

Anticariogenic Potential of Korean Native Plant Extracts against *Streptococcus mutans*

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ABSTRACT

Numerous chemically synthesized compounds are widely used in oral hygiene products. However, due to their potential risk, there is a need to improve the safety and quality of dental care by seeking alternative control agents such as those naturally found in plant materials. Here we assessed antibacterial potentials of extracts from 100 species of Korean native plants against *Streptococcus mutans* on cariogenesis. Among those, extracts from five plants (*Arctii Fructus*, *Caryopteris incana*, *Aralia continentalis*, *Symplocarpus renifolius*, and *Lamium amplexicaule*) showed a growth inhibition of *S. mutans*. The five extracts were further individually evaluated for their minimal inhibitory concentration and minimal bactericidal concentration. Interestingly, a synergistic antibacterial activity was observed with the combination of sodium fluoride and the plant extracts. To determine the anti-biofilm activity of plant extracts, *S. mutans* was treated with increasing concentrations of the extracts in the range from 1250 to 3750 µg/mL. When *S. mutans* was grown in the defined biofilm medium containing the individual extracts of 47 species, the biofilm amount markedly decreased compared to that of a negative control. Notably, the extract of *S. renifolius* significantly downregulated the *gtf* and *spaP* genes for synthesis of glucan and adhesive proteins in *S. mutans*, and *L. amplexicaule* decreased the expression of *gtfD* gene. Therefore, these results demonstrate that the five plant extracts modulate survival and pathogenesis of *S. mutans* by growth inhibition and downregulation of the gene(s) implicated in biofilm formation.

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Introduction

As societies worldwide become more developed and aged, oral health is increasingly perceived as one of the most globally prevalent concerns. In particular, dental caries, which is the most prevalent and significant oral disease, occurs in many people regardless of age and gender. Various scientific evidence supports that the prevalence of dental caries is correlated with the development

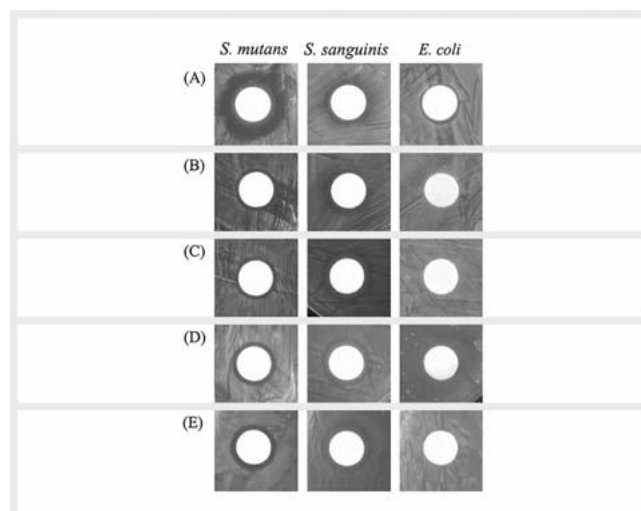
of food processing and frequency of carbohydrate intake [1, 2]. In general, dental caries occurs due to demineralization of enamel and dentine by organic acids produced from sugar fermentation by oral microorganisms residing in dental plaque. Studies about the human oral microbiome have shown that approximately 800 bacterial species inhabit the oral cavity, and only a small fraction of them are pathogenic [3].

Streptococcus mutans has long been appreciated as one of the bacteria species responsible for the development of dental caries, in part, through products of multiple genes or operons involved in colonization, fermentation acid production, acid tolerance (aciduricity), and biofilm formation in this bacteria. Following initial attachment to a surface, biofilm formation is proceeded by the activity of the glucosyltransferases (GTFs) that convert dietary sucrose to glucans, a main component of extracellular polymeric substances (EPS) [4]. GTFs (GtfB, C, and D) catalyze biosynthesis of water-insoluble and -soluble glucans [5–7] that promote tight adherence to the tooth surface and formation of microcolonies [8]. In dental plaques, *S. mutans* rapidly produces lactic acid through carbohydrate fermentation, and thus displays an increased acidogenicity in the presence of carbohydrates [9]. Consequently, elevated pathogen colonization of *S. mutans* causes demineralization of the tooth enamel layer, and the resulting acidic environment alters the bacterial diversity [10, 11].

Oral hygiene and prophylactic medicines containing a variety of chemical ingredients are generally used to minimize the risk of oral infection. For example, one of the commonly prescribed gargle products after surgical procedures, such as periodontal surgery and implant surgery in dentistry, is chlorhexidine-containing mouthwashes. These mouthwashes have a very powerful disinfectant and antiseptic properties to suppress plaque formation by oral microorganisms. Despite this beneficial effect, their prolonged application at high concentrations presents a potential risk of side effects to oral health. Besides, bodily accumulation of the chemicals found in these mouthwashes can have detrimental effects on the nervous system and various organs [12, 13]. Even with short-term usage, chlorhexidine-containing mouthwashes cause some temporal side effects, such as tooth discoloration, burning sensation, dry oral mucosa, and dysgeusia [14].

Similar to chlorhexidine, fluoride is also the most effective caries-preventive agent and functions to induce remineralization of decalcified teeth. Moreover, it has been shown that fluoride interferes with the acid production and secretion of GTFs in *S. mutans* and inactivates the virulence factors of cariogenic bacteria in dental plaque [15]. As shown in multiple studies, fluoride-resistant strains, including *S. mutans*, *Streptococcus sobrinus*, *Streptococcus salivarius*, and *Enterobacter cloacae*, have also been generated under laboratory conditions via prolonged exposure to high concentrations of sodium fluoride [16–20]. Importantly, some investigations showed that fluoride resistance can be a permanently acquired trait due to chromosomal alterations rather than a transient consequence [19–22]. Using a whole-genome shotgun (WGS) sequencing technique, Liao et al. [19] discovered single nucleotide polymorphisms (SNPs) were caused by nonsynonymous substitution in five genes (*smc*, *furR*, *pyk*, *holaA*, and *perB*) and in the intergenic regions of genes related to a fluoride antiporter in a fluoride-resistant *S. mutans* strain. Although such genetic studies using fluoride-resistant strains generated *in vitro* have been conducted, there have not been many clinical studies involving a fluoride-resistant strain directly isolated from caries patients. Therefore, novel approaches are needed to overcome the health hazard of preventive agents and emergence of fluoride-resistant bacteria.

A large number of studies have been conducted to identify traditional plants that present with a broad spectrum of uses against



► **Fig. 1** The antibacterial effects of the five plant extracts. Paper disks were placed on spread plates of a culture grown to the early exponential phase. A 20 μ L aliquot of the individual plant extracts, (A) *Arctii Fructus*, which is the dried seed of *A. lappa*, (B) *C. incana*, (C) *A. continentalis*, (D) *S. renifolius*, and (E) *L. amplexicaule*, was added onto each disk, and then the plates were incubated at 37 $^{\circ}$ C with 5% CO_2 for 24 h. The results show the inhibitory effect around the 8 mm diameter disks. Shown are representatives from three independent experiments conducted with 50 mg/mL plant extracts.

diseases. Additionally, various medicinal plants have antimicrobial and anti-biofilm activities against *S. mutans* [23–25]. Recently, the focus of the studies has been on the identification of caries-preventive natural substances that synergize with fluoride. Previous studies revealed that that application of a substance obtained from *Polygonum cuspidatum* and propolis enhances the inhibitory properties of fluoride on dental biofilms [26,27]. Here, we screened Korean traditional plant extracts to identify those that can inhibit the growth of *S. mutans* and evaluate their antibacterial and anti-biofilm activities. Our findings show that five plant extracts substantially alleviate the cariogenic activity of *S. mutans*, and this effect is correlated with the downregulation of the *gtf* and *spaP* genes in *S. mutans*. Furthermore, these results are expected to provide new approaches for designing combinatorial oral hygiene practices with nontoxic oral care products and plant extracts.

Results

Previous reports revealed that numerous natural plant extracts have antimicrobial activity against opportunistic oral pathogens [23–25]. Based on the literature, we selected 100 Korean native plant species to evaluate their potential antibacterial effects against the oral pathogen *S. mutans* and commensal *Streptococcus sanguinis* alongside the gram-negative bacteria control *Escherichia coli*. The antibacterial activities of these extracts against *S. mutans*, *S. sanguinis*, and *E. coli* were tested using disk diffusion assays (► **Fig. 1**). The results of these assays and information about the plants are summarized in ► **Table 1**. Growth inhibition of *S. sanguinis* was observed with 53 plant species, and 15 of those inhib-

► **Table 1** Information for Korean native plant extracts used in this study and evaluation of antibacterial activity of each extract against three bacterial species.

Botanical name	Part(s) ^a	Solvent for extraction	Antibacterial activity ^{b, c}		
			<i>S. mutans</i>	<i>S. sanguinis</i>	<i>E. coli</i>
<i>Chamaecyparis obtusa</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Chamaecyparis obtusa</i>	Stem	Methyl alcohol 99.9%	–	–	–
<i>Punica granatum</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	–	–
<i>Zingiber officinale</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	–	–
<i>Artemisia princeps var. orientalis</i>	Leaf, stem	Methyl alcohol 99.9%	–	–	+
Menthae Herba	N. I.	Ethyl alcohol 95.0%	–	+	–
Cinnamomi Cortex	N. I.	Ethyl alcohol 95.0%	–	–	–
Corni Fructus	N. I.	Ethyl alcohol 95.0%	–	+	–
Houttuyniae Herba	N. I.	Ethyl alcohol 95.0%	–	–	+
<i>Trachelospermi cauilis</i>	N. I.	Distilled water	–	–	–
<i>Pleuropteris cilinervis</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Isodon japonicas</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Sparganium stoloniferum</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Arctium lappa</i>	Leaf, stem	Methyl alcohol 99.9%	–	++	–
<i>Thesium chinense</i>	Whole plant	Methyl alcohol 99.9%	+ ^a	+++ ^b	– ^c
<i>Liriope platyphylla</i>	Root	Methyl alcohol 99.9%	–	++	–
<i>Allium macrostemon</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Aralia continentalis</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	++	+++	–
<i>Lonicera japonica</i>	Flower	Methyl alcohol 99.9%	+	+	–
<i>Dryopteris crassirhizoma</i>	Root	Methyl alcohol 99.9%	–	–	–
<i>Bidens biternata</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Datura metel</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	++	–
<i>Mellilotus alba</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	++	+
<i>Solanum nigrum</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	+	–
<i>Aster ageratoides</i>	Root	Methyl alcohol 99.9%	–	++	–
Phragmitis Rhizoma	N. I.	Ethyl alcohol 95.0%	+	–	–
Pulsatillae Radix	N. I.	Ethyl alcohol 95.0%	–	–	–
Crataegi Fructus	N. I.	Ethyl alcohol 95.0%	–	–	–
Bupleuri Radix	N. I.	Ethyl alcohol 95.0%	–	–	++
Schizandrae Fructus	N. I.	Ethyl alcohol 95.0%	+	++	+
Arctii Fructus (<i>Arctium lappa</i>)	Seed	Ethyl alcohol 95.0%	+++	++	+
Asiasari Radix	N. I.	Ethyl alcohol 95.0%	–	–	+
Asparagi Tuber	N. I.	Ethyl alcohol 95.0%	–	++	–
Forsythiae Fructus	N. I.	Distilled water	–	–	–
Akebiae Caulis	N. I.	Distilled water	–	–	–
<i>Lythrum anceps</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Solanum lyratum</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Dictamnus dasycarpus</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	++	–
<i>Abutilon avicennae</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Cayratia japonica</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	+	–
<i>Indigofera pseudotinctoria</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Patrinia scabiosaeifolia</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Aconitum jaluense</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Aphananthe aspera</i>	Stem	Methyl alcohol 99.9%	–	+	–

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► **Table 1** Continued

Botanical name	Part(s) ^a	Solvent for extraction	Antibacterial activity ^{b, c}		
			<i>S. mutans</i>	<i>S. sanguinis</i>	<i>E. coli</i>
<i>Elsholtzia ciliate</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Hovenia dulcis</i>	Fruit	Methyl alcohol 99.9%	–	–	–
<i>Dracocephalum argunense</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Clinopodium chinense</i> var. <i>parviflorum</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Clinopodium gracile</i> var. <i>multicaule</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Mosla japonica</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Xanthium strumarium</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	+	–
<i>Chenopodium album</i> var. <i>centrorubrum</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	++	–
<i>Elaeagnus umbellata</i>	Leaf	Methyl alcohol 99.9%	–	+	+
<i>Rhus chinensis</i>	Leaf	Methyl alcohol 99.9%	+	++	–
<i>Filipendula formosa</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Tetragonia tetragonoides</i>	Whole plant	Methyl alcohol 99.9%	+	+++	–
<i>Euonymus trapococcus</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Caryopteris incana</i>	Flower	Methyl alcohol 99.9%	++	++	–
<i>Houttuynia cordata</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Isodon excisus</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Picrasma quassioides</i>	Stem	Methyl alcohol 99.9%	–	+	–
<i>Teucrium veronicoides</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Agastache rugosa</i>	Whole plant	Methyl alcohol 99.9%	–	+	+
<i>Eleocharis kuroguwai</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Mollugo verticillata</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Scutellaria indica</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Hepatica maxima</i>	Whole plant	Methyl alcohol 99.9%	+	++	–
<i>Ranunculus sceleratus</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Rhamnus koraiensis</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Pinus densiflora</i>	Root	Methyl alcohol 99.9%	+	++	–
<i>Phellodendron amurense</i>	Leaf, stem	Methyl alcohol 99.9%	–	+++	++
<i>Salix pseudo-lasiogyne</i>	Leaf, stem	Methyl alcohol 99.9%	–	–	–
<i>Spergularia marina</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Momordica charantia</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Luffa cylindrica</i>	Leaf, stem	Methyl alcohol 99.9%	+	+++	–
<i>Hosta capitata</i>	Seed	Methyl alcohol 99.9%	–	–	–
<i>Lamium amplexicaule</i>	Whole plant	Methyl alcohol 99.9%	++	++	–
<i>Perilla frutescens</i> var. <i>acuta</i>	Whole plant	Methyl alcohol 99.9%	–	++	–
<i>Potentilla cryptotaeniae</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Epilobium pyrricholophum</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Prunus armeniaca</i> var. <i>ansu</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Spinacia oleracea</i>	Whole plant	Methyl alcohol 99.9%	–	+	–
<i>Ixeris dentata</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Pinus thunbergii</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Polygonatum odoratum</i> var. <i>pluriflorum</i>	Whole plant	Methyl alcohol 99.9%	–	–	–
<i>Asparagus officinalis</i>	Leaf, stem	Methyl alcohol 99.9%	–	–	–
<i>Symplocarpus renifolius</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	++	++	+
<i>Humulus japonicus</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	+	–
<i>Cinnamomum camphora</i>	Leaf	Methyl alcohol 99.9%	–	–	–

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► Table 1 Continued

Botanical name	Part(s) ^a	Solvent for extraction	Antibacterial activity ^{b, c}		
			<i>S. mutans</i>	<i>S. sanguinis</i>	<i>E. coli</i>
<i>Ficus nipponica</i>	Branch	Methyl alcohol 99.9%	–	–	–
<i>Raphanus sativus var. hortensis for. acanthiformis</i>	Root	Methyl alcohol 99.9%	–	+	–
<i>Prunus persica</i>	Fruit	Methyl alcohol 99.9%	–	–	–
<i>Alnus firma</i>	Branch	Methyl alcohol 99.9%	–	–	–
<i>Eurya japonica</i>	Branch	Methyl alcohol 99.9%	–	–	–
<i>Eurya japonica</i>	Leaf	Methyl alcohol 99.9%	–	++	+
<i>Cryptomeria japonica</i>	Branch	Methyl alcohol 99.9%	+	++	–
<i>Hypochoeris radicata</i>	Leaf, stem, fruit	Methyl alcohol 99.9%	–	+	–
<i>Aucuba japonica</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Viburnum awabuki</i>	Leaf	Methyl alcohol 99.9%	–	–	–
<i>Carex dimorpholepis</i>	Whole plant	Methyl alcohol 99.9%	–	+	–

^a N. I., no information. ^b The number of “+” symbol represents the intensity of antibacterial activity. ^c The “–” symbols indicate that no growth inhibition was detected with the extract treatment

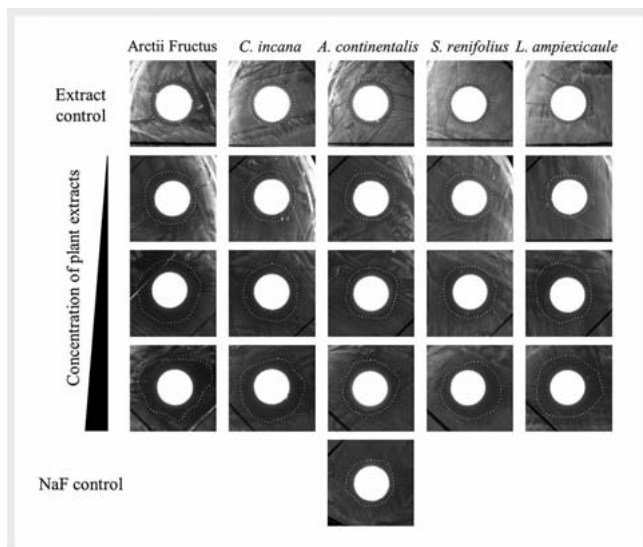
ited the growth of *S. mutans* as well. Additionally, 12 plant species appeared to inhibit the growth of *E. coli*. Through screening, we found that the extracts of five plants (Arctii Fructus [the dried seed of *Arctium lappa*], *Caryopteris incana*, *Aralia continentalis*, *Symplocarpus renifolius*, and *Lamium amplexicaule*) had higher antibacterial activity against *S. mutans* relative to that of other species. Among these extracts, the extract of Arctii Fructus showed the highest antibacterial activity against *S. mutans* (4 mm inhibitory diameter), whereas the others showed smaller inhibition zones (1–1.5 mm in diameter). Of particular note, Arctii Fructus and *A. continentalis* extracts exhibited relatively higher specificity against *S. mutans*.

Fluoride is generally found in various oral healthcare products due to its antimicrobial effects in caries prevention. The American Dental Association recommends < 120 mg fluoride (equivalent to 264 mg sodium fluoride [NaF]) in a single usage [28]. However, given that bodily accumulation of fluoride leads to toxicity, especially in children < 6 years old [29], inclusion of the minimum effective amount of fluoride (0.02–0.10 mg/kg/day) in dental hygiene products is essential for the minimum bodily accumulation [30]. Thus, we hypothesized that a reduction in the fluoride dosage can be compensated by the plant extracts. To assess whether combinations of NaF with the plant extracts could have synergistic effects against *S. mutans*, the antimicrobial activities were evaluated in the presence of combinations of NaF and each extract using a disk diffusion assay (► Fig. 2). The five plant extracts evaluated with different concentrations (5 mg/mL, 15 mg/mL, and 25 mg/mL) showed a concentration-dependent synergistic inhibitory effect with 25 mg/mL NaF. Interestingly, while the inhibitory zone by the plant extracts showed a clear circle, the shape of the inhibitory zone by combinations of NaF and each extract had a comparatively irregular circle. In the presence of NaF alone, the inhibitory zone displayed the same as presented on the plates containing the combinations. Thus, the combinatorial application of

NaF and the selected extracts had a significantly more antibacterial effect than that of NaF or each extract alone.

Minimal bactericidal concentration (MBC) is complementary to minimal inhibitory concentration (MIC). While the MIC test determines the lowest level of antimicrobial agents that significantly inhibit the microbial growth (bacteriostatic activity), the MBC test is intended to determine the lowest level of antimicrobial agents that result in microbial cell death (bactericidal activity) [31]. The MIC test was performed by preparing serial twofold dilutions of each extract up to a 2 µg/mL final concentration in BHI medium, inoculating it with the number of *S. mutans* cells grown to the exponential phase, and incubating the samples at 37 °C in a 5% CO₂ atmosphere for 24 h (► Table 2). The MIC of both Arctii Fructus and *A. continentalis* extracts was found to be 700 µg/mL. The MICs of *S. renifolius* and *L. amplexicaule* extracts were 1800 µg/mL and 1500 µg/mL, respectively. However, the MIC of *C. incana* could not be measured because of high turbidity of the plant extract due to its pigment. Following the MIC test, the MBC was determined by inoculating BHI solid medium with the cultures used in the MIC test (► Table 2). The MBC of Arctii Fructus and *A. continentalis* was 2048 µg/mL. The MBC of *C. incana*, *S. renifolius*, and *L. amplexicaule* extracts was 4096 µg/mL. Surprisingly, *A. continentalis* extract exhibited a lower inhibitory effect than that of Arctii Fructus, but the MIC and MBC values were almost the same in the both extracts.

Biofilm formation is an indispensable characteristic of *S. mutans*, enabling survival and existence of *S. mutans* in the human oral cavity. To evaluate anti-biofilm effects of the extracts on *S. mutans*, biofilm formation was quantitatively measured in BM medium containing each extract (► Fig. 3). Of the 100 plant extracts, 47 extracts that significantly reduced the absorbance of the biofilm (OD₅₉₅ ≤ 0.6; approximately > 80% reduction) relative to negative control (DMSO) were selected. Again, the extracts were classified into two groups as follows: those showing a signifi-



► **Fig. 2** Combinational effect of NaF and the plant extracts against *S. mutans*. The antibacterial effects of mixtures of NaF and each plant extract were evaluated using a disk diffusion assay. A solution of NaF (50 mg/mL) was mixed at a 1 : 1 (v/v) ratio with different concentrations (10 mg/mL, 30 mg/mL, and 50 mg/mL) of each plant extract. The final concentration of the plant extract in the mixture was 5 mg/mL, 15 mg/mL, and 25 mg/mL with 25 mg/mL NaF in each. The dotted lines represent the inhibitory zone formed by the extract control (10 mg/mL), NaF alone or mixture.

cant anti-biofilm effect at all tested concentrations (► **Fig. 3A**) and those whose inhibitory effects were concentration dependent (► **Fig. 3B**). Of the 47 extracts, 24 extracts were able to inhibit biofilm formation at the lowest concentration tested (► **Fig. 3A**). Notably, a significant decrease in biofilm formation was observed when the *S. mutans* cells were subjected to the *L. amplexicaule* extract at a lower concentration than the MIC. Thus, we suggest that the extracts in this group selectively suppress biofilm formation rather than inhibiting bacterial growth. Given that surface adhesion is the most important step in early biofilm formation [32, 33], this group of extracts might suppress the bacterial surface adhesion. On the other hand, the anti-biofilm effects of the 23 extracts of the second group were clearly dependent on the concentrations (► **Fig. 3B**). This inhibitory pattern is presumably related to decreased viability, in addition to surface adhesion, of the bacteria. In fact, the biofilm formation of *S. mutans* was markedly decreased ($p < 0.05$) when grown in BM supplemented with the *S. renifolius* extract at the final concentration of 2500 $\mu\text{g/mL}$, which is lower than its MIC.

GTFs (glucosyltransferase B, C, and D) are encoded by *gtf* genes (*gtfB*, *gtfC*, and *gtfD*) and function in the synthesis of water-insoluble or -soluble glucans in a sucrose-dependent manner [32, 33]. In addition, SpaP (coded by *spaP*), also known as P1 and Pac1, is a cell surface protein that functions in sucrose-independent adhesion of *S. mutans* [34]. We examined whether the expressions of *gtf* and *spaP* genes in *S. mutans*, two genes linked to biofilm formation, were affected by the five plant extracts (► **Fig. 4**). The results revealed that the *gtfB* gene was significantly

► **Table 2** The MIC and MBC values against *S. mutans*.

Botanical name	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
Arctii Fructus	700	2048
<i>C. incana</i>	ND ^a	4096
<i>A. continentalis</i>	700	2048
<i>S. renifolius</i>	1800	4096
<i>L. amplexicaule</i>	1500	4096

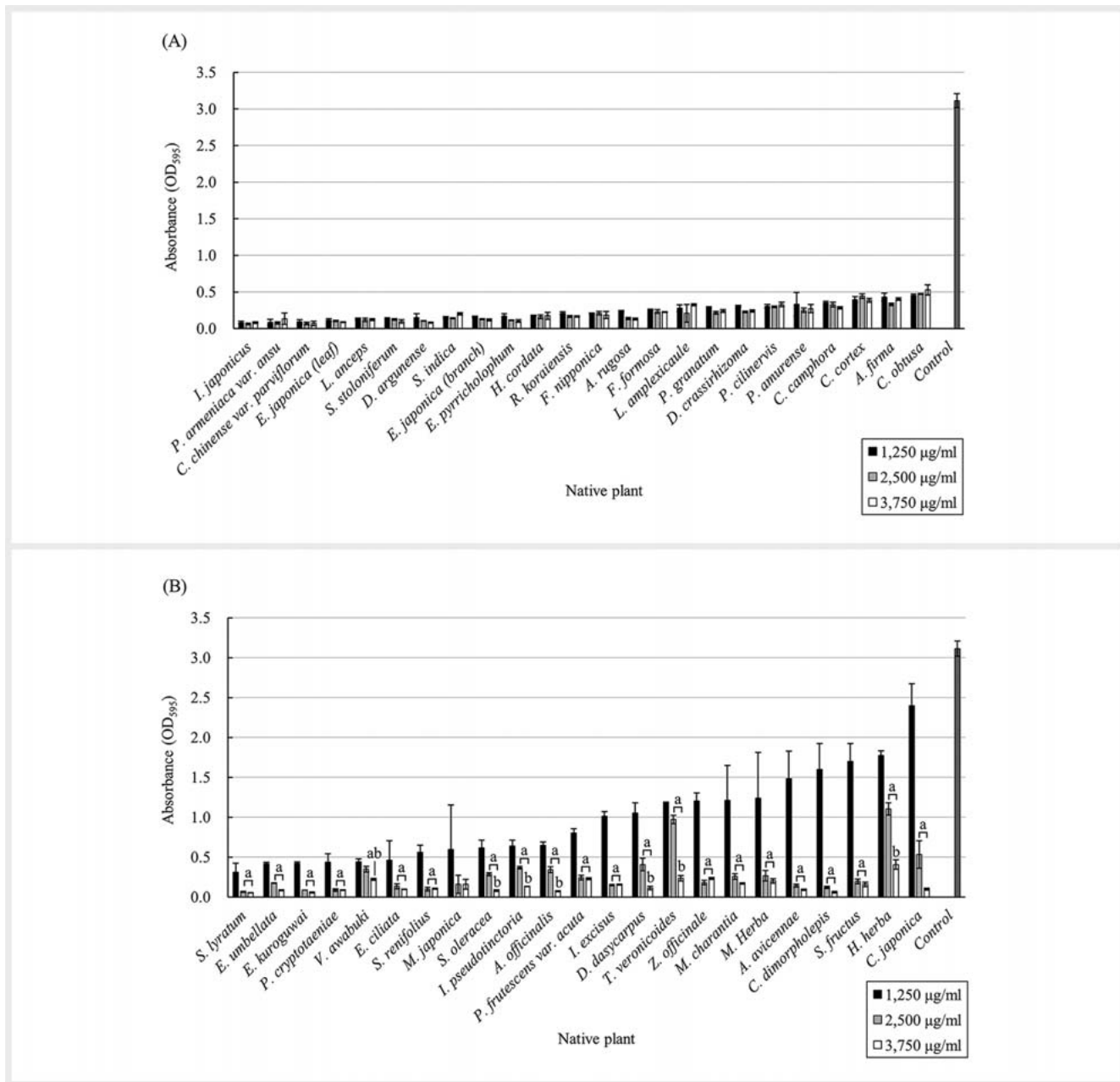
^a Not determined

downregulated by the extract of *S. renifolius* (2.08-fold change, $p < 0.05$), whereas it was slightly upregulated by the extract of Arctii Fructus (1.67-fold change, $p < 0.05$) (► **Fig. 4A**). Consistent with *gtfB* expression, a similar pattern in the mRNA levels of *gtfC* were observed when *S. mutans* was treated with the extracts of Arctii Fructus and *S. renifolius* (1.5-fold changes, respectively; $p < 0.05$) (► **Fig. 4B**). The *gtfD* gene was significantly downregulated by three extracts (*A. continentalis*, *S. renifolius*, and *L. amplexicaule*), whereas the extract of Arctii Fructus and *C. incana* induced no change (► **Fig. 4C**). Again, the *spaP* gene was significantly downregulated upon treatment with the *S. renifolius* extract (2.05-fold change, $p < 0.05$) (► **Fig. 4D**). Taken together, the *S. renifolius* extract inhibited the biofilm formation of *S. mutans* by downregulating major genes involved in glucan synthesis, regardless of sucrose dependency.

Discussion

The prevalence of dental caries is positively correlated with a high consumption of food rich in free sugars. Dental caries is a costly disease, which consumes 5–10% of healthcare budgets in industrialized countries [35]. In order to reduce the economic loss resulting from dental treatments, development of dental hygiene products that are both safe and effective must be taken into consideration. Incomplete understanding of the mechanisms underlying the anti-caries effects of chemically diverse plant extracts constitutes a major barrier in the development of broadly applicable natural anti-caries agents. The purpose of this study was to discover highly active and biocompatible antibacterial plant extracts against the caries pathogen *S. mutans* and to establish whether such plant extracts could be used to compromise the pathogenic potential of *S. mutans* without jeopardizing human health. In fact, the results presented here demonstrate that some plant extracts appear to contain an active ingredient(s) that diminished the virulence and survival of *S. mutans*. It is also notable that the plant extracts and NaF showed synergistic effects when combined, suggesting the potential clinical efficacy of the plant extracts as preventive agents against dental caries.

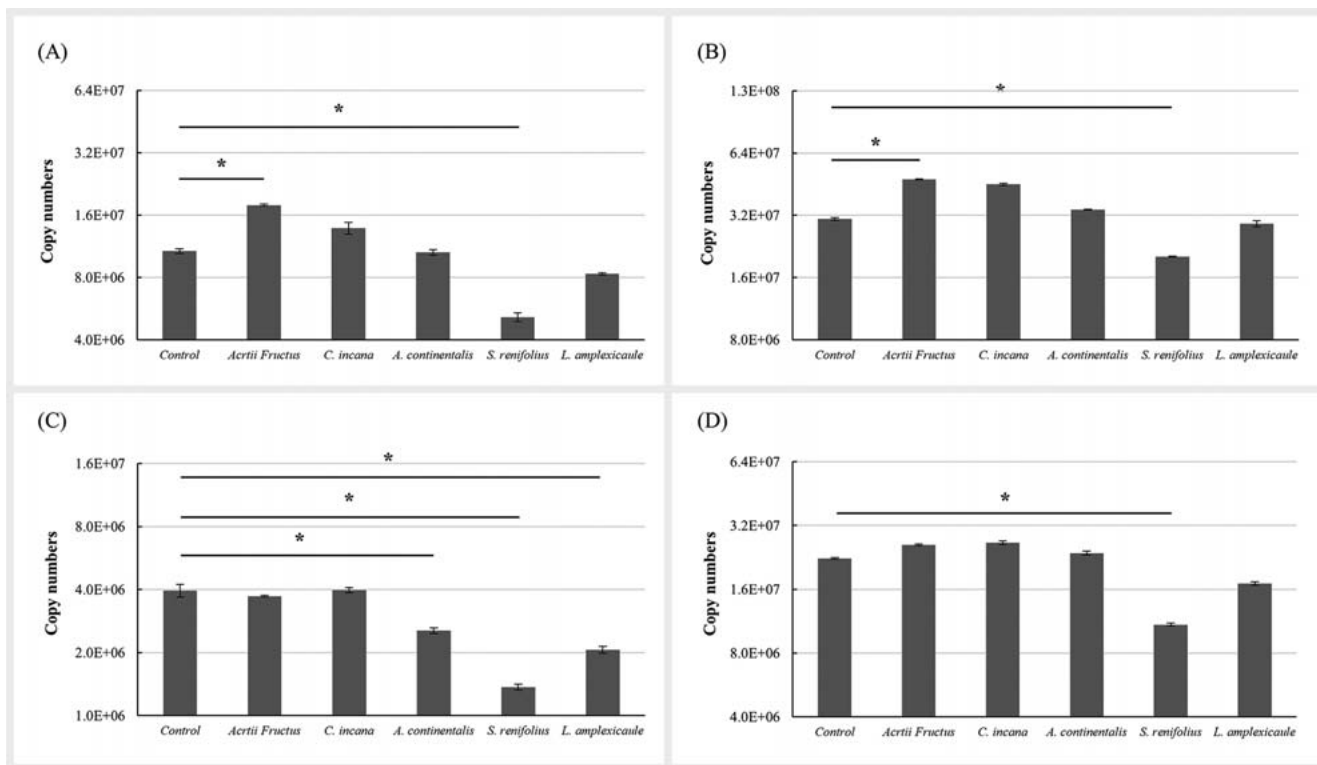
Studies on the efficacy and function of herbal medicine extracts have been progressing to some extent, and there has been scientific evidence of antimicrobial activity against various infectious bacteria, including *S. mutans*. For example, Arctii Fructus has been traditionally known to have anti-inflammatory, antimicrobial, and diuretic effects. *C. incana* is also used to treat various



► **Fig. 3** Anti-biofilm activity of the plant extracts against *S. mutans*. To quantify biofilm formation, the plates were washed twice with PBS, stained with 0.1% crystal violet, and resuspended with an ethanol:acetone (8:2 [v/v]) mixture. The optical density of the stained biofilm was measured at a wavelength of 595 nm. **A** Of the 100 plant extracts, 24 extracts inhibited biofilm formation of *S. mutans* at all concentrations tested. **B** The graph shows that the reduction in biofilm amount is dependent on the concentration of the 23 extracts. Results shown are the mean and standard deviation (error bars) of three separate isolates assayed in triplicate. Statistical significance was determined by one-way ANOVA. The marks “a” and “b” indicate a significant difference ($p < 0.05$) when 1250 µg/mL and 2500 µg/mL were used, respectively. The mark “c” indicates no significant difference in comparison with the control.

diseases, such as eczema, sore throat, arthritis, bronchitis, pertussis, tussis, and bacterial infections [36]. Previous studies by other groups demonstrated that *A. continentalis* inhibits growth, biofilm formation, and adherence of *S. mutans* [37,38]. Of particular note, Jeong et al. indicated that kaurenoic acid isolated from *A. continentalis* extract by phase separation is an active compound that exhibits antibacterial activity against *S. mutans* [37]. In addition,

continentalic acid and caryocanolide extracted from the same plant inhibited growth of methicillin-resistant *Staphylococcus aureus* and *Bacillus subtilis*, respectively [39,40]. It is interesting that *S. renifolius* has been used as a medicine by American Indians as a sedative, diuretic, and antiemetic agent despite its toxicity to humans [41]. However, the results of our cell viability assays indicated that all the extracts from four plants (Arctii Fructus,



► **Fig. 4** The effects of the plant extracts on the expression of *gtf* and *spaP* genes in *S. mutans*. The graphs show the mRNA levels of (A) *gtfB*, (B) *gtfC*, (C) *gtfD*, and (D) *spaP*. The results are the means \pm standard deviations derived from three independent experiments, each analyzed in triplicate. Statistical significance was determined by one-way ANOVA. The asterisk (*) marks those that differ from the negative control at $p < 0.05$ ($n = 3$).

A. continentalis, *S. renifolius*, and *L. amplexicaule*) had no cytotoxicity on human oral keratinocytes (Fig. 1S, Supporting Information). In-depth chemical analyses of these extracts are currently in progress to identify the active compound(s) that interfere with *S. mutans*. The results obtained by far suggest that the antibacterial effect(s) localizes to the hexane layer when the extracts are dissolved in organic solvents.

Interestingly, we observed that the plant extracts and NaF have combinatorial effects on inhibiting the growth of *S. mutans*. A previous study has demonstrated that apigenin and *tt*-farnesol, which are derived from propolis, are able to enhance the protective effects of fluoride against *S. mutans* [27]. Apigenin is a potent inhibitor of water-insoluble glucan synthesis, and *tt*-farnesol increases proton permeability while downregulating acid products in *S. mutans* biofilms. Notably, the authors discussed that these compounds cooperatively work with fluoride to reduce the proton motive force by enhancing proton permeability and discharging ΔpH across the cell membrane [27, 42]. Furthermore, they proposed that, these compounds synergize with fluoride and decrease secretion of GTFs, which in turn decreases extracellular insoluble glucan levels in *S. mutans* biofilms. Although we have no direct evidence that indicates any change in GTF activity or secretion, our finding that the *S. renifolius* extract markedly downregulated the *gtf* and *spaP* genes and decreased biofilm formation may be partially explained as described above. We further suggest that the combinatorial effects could be derived from distinct actions of NaF and plant extracts on *S. mutans* growth inhibition. Indeed,

analysis of RNA-seq data revealed that individual extracts differentially modulated the expression of different regulons that are involved in amino acid biosynthesis, protein translation, and carbon metabolism (unpublished data).

Although antibacterial effects of five extracts were clearly observed, only two of them (*S. renifolius* and *L. amplexicaule*) significantly reduced the biofilm formation of *S. mutans*. Even though the other three extracts also statistically decreased the biofilm amount, they were excluded from the graphs because the satisfaction for either our criteria for absorbance ($OD_{595} = 0.6$) or the concentration-dependent pattern was not met. A previous study on anti-biofilm activity of *A. continentalis* extract showed a positive correlation between a decrease in biofilm formation and decreased levels of water-insoluble glucans produced by GTFase [38]. Moreover, the addition of kaurenic acid from the extract of *A. continentalis* leads not only to inhibitory effects on growth and biofilm formation, but also to the downregulation of genes that contribute to virulence-related traits of *S. mutans* [37]. Although not dramatically, our qRT-PCR results showed the downregulation of the *gtfD* gene in response to the crude extract of *A. continentalis*. Adhesion to the tooth surface is one of the key stages during early biofilm formation of *S. mutans* and is mediated by either a sucrose-dependent or -independent mechanism [32, 33]. GTFs are responsible for the synthesis of water-soluble or -insoluble glucans in the presence of sucrose as the sole carbon source, while SpaP facilitates biofilm formation through the sucrose-independent pathway. As shown in the qRT-PCR results, a

reduction of *gtfD* expression by the addition of either the *A. contortalis* or *L. amplexicaule* extract is apparently presumed to decrease the amount of biofilms produced in a sucrose-dependent manner.

Natural products have been estimated to be a valuable source for developing antibacterial agents against microbial infection. In this study, extracts from 100 Korean native plants were tested to determine whether each could affect virulence properties of the oral pathogen *S. mutans*. In fact, five extracts were identified to exhibit antibacterial potentials against *S. mutans*, and they are ultimately expected to be used in lieu of the synthetic compounds in conventional dental hygiene products. It is possible that the anticariogenic actions of the selected extracts are not identical. The antibacterial mechanisms involving the target genes affected by the extracts is currently under investigation through transcriptomics analysis. Further results from this analysis will provide evidence for a molecular link between the crude extracts and target gene/operon(s), presumably explaining the underlying antibacterial and anticariogenic mechanisms.

Materials and Methods

Collection of plant extracts

One hundred Korean plant extracts known to have medicinal effects, such as for dental caries and toothaches, were selected to evaluate their antibacterial and anti-biofilm activities. The freeze-dried samples that were extracted using a solvent (methanol, ethanol, or distilled-water) were obtained from the Korea plant extract bank (<http://extract.kribb.re.kr>). The plant extracts were dissolved in 99% DMSO (SAMCHUN Chemical) to a final concentration of 50 mg/mL.

Bacterial strains and growth condition

S. mutans UA159, *S. sanguinis* DSS-10 (ATCC 10556), and *E. coli* DH10B were used in this study as the caries-causing bacteria of the normal oral flora alongside a representative strain of gram-negative bacteria. The *E. coli* DH10B and *S. mutans* UA159 strains were obtained from Dr. Robert Burne at the University of Florida as a generous gift. The *S. sanguinis* DSS-10 strain was purchased from the ATCC. *S. mutans* and *S. sanguinis* were maintained in brain heart infusion (BHI) medium (BD Biosciences) at 37°C in a 5% CO₂ atmosphere, and the *E. coli* strain was aerobically cultured in Luria-Bertani (LB) medium (BD Biosciences) at 37°C.

Determination of antibacterial activity

The antibacterial activities of the plant extracts were evaluated on the above strains by disk diffusion assays. Briefly, an overnight culture of each bacterial strain was diluted 1:100 into fresh BHI (for *S. mutans* and *S. sanguinis*) or LB (for *E. coli*) medium and grown to the early exponential phase (optical density at 600 nm [OD₆₀₀] = 0.3) at 37°C in a 5% CO₂ atmosphere. Next, BHI (or LB for the *E. coli* strain) agar plates were spread with 150 µL aliquots of the individual bacterial cultures. Paper disks (8 mm diameter; ADVANTEC) were placed onto the plates at regular distances. The plant extract (20 µL aliquots of 50 mg/mL stock solution) under investigation was added onto the paper disks, and plates were incubated

aerobically (for *E. coli*) or anaerobically (for *S. mutans* and *S. sanguinis*) at 37°C for 24 h. DMSO (99%) was used as the negative control.

Evaluation of the combinatorial effects of the plant extracts and sodium fluoride

The combinatorial effects of the plant extracts and NaF (Junsei Chemical Co., Ltd.) were evaluated by disk diffusion assays as explained above. Briefly, a *S. mutans* strain was grown in BHI medium to the early exponential phase (OD₆₀₀ = 0.3) at 37°C in a 5% CO₂ atmosphere. A 150-µL aliquot of the culture was spread on BHI agar plates, and the paper disks were placed on the plates. NaF stock solution was prepared by dissolving in distilled water to a final concentration of 50 mg/mL. Mixtures of NaF solution and a gradient concentration of the plant extracts (10 mg/mL, 30 mg/mL, and 50 mg/mL) were prepared. A 20-µL aliquot of each mixture was added onto the paper disks. The plates were then incubated anaerobically at 37°C for 24 h. The antibacterial activity of each combination was determined by measuring the diameter of the inhibitory zone around the disks.

Quantitative comparison of biofilm formation

The biofilm formation capacity of *S. mutans* in the presence of the plant extracts was evaluated using crystal violet as previously described [43]. Briefly, an early exponential culture of *S. mutans* grown in BHI was diluted 1:50 in semi-defined biofilm medium (BM) [44] including 20 mM glucose and 5 mM sucrose as the carbohydrate source. Plant extracts (1250 mg/mL, 2500 mg/mL, and 3750 mg/mL) were added into the individual samples in a 96-well polystyrene plate (SPL Life Sciences). Following 24 h incubation under the conditions described above, each well was washed with PBS, and stained with 50 µL of 0.1% (w/v) crystal violet for 15 min at room temperature. The stained cells were washed twice with PBS and then air-dried. The retained dye was eluted from the cells using a 4:1 (v/v) ethanol-acetone solution, and the biofilms were quantified by measuring their absorbance at a wavelength of 595 nm using a model 680 microplate reader (Bio-Rad).

Determination of minimal inhibitory concentration and minimum bactericidal concentration

The five extracts that showed antibacterial activity against *S. mutans* according to the disk diffusion assays were subjected to the modified dilution technique for determining their MICs [45]. Briefly, the plant extracts were diluted twofold in a range from 4096 µg/mL to 2 µg/mL in 96-well plates containing 200 µL of BHI per well. Overnight culture of each strain was 1:100 diluted into fresh BHI (for *S. mutans* and *S. sanguinis*) or LB (for *E. coli*) medium and grown to the early exponential phase (OD₆₀₀ = 0.3) at 37°C. These cultures were 1:100 diluted and placed into each well of the 96-well plates containing the extracts. Afterward, they were cultured at 37°C in a 5% CO₂ atmosphere for 24 h. MIC was considered the lowest concentration at which no growth of *S. mutans* was visually detected. Determination of the MBCs was performed by inoculating 10 µL of the individual samples used in the MIC test. Aliquots (10 µL) of each sample were spotted on BHI agar plates and incubated at 37°C in a 5% CO₂ atmosphere for 24 h. The lowest concentrations of the plant extracts that no bac-

► **Table 3** Oligonucleotide primers used in this study.

Primer name	Sequences (5' to 3')	Purpose
16 s rRNA-FP	TGTCGTGAGATGTTGGGTTAAG	qRT-PCR reaction
16 s rRNA-RP	CCACCTTCCTCCGGTTTATTAC	qRT-PCR reaction
<i>gtfB</i> -FP	GTGTCTTCAACAGATGGTTCTTTC	qRT-PCR reaction
<i>gtfB</i> -RP	CATCGGCTGTCCCGTATTTAT	qRT-PCR reaction
<i>gtfC</i> -FP	GGTTACGTCTTTCCTTGCTTTATT	qRT-PCR reaction
<i>gtfC</i> -RP	GCGGCAGTTTCAGCATTATC	qRT-PCR reaction
<i>gtfD</i> -FP	GCTTTACAGCAACAGCGATAAG	qRT-PCR reaction
<i>gtfD</i> -RP	GAAGTCATAGCCACCAGAAGAA	qRT-PCR reaction
<i>spaP</i> -FP	GAAGCTGCACTCAAGCAATATG	qRT-PCR reaction
<i>spaP</i> -RP	GAGCGAGCTCTGTTTGATAGG	qRT-PCR reaction

terial growth was visible on the plate were determined. Concentrations of the plant extracts were further adjusted for fine-tuning the MICs.

Quantitative real-time PCR

Cells were grown to the late exponential phase ($OD_{600} = 0.7$) in BHI at 37 °C in a 5% CO₂ aerobic atmosphere. The cells were treated with the MIC concentrations of the plant extracts (see Results section) and incubated at 37 °C in a 5% CO₂ atmosphere for 5 min. The cells were harvested and resuspended with 1 mL of RNeasy Protect Bacteria Reagent (Qiagen) and incubated for 10 min at room temperature. They were then resuspended in 10 mM Tris-EDTA buffer (10 mM Tris, and 1 mM EDTA; pH 7.5) and subjected to mechanical lysis in a Bead Beater-16 (BioSpec Products, Inc.). The total RNA was isolated using the RNeasy Mini Kit (Qiagen), and the RNA concentration was determined with NanoDrop 2000 (Thermo Fisher Scientific). Next, cDNA was synthesized from 1 µg of the total RNA using SuperScript IV First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The target specific primers used for the qPCR were designed with PrimerQuest Tool software (Integrated DNA Technologies). Their sequences are listed in ► **Table 3**. qRT-PCR was performed using 2 × qPCR MasterMix (with EvaGreen, high ROX) (Coregen) and the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) as follows: one cycle of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, and 60 °C for 30 s. All the expression data were normalized to 16S rRNA copy number in each sample.

Statistical analysis

All graphical data display the mean values and standard deviations derived from three biological replicates ($n = 3$). All data were analyzed with one-way analysis of variance (ANOVA) using Prism 8 (GraphPad Software), and $p < 0.05$ was considered statistically significant.

Supporting information

Toxicity evaluation of extracts on human oral keratinocytes are available as Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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