 5,7-dihydroxy-4′-methoxyisoflavone (**6**) [13], and daidzein (**7**) [14] (Fig. 1).

The preparation of recombinant SrtA from *S. mutans* OMZ65 was performed in accordance with a previous literature protocol [15]. The region of the *srtA* gene was PCR-amplified from genomic DNA using forward (5′-GGC GAATTCGCTTGGAATACCAATAGA-3′, EcoRI) and reverse (5′-GAAGTCGACTTAAAATGATATTTGATTATAGGACTG-3′, SalI) primers. The ability of compounds **1–7** to inhibit SrtA activity was evaluated by using a fluorescent peptide (Dabcyl-QALPETGEE-Edans) [16]. The inhibitory potencies (IC_{50} values) of isolated flavonoids against *S. mutans* SrtA are summarized in Table 1 alongside the known SrtA inhibitors curcumin (45.8 μM) [17] and berberine chloride (56.9 μM) [18]. Compound **1** showed almost the same inhibitory activity (IC_{50} = 46.1 μM) as the control compound curcumin. Interestingly, compound **3** was not active against *S. mutans* SrtA at the highest concentration tested (IC_{50} > 533.2 μM). A SrtA inhibitory activity study of compounds **1–3** revealed that the methoxy group at the C-6 position and the *R*-configured B ring at the C-2 position of compound **1** were important (Fig. 1). We found that compound **4** (IC_{50} = 41.8 μM) was more potent than the positive control compounds. Methoxylation at the C-6 position or hydroxylation of the 4′-methoxy group in the B ring of compound **4**, as found in compounds **5** and **7**, respectively, led to lower inhibitory activities than that of compound **4**. Interestingly, hydroxylation at the C-5 position of compound **4**, as in compound **6**, led to a total loss of activity against SrtA (IC_{50} > 450.6 μM). These results suggest that the co-occurrence of the hydroxyl group at C-7 (A ring) and methoxy group at C-4′ (B ring) is essential for strong SrtA inhibitory activity by flavonoid compounds.

Table 1. Inhibitory effects of compounds **1–7** on the activity of SrtA enzyme and bacterial growth of *S. mutans* strain OMZ65.

Compound	IC_{50} (μM)	MIC (μM)
1	46.1 ± 1.6	>473.9
2	210.4 ± 3.3	>447.4
3	>533.2	>533.2
4	41.8 ± 2.1	>477.5
5	76.9 ± 1.4	>429.4
6	>450.6	>450.6
7	144.7 ± 2.9	>503.8
Curcumin	45.8 ± 1.7	>694.9
Berberine chloride	56.9 ± 2.3	>344.3

Curcumin and berberine chloride were used as reference inhibitors of SrtA. IC_{50} values are the mean ± SD (n = 3).

SrtA inhibitors should act as anti-infective agents and disrupt the pathogenesis of bacterial infections without affecting microbial viability [19]. Therefore, we investigated the effect of test compounds on *S. mutans* OMZ65 cell growth by the microtiter broth dilution method [20] and determined the MICs of these compounds. As shown in Table 1, these compounds exhibited no growth-inhibitory activity. Based on the combined bioactivity test, we next investigated the effect of compounds **1** and **4** on saliva-induced aggregation of wild-type *S. mutans* strain NG8, as well as its isogenic knockout mutants [5]. Aggregation was assessed based on a reduction in optical density (OD) at 700 nm. Both NG8 and the *srtA*-complemented mutant, but not the *srtA*-deletion mutant, aggregated upon incubation

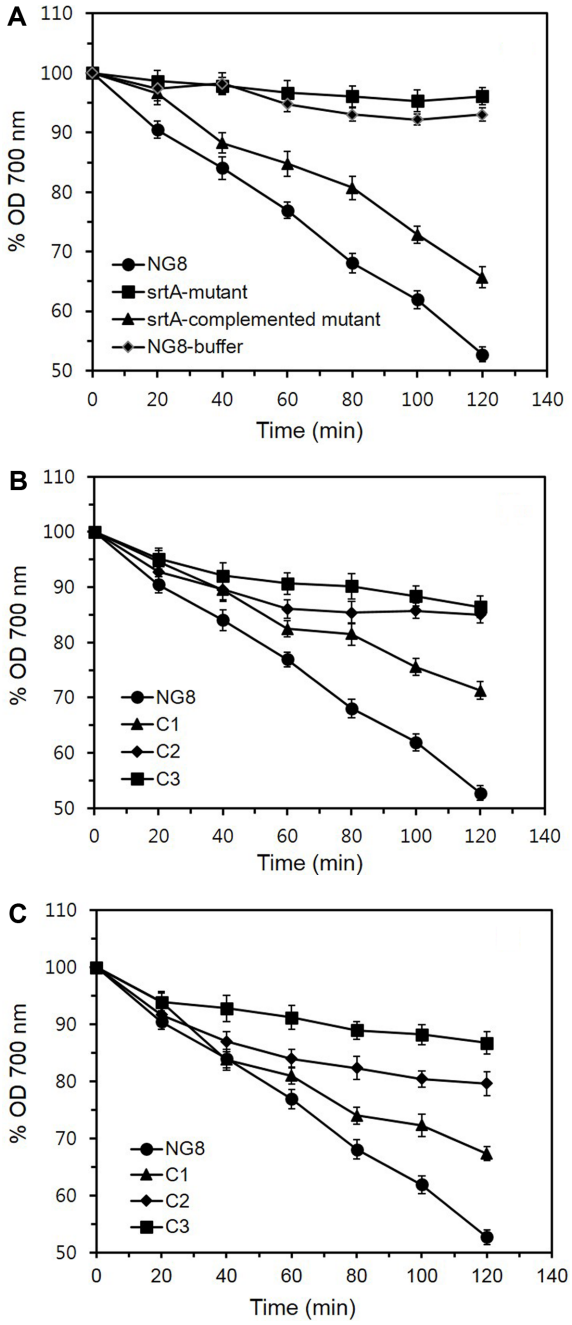

Fig. 2. Effects of compounds **1** and **4** on saliva-induced aggregation of *S. mutans* NG8 mutants.

(**A**) Saliva-induced aggregation of *S. mutans* NG8 (wild-type), *srtA*-defective mutant, and *srtA*-complemented mutant cells without addition of inhibitors. NG8-buffer refers to the aggregation assay performed with *S. mutans* NG8 in the absence of saliva. (**B**) *S. mutans* NG8 treated with compound **1**. C1, C2, and C3 refer to the aggregation assay performed with *S. mutans* NG8 in the presence of 23.0 (1/2 × IC_{50}), 46.1 (1 × IC_{50}), and 92.2 μM (2 × IC_{50}), respectively. (**C**) *S. mutans* NG8 treated with compound **4**. C1, C2, and C3 refer to the aggregation assay performed with *S. mutans* NG8 in the presence of 20.9 (1/2 × IC_{50}), 41.8 (1 × IC_{50}), and 83.6 μM (2 × IC_{50}), respectively.

with saliva (Fig. 2A). As expected, treatment of NG8 with compounds **1** (Fig. 2B) and **4** (Fig. 2C) significantly reduced bacterial cell aggregation in a dose-dependent manner. It is important to note that both the onset and the magnitude of aggregation inhibition in NG8 treated with compounds **1** and **4** (2 × IC_{50}) were comparable to those of an untreated *srtA*-deletion mutant. These results were consistent with the observations that compounds **1** and **4** strongly inhibited SrtA.

In this study, seven flavonoids were isolated from the stem of *S. suberectus*, and the inhibitory activity toward *S. mutans* SrtA was investigated. These studies led to the identification of 7-hydroxy-6-methoxyflavanone (**1**) and formononetin (**4**) as potent SrtA inhibitors. Saliva-induced aggregation activity data revealed the potential of these compounds for the treatment of *S. mutans* infections via inhibition of SrtA activity.

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