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RESEARCH NOTE

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Anti-biofilm Activity of Xanthorrhizol Isolated from *Curcuma xanthorrhiza* Roxb. against Bacterial Biofilms Formed by Saliva and Artificial Multi-species Oral Strains

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Abstract Xanthorrhizol, a sesquiterpene isolated from *Curcuma xanthorrhiza* Roxb., was used to investigate its effect on reducing the saliva and multi-species oral biofilms consisting of *Streptococcus mutans*, *Streptococcus sanguis*, and *Actinomyces viscosus* by anti-biofilm and confocal laser scanning microscopy (CLSM) assays. Xanthorrhizol exhibited significant anti-biofilm activity in the dose- and time-dependent manners. Exposure to 2 and 5 μg/mL xanthorrhizol for 30 min remained <50% of saliva and multi-species biofilms formed for 24 hr. In addition, exposure to 10 μg/mL xanthorrhizol for 30 min reduced 65 and 77% of 24 hr saliva and multi-species oral biofilms, respectively. CLSM results visually demonstrated that xanthorrhizol reduced bacterial viability in the saliva and multi-species oral biofilms. These results suggest that *C. xanthorrhiza* Roxb. containing xanthorrhizol with strong anti-biofilm activity can be employed as a plant source for oral care functional foods.

Keywords: anti-biofilm activity, *Curcuma xanthorrhiza* Roxb., xanthorrhizol, oral biofilm

Introduction

Oral risk factors include the increased numbers of decayed teeth, the presence of decay-causing organisms, and the presence of periodontal disease-associated dental plaque organisms. Dental plaque is a multi-species biofilm consisting of more than 700 species of oral bacteria on tooth surfaces, and mutans streptococci such as *Streptococcus mutans, Streptococcus sanguis*, and *Streptococcus sobrinus* are the major group of early primary colonizers (1). Mutans streptococci act as primary initiators of tooth decay upon carbohydrate exposure in the formation of oral biofilm (2,3). An effective approach to control dental plaque is to remove the existing biofilm from the system by using anti-biofilm agents. Most studies on oral biofilms have been conducted using single-species oral biofilms rather than multi-species of dental plaques. In *in vitro* biofilm experimental models, the efficacy of an anti-biofilm agent is dependent on its capability to desorb either single- or multi-species biofilms from the test surface.

A majority of oral biofilm researches have been focused on looking for natural anti-biofilm agents for the treatment of plaque-related diseases such as caries or periodontitis. However, only few studies have reported for the susceptibility of single- and multi-species oral biofilms to natural anti-biofilm agents (4-6). Xanthorrhizol, a sesquiterpene compound isolated from an edible *Curcuma xanthorrhiza* Roxb., has shown strong anti-cariogenic effect on several oral pathogens and anti-biofilm activity against single *S. mutans in vitro* (5,7,8). Based on these previous observations, we tested anti-biofilm activity of xanthorrhizol on reducing the existing saliva and artificial multi-species oral biofilms *in vitro*.

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Materials and Methods

Microorganisms and growth conditions The oral microorganisms used in this study were *S. mutans* ATCC 25175, *S. sanguis* ATCC 10556 (American Type Culture Collection, Rockville, MD, USA), and *Actinomyces viscosus* KCCM 12074 (Korean Culture Center of Microorganisms, Seoul, Korea). All strains were cultured in brain heart infusion (BHI; Difco, Detroit, MI, USA) broth or BHI supplemented with 1.5% of bacteriological agar (BHIA) at 37°C for 24 hr aerobically.

Preparation of samples and artificial saliva Xanthorrhizol (Fig. 1) was isolated and purified (purity>98%) from the ethyl acetate fraction of *C. xanthorrhiza* Roxb. according to the method of Hwang *et al.* (8). Xanthorrhizol was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted to the final concentrations of 1, 5, 10, 25, and 50 μg/mL using 10% DMSO. A commercial antimicrobial agent, chlorhexidine digluconate (1,1'-hexamethylenebis[5-*p*-chlorphenyl]biguanide) (#9394; Sigma-Aldrich, St. Louis, MO, USA), was diluted to the same concentration with xanthorrhizol in sterile distilled water. The artificial saliva, 1%(w/v) mucin from porcine stomach type III (Sigma-Aldrich), was diluted with adherence buffer (10 mM KPO_4, 50 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 7.0) and autoclaved at 121°C for 15 min.

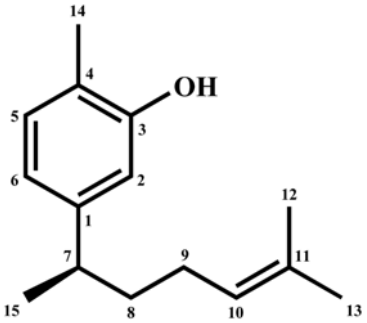

Fig. 1. Structure of xanthorrhizol.

Preparation of human saliva and multi-species oral bacterial inocula For human saliva inoculum cell, whole saliva was stimulated for collection by chewing parafilm. Donors abstained from oral hygiene for 24 hr prior to saliva collection. The saliva was diluted 1:2 in 50 mM phosphate-buffered saline (PBS) pH 7.2, and centrifuged at 2,000×g for 10 min. The supernatant was then filtered using a 0.22-μm sterilized Millex-GP syringe filter (Millipore, Billerica, MA, USA) and stored at 4ºC. The saliva inoculum gave a final concentration of 1×10^7 colony forming unit (CFU)/mL.

For multi-species bacterial inoculum, all bacterial cells were cultured at 37ºC for 24 hr in BHI broth with shaking agitation of 200 rpm. Each 1 mL culture was centrifuged at 2,000×g and 4ºC for 5 min, followed by resuspending the pellet twice with 1 mL of 50 mM PBS pH 7.2 as a washing step to remove debris-free cells. The pellets of each strain were resuspended in 1 mL of adherence buffer to give a final concentration of 1×10^6 CFU/mL. Multi-species inoculum cells contained a mixture *S. mutans*, *S. sanguis*, and *A. viscosus* inocula with similar ratio and approximate cell density of 1×10^6 CFU/mL. A standard curve of turbidity against CFU was used to obtain the number of cells.

***In vitro* oral biofilm formation** Oral biofilms were prepared in the presterilized, polystyrene, flatbottom 96-well tissue culture microtiter plate (SPL Plastic Labware, Pocheon, Korea) by the method of Stepanovic *et al.* (9) with slight modification. Briefly, the plate was conditioned with 200 μL mucin, incubated and shaken gently at room temperature for 3 hr. Then, excess mucin was removed and the plate was air-dried for overnight. Oral biofilms were initially grown using BHI broth supplemented with 3%(w/v) sucrose (BHIS). For testing the positive control (chlorhexidine gluconate) and treatment (xanthorrhizol), 20 μL inocula with approximate cell density of 1×10^6 CFU/mL (for multi-species inoculum) or 1×10^7 CFU/mL (for saliva inoculum) was added to each well with 180 μL BHIS media to give 10-fold dilution. Thus, the final cell density of each culture was calculated about 1×10^5 CFU/mL (for multi-species inoculum) or 1×10^6 CFU/mL (for saliva inoculum). For the negative control (untreated), the steps were taken only using BHIS broth (200 μL) without xanthorrhizol and inoculum cell addition. The plate was incubated at 37ºC for 24 hr.

Biofilm treatment and quantification Oral biofilms were treated with 50 μL xanthorrhizol (1, 5, 10, 25, and 50 μg/mL as prepared above), resulting in the final concentrations of 0.2, 1, 2, 5, and 10 μg/mL, respectively. The xanthorrhizol exposure times were 5, 10, 15, and 30 min. Biofilm cell was quantified by using a crystal violet assay as described by Djordjevic *et al.* (10). After biofilm formation and sample treatment, the plate was gently shaken to remove all unbound bacteria. The remaining adherent bacteria were washed twice with 200 μL of 50 mM PBS (pH 7.2) and air-dried for 1 hr. Each well was stained with 110 μL of 0.4%(v/v) crystal violet solution for 1 hr, washed twice using 300 μL sterile distilled water, and immediately destained with 200 μL ethanol absolute. Destaining solution (100 μL) was transferred to the new well and the amount of remaining crystal violet stain in the destaining solution was measured at 596 nm with a tunable microplate reader (Versa Max, Sunnyvale, CA, USA). Xanthorrhizol activity was defined as the percentage of the absorbance of biofilm remaining after xanthorrhizol treatment in comparison with the untreated control. All experiments were performed in triplicates with 2 repeats.

Confocal laser scanning microscopy (CLSM) Effect of xanthorrhizol on removing saliva and artificial multi-species oral biofilms was also visualized through CLSM by the modified method of Zaura-Arite *et al.* (11). After exposure with xanthorrhizol and chlorhexidine gluconate at 2 and 10 μg/mL for 30 min at 37ºC, the biofilm cells were washed gently 3 times with 25 mM PBS (pH 7.2). To differentiate the live bacterial organisms in the biofilm from the dead ones, the biofilm was visualized by fluorescent staining with the Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR, USA). The biofilm on the cover glass was stained with the mixtures of 1.5 μL of 3.34 mM SYTO-9 for the live organisms and 1.5 μL of 20 mM propidium iodide (PI) for the dead organisms in a total volume of 1 mL of 50 mM PBS (pH 7.2), followed by incubation the biofilm at dark room temperature for 30 min. The live organisms, freshly cultured and subsequently harvested, were used for control staining. After staining treatment, a drop of mounting oil was added to the slide glass, followed by covering the biofilm cell inside the cover glass. All confocal images were visualized using confocal laser scanning microscope (Bio-Rad, Hertfordshire, UK). At least 2 random fields were analysed in triplicate experiments.

Statistical analysis Data were expressed as mean (n= 3×2) and standard deviation (SD) by computational analysis. Statistical comparisons of untreated-, xanthorrhizol-treated and chlorhexidine gluconate-treated saliva and multi-species oral biofilms were determined by analysis of variance (SPSS 11.0 for Windows). The level of significance was taken at $p<0.05$.

Results and Discussion

Effects of xanthorrhizol on saliva and multi-species oral biofilms Saliva and multi-species oral biofilms at 24 hr showed similar trends on removing the amount of biofilm cells after treatment with xanthorrhizol and chlorhexidine gluconate at various concentrations and time exposures

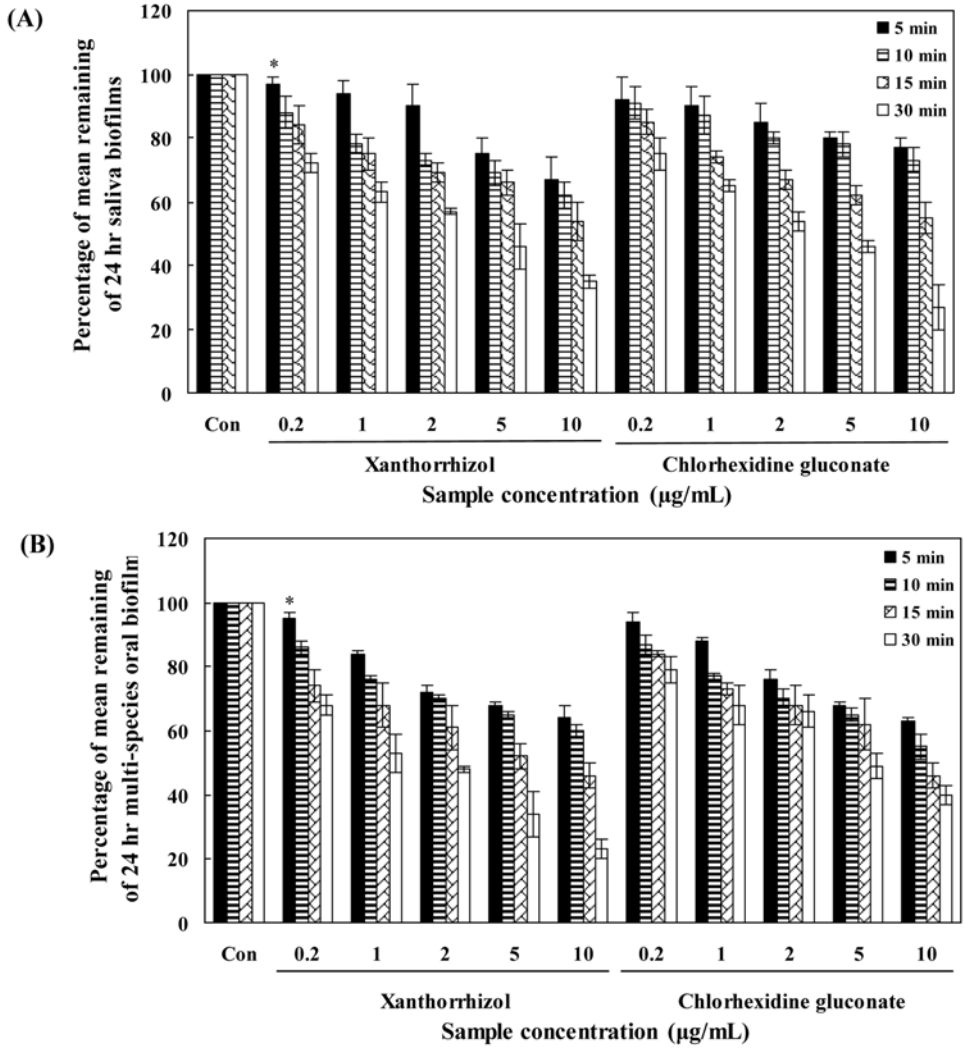

Fig. 2. Effects of xanthorrhizol at different concentrations and exposure times on saliva (A) and multi-species oral biofilms (B). Chlorhexidine gluconate was used as a positive control. Values are expressed as the mean percentage of absorbance (596 nm) of cells in treated wells compared with that in untreated control wells (considered to be 100%). Error bar is a standard deviation of the mean percentage of absorbance (596 nm) of cells derived from 3 times of experiments and 2 replicates/experiment. *Not significantly different from the control treatment. All entries in the figure are significantly different ($p<0.05$) from the controls unless otherwise indicated.

(Fig. 2). Exposure to 2 and 5 μg/mL xanthorrhizol for 30 min remained <50% of saliva and multi-species oral biofilms formed for 24 hr ($p<0.05$). It was observed that exposure to 10 μg/mL xanthorrhizol for 30 min reduced 65 and 77% of 24 hr saliva and multi-species oral biofilms, respectively ($p<0.05$). Xanthorrhizol performed anti-biofilm activity against bacterial biofilms in saliva and artificial multi-species strains in the dose- and time-dependent manners. It was also noted that xanthorrhizol was more active to reduce multi-species biofilm cell compared to that of saliva biofilms. Moreover, its activity was almost comparable with chlorhexidine gluconate.

CLSM images The effects of xanthorrhizol and chlorhexidine gluconate on reducing the viability of 24 hr saliva and multi-species oral biofilm cells were also visualized by CLSM analysis. The results demonstrated that xanthorrhizol and chlorhexidine gluconate reduced bacterial viability in a dose-dependent manner (Fig. 3). As compared with untreated control, exposure to xanthorrhizol at 2 and 10 μg/mL for 30 min did not remove biofilm cells completely, as indicated by yellow color of stained cells. However, when exposed to 1,000 μg/mL xanthorrhizol, all biofilm cells were completely killed as revealed by red color (data not shown). The results indicate that xanthorrhizol provides anti-biofilm properties by eradicating microbial viability in both saliva and multi-species oral biofilms.

Human oral biofilms consist of major oral strains that are common to saliva and plaque, such as *Streptococcus*, *Actinomyces*, *Fusobacterium*, *Prevotella*, and *Veillonella* species (12). To control human oral biofilms, several approaches based on various underlying rationales have been used, such as preventing biofilm formation, disrupting existing biofilms, preventing further biofilm growth, and killing microorganisms in the biofilm (13). The use of anti-biofilm agents with dual functionalities that not only reduce the viability, but also control the colonialization and

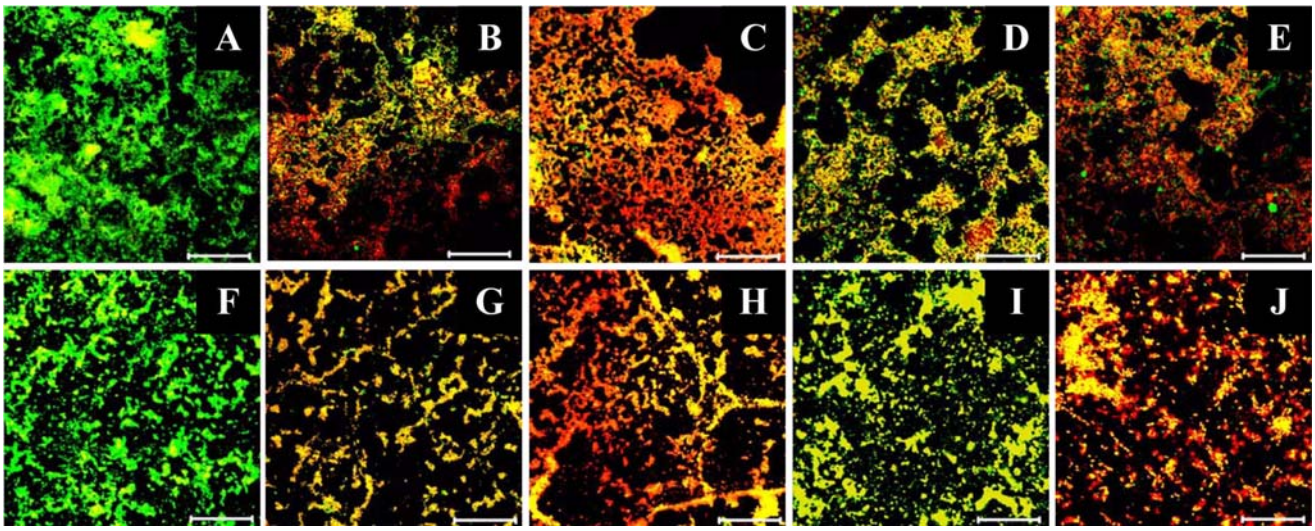

Fig. 3. Confocal laser scanning microscopy (CLSM) images of saliva (A-E) and multi-species oral biofilms (F-J) after treatment with xanthorrhizol and chlorhexidine gluconate. The experiments were repeated 3 times with at least 2 random fields for each experiment. A, untreated control; B-C, xanthorrhizol 2 and 10 μg/mL; D-E, chlorhexidine gluconate 2 and 10 μg/mL; F, untreated control; G-H, xanthorrhizol 2 and 10 μg/mL; I-J, chlorhexidine gluconate 2 and 10 μg/mL. Magnification of 400×. Bars represent 50 μm.

accumulation of oral pathogen bacteria on the tooth surface could be more effective. For decade, chlorhexidine gluconate has been used for dental plaque therapy since it strongly binds to oral surfaces, thereby prolonging its effectiveness in comparison with other antimicrobial agents (14). However, the routinely using of chlorhexidine gluconate at high concentration is particularly unsafe and sensitive against mutans streptococci (15,16). Recent trend on natural antimicrobial compounds are increasingly demanding novel anti-biofilm agents. Macelignan isolated from *Myristica fragrans* Houtt., xanthorrhizol isolated from *Curcuma xanthorrhiza* Roxb., salvipisone diterpenoids isolated from *Salvia sclarea* L., and *Nidus vespae* extract also demonstrated their anti-biofilm activities to reduce the existing single oral biofilms (5,6,17-19). However, only few studies employed natural anti-biofilm agents for the susceptibility of multi-species oral biofilms.

In this research, we employed xanthorrhizol on removing of bacterial biofilms in saliva and artificial multi-species oral strains. Our results demonstrated that xanthorrhizol possessed stronger anti-biofilm activity against artificial multi-species oral biofilms compared to that of natural saliva biofilms. Saliva has several major functions in relation to oral biofilm, i.e., medium for transporting planktonic bacteria within and between mouths, prevent reattachment to surfaces, form conditioning films on all oral surfaces, etc (20). Bacterial biofilms in natural saliva are more complex and difficult to remove compared to that of artificial multi-species biofilms consisting of 3 oral primary strains (*S. mutans, S. sanguis*, and *A. viscosus*). Furthermore, exposure time and sample concentration significantly affected the ability of xanthorrhizol to reduce and disrupt the existing biofilms. CLSM data also showed the different appearances for both saliva and multi-species oral biofilms formed at 24 hr. It appeared that saliva biofilms had more rigid, thick, and complex biofilm structures compared to that of artificial multi-species oral biofilms. The ability of xanthorrhizol on attacking and disrupting the biofilms is quantitatively affected by these factors, however, in this experiment, we only analyzed the xanthorrhizol effect qualitatively against the biofilms.

It is noteworthy that anti-biofilm properties against single- and multi-species biofilms do not represent similar pattern. In general, multi-species biofilms were more resistant and difficult to reduce in comparison to single-species biofilms. Dual-species biofilms of *S. mutans* and *Veillonella parvula* were reported to be more resistant to 0.1 and 0.4 mg/mL chlorhexidine gluconate than single-species biofilms of either strains (21). Exposure to 0.2% chlorhexidine gluconate for 1 or 5 min had no effect on multi-species oral biofilms, but a 60 min exposure resulted in bactericidal effect of biofilm colonizers (22). In other reports, exposure of multi-species biofilms to 0.07 mM triclosan for 1 hr reduced approximately 40% of the oral bacteria present (23) and 2.23 mM chlorhexidine gluconate removed approximately 40% of multi-species oral biofilms (24). The efficacy of an anti-biofilm agent is dependent on its capability to detach the biofilm cells from the test surface. However, simple comparisons are difficult to do because of differences in the biofilm compositions, the anti-biofilm agents and their concentrations used.

In summary, our findings suggest that xanthorrhizol significantly reduced both saliva and multi-species oral biofilms in the dose- and exposure time-dependent manners. The exact mechanism for anti-biofilm effect of xanthorrhizol on disrupting the existing saliva and artificial multi-species biofilms is unknown. As biofilm is composed of immobilized multi-species colonizers, the action mode of xanthorrhizol might be considered by attacking and killing the oral microbes on the biofilm cell surface. The release of the dead cells from the biofilm was confirmed as reduction of biofilm quantity. Taken together, it is anticipated that xanthorrhizol could be used as a functional ingredient for oral care foods.

Acknowledgments

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