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ORIGINAL ARTICLE

Determination of optimal concentration of deglycyrrhizinated licorice root extract for preventing dental caries using a bacterial model system

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Abstract *Background/purpose:* In prior studies, we induced the antimicrobial activity of deglycyrrhizinated licorice root extract (DG-LRE) by inhibiting the growth and biofilm formation of *Streptococcus mutans* UA159. Here, we used clinical strains of mutans streptococci (MS) collected from Koreans to determine the optimal concentration of DG-LRE for oral hygiene products to prevent dental caries.

Materials and methods: Antimicrobial effects of DG-LRE against 14 clinical strains of MS were evaluated through the minimum inhibitory concentration, minimum bactericidal concentration, time-kill assay, and biofilm-forming assay.

Results: Minimum inhibitory concentration and minimum bactericidal concentration values of DG-LRE against the clinical strains of MS ranged from 4 μg/mL to 8 μg/mL and from 8 μg/mL to 16 μg/mL, respectively. Time-kill assay demonstrated that the antimicrobial effects of DG-LRE primarily resulted from bactericidal activity. DG-LRE significantly decreased the biofilm formation of *S. mutans* ranging from 57.6% to 92.8% at 16 μg/mL.

Conclusion: These findings reveal that a DG-LRE concentration of 16 μg/mL may be used to prevent dental caries in Koreans.

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Introduction

Mutans streptococci (MS) have been implicated as a causative agent of dental caries. Inhibition of MS growth and biofilm formation, therefore, remains an intriguing target for the prevention of dental caries.[1,2] MS represents seven species characterized by the 16S ribosomal RNA gene: *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus downei*, *Streptococcus rattus*, *Streptococcus cricetus*, *Streptococcus ferus*, and *Streptococcus macacae*.[3] Of these, *S. mutans* and *S. sobrinus* are commonly found in the human oral cavity and have been implicated as primary agents in human dental caries.[1,4]

Many antimicrobials have been used to prevent dental caries. Chlorhexidine is generally accepted as the gold standard in the dental field due to its clinical efficacy on a wide range of oral microorganisms. Regular use of chlorhexidine as an antimicrobic, however, is limited due to its common side effects, such as the formation of extrinsic tooth and tongue staining.[5,6] As a result, considerable interest exists in developing new antimicrobial agents for the control of dental caries.

Recently, several plants have been examined for their potential in preventing dental caries.[7,8] Licorice (*Glycyrrhiza glabra*, *Glycyrrhiza inflata*, and *Glycyrrhiza uralensis*) is one such plant. Traditionally, licorice root has been used in China and Italy to purportedly lengthen lifespan, improve overall health, cure injury and swelling, and detoxify.[9] Licorice root extract contains significant amounts of glycyrrhizin, a triperpenoid saponin.[10] Glycyrrhizin is converted by human intestinal bacteria to glycyrrhetic acid, which can induce severe hypertension, hypokalemia, and mineralocorticoid excess by preventing renal conversion of cortisol to cortisone.[11] It is therefore important to prepare licorice extract without glycyrrhizin in order to achieve safe use in humans. In a previous study, we prepared roots of *G. uralensis* using a specific resin filter and obtained an extract without detectable glycyrrhizin.[8]

Generally, only certain strains of MS have been used to test the antimicrobial activity of various natural extracts.[8,12] Prior evidence has indicated, however, that antimicrobial activity differs depending on the MS strain studied.[13,14] This suggests that clinically-relevant strains of MS in Koreans need to be investigated to aid in preventing dental caries in Korea. We introduced a bacterial screening system for preventing dental caries in the Korean population with the goal of determining the optimal concentration of anti-caries candidate substances and subsequently developing oral hygiene products.[14] The purpose of this study was to determine the optimal concentrations of deglycyrrhizinated licorice root extract (DG-LRE) in preventing dental caries using the aforementioned bacterial screening system.

Materials and methods

Preparation of deglycyrrhizinated licorice root extract

Licorice root derived from *G. uralensis* was purchased from a herbal drug market (Youngju, Korea). DG-LRE was then prepared as previously described.[8] Briefly, dried roots were cut into thin slices, mixed with distilled water (distilled water:dried root ratio of 20:1 [v/w]) and heated for 2 hours in a round-bottom flask. Distilled water was removed, and 95% ethanol was added to the flask (95% ethanol:residue ratio of 15:1 [v/w]). The mixture was heated at 78°C for 2 hours, and the extract was evaporated. After adding 99% ethanol, the solution was filtered using a custom-made column (6.5 cm × 60 cm) filled with Diaion HP-20 adsorbent (Mitsubishi Chemical Corporation, Minato-ku, Tokyo, Japan). The final DG-LRE was passed through the column, evaporated, and used for subsequent assays. Extracts were suspended in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA) at 50 mg/mL for further assays. Absence of glycyrrhizin was confirmed using high-performance liquid chromatography (data not shown).

Bacterial strains and culture condition

Clinical strains of *S. mutans* (KCOM 1054, KCOM 1111, KCOM 1113, KCOM 1116, KCOM 1126, KCOM 1128, KCOM 1136, KCOM 1197, KCOM 1202, KCOM 1207, and KCOM 1217) as well as *S. sobrinus* (KCOM 1157, KCOM 1196, and KCOM 1221) were obtained from the Korean Collection for Oral Microbiology (KCOM, Gwangju, Korea). All strains were cultured on a Todd Hewitt (TH, Difco, Franklin Lakes, NJ, USA) broth in a 37°C incubator in air containing 5% CO_2.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were calculated using a microdilution assay according to the Clinical and Laboratory Standards Institute.[15] Bacterial strains were cultured in TH broth at 37°C in an incubator for 24 hours and added into a 96-well plate to a final concentration of 1×10^6 CFU/mL. DG-LRE solutions were then added to each well to final concentrations of 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL, 2 μg/mL, or 1 μg/mL. The final DMSO concentration in each well was 1%. Ampicillin 100 μg/mL was used as the positive control and the culture medium of 1% DMSO was used as the double-negative control. After 24 hours of incubation under suitable conditions, the lowest concentration of DG-LRE that inhibited visible growth was considered to be the MIC. To determine the MBC, 10 μL of bacterial culture solution from each well at the MIC was then diluted 100- or 10,000-fold and plated onto a TH agar plate for each bacterial strain. The agar plate was incubated in a 37°C incubator for 48 hours and the bacterial colonies were then counted. The concentration that killed 99.9% of the bacteria was considered the MBC. All experiments were carried out in triplicate.

Time-kill kinetic assay

Time-kill kinetic assay was used to determine the bactericidal or bacteriostatic effects of the same MS strains under varying DG-LRE concentrations. Time-kill curves were assessed at the following DG-LRE concentrations: 32 μg/

mL, 16 μg/mL, 8 μg/mL, and 4 μg/mL. Control curves were obtained in the culture medium for each strain. Bacteria were inoculated in TH broth and incubated overnight in a 37°C incubator. Liquid media containing the aforementioned DG-LRE concentrations were inoculated with 1×10^6 CFU/mL of an overnight culture and incubated in a 37°C incubator. At 0 hours, 3 hours, 6 hours, 9 hours, 12 hours, and 24 hours after inoculation with bacteria, each bacterial culture solution was diluted 100- or 10,000-fold and plated onto a TH agar plate. The agar plate was then incubated at a 37°C for an additional 48 hours before the bacterial colonies were counted.

Biofilm formation assay

Unstimulated whole saliva was collected by the spitting method from healthy volunteers as previously described.[16] Each individual consented to the research protocol that had been reviewed and approved by the institutional review board of Seoul National University Hospital (Seoul, Korea). Each saliva sample was centrifuged at 3500×*g* for 10 minutes to remove any cellular debris and the resulting supernatant was used after filter-sterilization through a Stericup and Steritop (Millipore, Billerica, MA, USA).

To assess biofilm formation, each strain was grown in a semi-defined biofilm medium with 18 mM glucose and 2 mM sucrose as carbohydrate sources. Biofilm assays were done using polystyrene 96-well (flat-bottom) cell culture clusters (Costar 3595; Corning Inc, NY, USA). An overnight culture of each strain was transferred to pre-warmed brain heart infusion (BHI, Difco) broth and grown at 37°C in a 5% CO_2, aerobic atmosphere to the mid exponential phase (OD_{600} = 0.5). The cultures were then diluted 100-fold in pre-warmed biofilm medium containing DG-LRE at various concentrations. A cell suspension containing only 1% DMSO was used as the negative control.

Biofilm formation assays were performed with saliva-coating. Each well was conditioned with 100 μL of unstimulated whole saliva. The plates were incubated at 37°C for 2 hours with gentle shaking and then washed three times with phosphate-buffered saline (pH = 7.2). Immediately after air drying for 30 minutes, the wells were inoculated with 200 μL of the cell suspensions containing various concentrations of DG-LRE (16 μg/mL, 8 μg/mL, 4 μg/mL, or 2 μg/mL). After inoculation, all plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The culture medium was then decanted and the plates were washed twice with 200 μL of sterile distilled water to remove planktonic and loosely-bound cells. Adherent bacteria were stained with 50 μL of 0.1% crystal violet for 15 minutes. After rinsing twice with 200 μL of water, the bound dye was extracted from the stained cells using 200 μL of 99% ethanol. Biofilm formation was then quantified by measuring the absorbance of the solution at 600 nm using a spectrophotometer (Helios Beta, Thermo Scientific, Madison, WI, USA). The relative biofilm formation was determined by the percentage rate using the following equation: relative biofilm formation (biofilm formation rate, %) = (biofilm formation with 16 μg/mL, 8 μg/mL, 4 μg/mL, or 2 μg/mL of DG-LRE/biofilm formation without DG-LRE) × 100. All experiments were carried out in triplicate.

Results

The MIC and MBC values of DG-LRE against the clinical strains of MS ranged from 4 μg/mL to 8 μg/mL, and from 8 μg/mL to 16 μg/mL, respectively (Table 1). MBC values against *S. mutans* and *S. sobrinus* were one to four times higher than the MIC values. Antimicrobial effects of DG-LRE increased in a concentration- and treatment time-dependent manner (Fig. 1). Data from the time-kill kinetic assay revealed that a DG-LRE concentration of 8 μg/mL had bactericidal effects on all MS strains except *S. mutans* KCOM 1128 and *S. mutans* KCOM 1136. In the case of *S. mutans* KCOM 1128 and *S. mutans* KCOM 1136, bactericidal effects were shown at a DG-LRE concentration of above 16 μg/mL.

The biofilm-inhibitory effects of DG-LRE were investigated in the presence of saliva coating in order to simulate the clinical situation. DG-LRE inhibited biofilm formation above 50% in seven strains of *S. mutans* (KCOM 1113, KCOM 1126, KCOM 1128, KCOM 1136, KCOM 1202, KCOM 1207, and KCOM 1217) and three strains of *S. sobrinus* (KCOM 1157, KCOM 1196, and KCOM 1221) at 8 μg/mL of DG-LRE. DG-LRE significantly decreased biofilm formation of MS ranging from 57.6% to 92.8% at 16 μg/mL (Fig. 2).

Discussion

The results showed that DG-LRE concentration of 16 μg/mL had bactericidal effect against MS strains used in this study (Table 1 and Fig. 1). In our previous study, the antimicrobial activity of DG-LRE was evaluated against *S. mutans* UA159, where we found that both the MIC and MBC values of DG-LRE were 8 g/mL.[8] This reflects that some MS strains isolated from the Korean population could be more resistant than the reference MS strain isolated from the Western population. Our prior work tested the antimicrobial efficiencies of methanol-extracts from three plants against

Table 1 MIC values of deglycyrrhizinated licorice root extract against clinical strains of *Streptococcus mutans* and *Streptococcus sobrinus* isolated from a Korean population.

Species and strains	MIC (μg/mL)	MBC (μg/mL)
S. mutans KCOM 1054	4	8
S. mutans KCOM 1111	4	8
S. mutans KCOM 1113	4	8
S. mutans KCOM 1116	8	8
S. mutans KCOM 1126	8	8
S. mutans KCOM 1128	4	16
S. mutans KCOM 1136	4	16
S. mutans KCOM 1197	4	8
S. mutans KCOM 1202	4	8
S. mutans KCOM 1207	8	8
S. mutans KCOM 1217	8	8
S. sobrinus KCOM 1157	4	8
S. sobrinus KCOM 1196	4	8
S. sobrinus KCOM 1221	4	8

MBC = minimum bactericidal concentration; MIC = minimum inhibitory concentration.

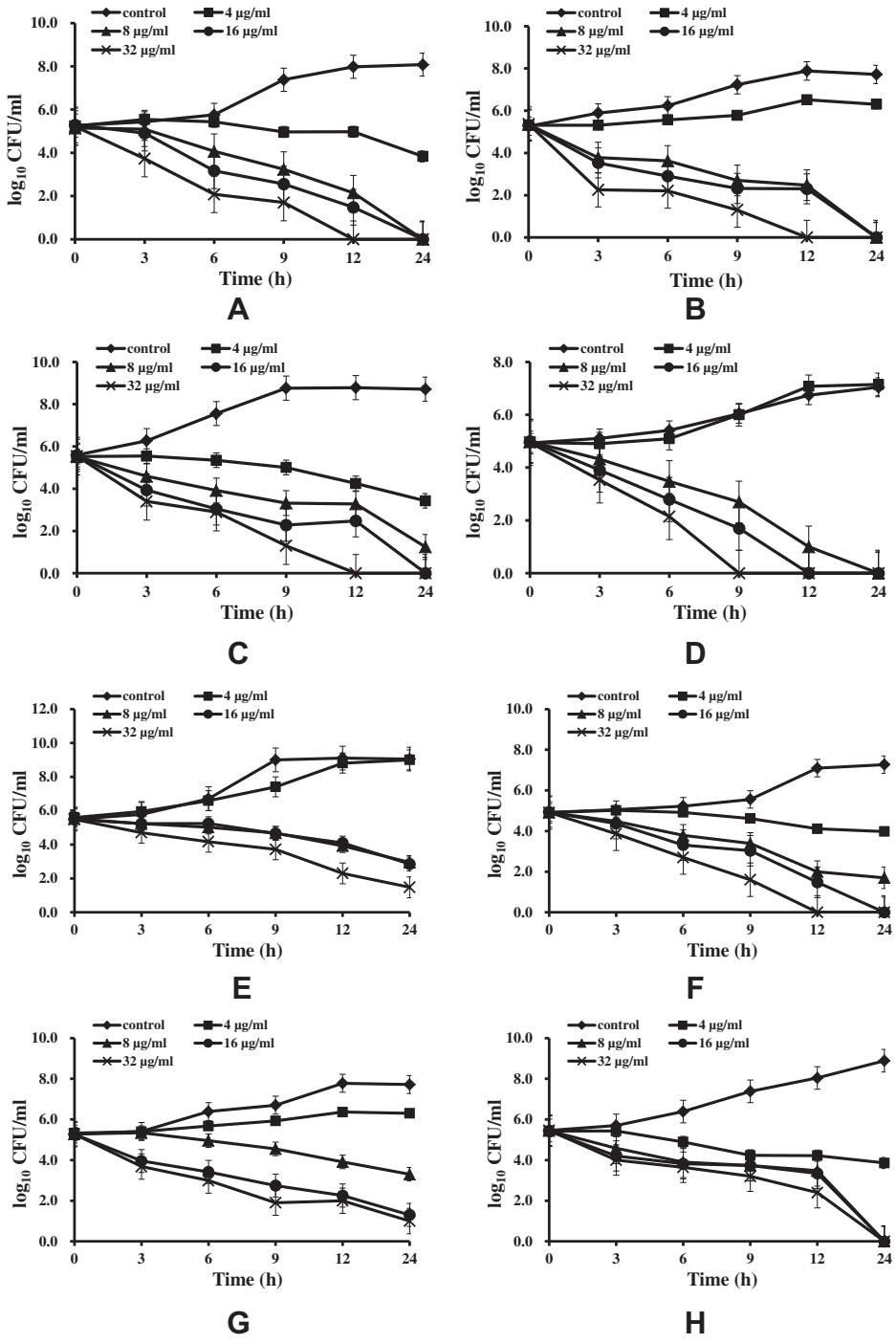

Figure 1 Time-kill curves of varying concentrations of deglycyrrhizinated licorice root extract against: (A) *Streptococcus mutans* KCOM 1054; (B) *S. mutans* KCOM 1111; (C) *S. mutans* KCOM 1113; (D) *S. mutans* KCOM 1116; (E) *S. mutans* KCOM 1126; (F) *S. mutans* KCOM 1128; (G) *S. mutans* KCOM 1136; (H) *S. mutans* KCOM 1197; (I) *S. mutans* KCOM 1202; (J) *S. mutans* KCOM 1207; (K) *S. mutans* KCOM 1217; (L) *Streptococcus sobrinus* KCOM 1157; (M) *S. sobrinus* KCOM 1196; and (N) *S. sobrinus* KCOM 1221.

type strains (*S. mutans* ATCC 25175^T and *S. sobrinus* ATCC 33478^T) as well as 55 clinical strains (40 strains of *S. mutans* and 15 strains of *S. sobrinus*). Interestingly, 11 clinical strains of *S. mutans* and three clinical strains of *S. sobrinus* had MBC_{90} values greater than two of three natural extracts.[14] We selected those 14 clinical strains for the bacterial model mentioned earlier. In addition, the detection percentages of *S. mutans* and *S. sobrinus* in a Korean sample ($n = 88$) were 88% and 20%, respectively.[17] This may help to explain the MS strain imbalance for the bacterial model. We previously quantified the optimal concentrations of three essential oils and sophoraflavanone G through this bacterial model.[18,19] These results—coupled with the data presented here—suggest that the bacteria model system could be useful in determining an optimal concentration of anti-caries agents in the development of oral

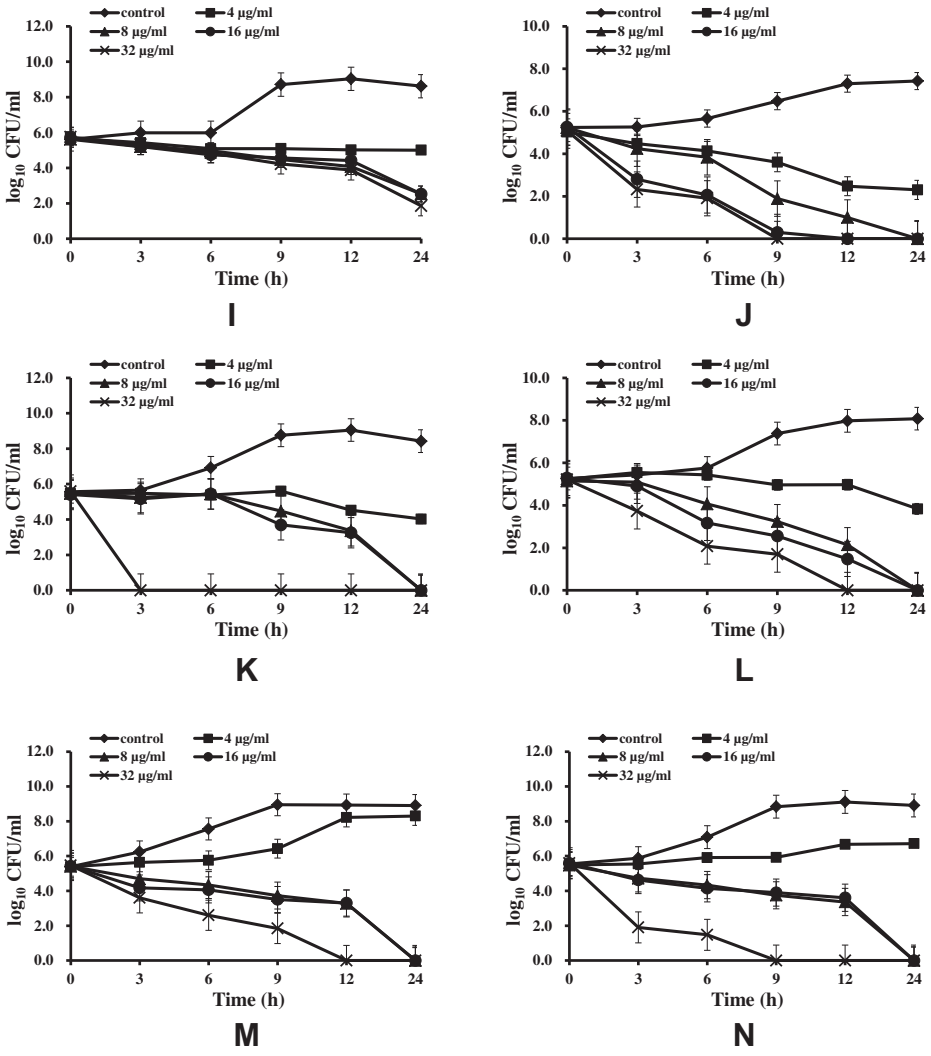

Figure 1 (*continued*).

hygiene products targeted specifically for the Korean population.

The reduced biofilm formation may be mainly due to the growth impairment of MS in the presence of DG-LFE, because the MIC values of DG-LRE against the clinical strains of MS ranged from 4 μg/mL to 8 μg/mL (Table 1). The DG-LRE concentrations that inhibited the biofilm formation of the MS, however, were higher than the MIC values. The different inhibitory concentrations of DG-LRE between biofilm formation and MIC might be due to the glucan synthesis capacities of MS. In the presence of sucrose, production of, and binding to, glucans effectively promotes the vigorous biofilm formation of MS and the bulk glucan layers constitute the biofilm's matrix.[16] In addition, saliva coating enhances the glucan synthetic activities of MS by facilitating the aggregation of glucosyltransferases by salivary components.[20] The polysaccharide biofilm matrices may not only prevent DG-LRE from direct contact with bacteria but also interfere in the diffusion of DG-LRE into the matrices, which may reduce the antimicrobial effects of DG-LRE against MS at a lower concentration of DG-LRE.

According to our previous data, the cell viability of normal human gingival fibroblast cells for DG-LRE concentrations at 8 μg/mL and 16 μg/mL were 80% and 67%, respectively.[8] A classification of cytotoxicity has been previously proposed; extracts were rated as severely, moderately, or slightly cytotoxic when the activity relative to controls was less than 30%, between 30% and 60%, or greater than 60%, respectively.[21] Considering the classification, the concentration at 16 μg/mL of DG-LRE is slightly cytotoxic to normal human gingival fibroblast cells. Generally, the cell viability test for natural extracts on human cells is performed *in vitro*. Human oral mucosa *in vivo* may be more resistant to the natural extracts than those *in vitro*. Such differences might be because oral tissue cells *in vivo* can continuously be supplied nutrients via blood supply, thereby enhancing repair mechanisms as compared cells *in vitro*.[13] DG-LRE concentrations of 16 μg/mL *in vivo* might therefore be useful in the prevention of dental caries. This, however, has to be proved by animal and/or clinical studies.

In summary, the results in this paper suggest that DG-LRE concentrations of 16 μg/mL could be useful in the

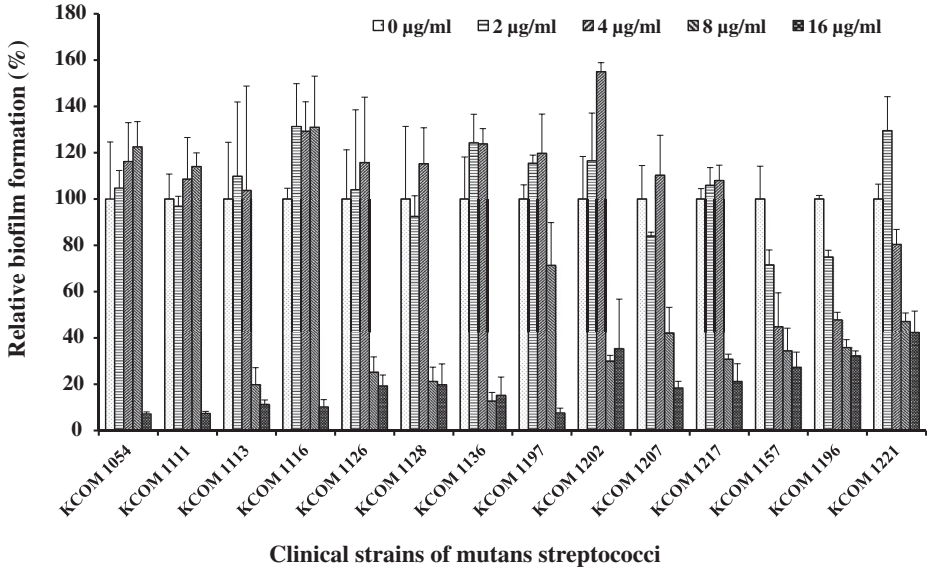

Figure 2 Inhibitory effects of deglycyrrhizinated licorice root (DG-LRE) extract on the biofilm formation of clinical strains of *Streptococcus mutans* (KCOM 1111, KCOM 1113, KCOM 1116, KCOM 1126, KCOM 1128, KCOM 1136, KCOM 1197, KCOM 1202, KCOM 1207, and KCOM 1217) and *Streptococcus sobrinus* (KCOM 1157, KCOM 1196, and KCOM 1221). The relative biofilm formation was determined by the percentage rate using the following equation: relative biofilm formation (biofilm formation rate, %) = (biofilm formation with each various concentration of DG-LRE/biofilm formation without DG-LRE) × 100.

development of oral hygiene products for the prevention of dental caries in the Korean population.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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