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Original Article

우식원세균 바이오필름 모델에서 다양한 농도의 *Galla Chinensis* 추출물의 항균효과

김은정[1], 진보형[2]

서울대학교 치의학대학원 [1]치학연구소, [2]예방치학교실

Antibacterial effect of different concentrations of *Galla Chinensis* extract on cariogenic bacteria in a biofilm model

Eun-Jeong Kim[1], Bo-Hyoung Jin[2]

[1]Dental Research Institution, [2]Department of Preventive & Social Dentistry, School of Dentistry, Seoul National University, Seoul, Korea

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Corresponding Author: Bo-Hyoung Jin
Department of Preventive & Social Dentistry, School of Dentistry, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Korea
Tel: +82-2-740-8783
Fax: +82-2-766-8781
E-mail: jjbh@snu.ac.kr
https://orcid.org/0000-0003-3526-6805

Objectives: *Galla chinensis* inhibited the adherence of planktonic oral bacteria and acid production by cariogenic bacteria. However, little is known about the relevant conditions of *Galla Chinensis* extract (GCE) exposure time and concentration and the effect of GCE on the structural and functional activity of cariogenic bacteria. The antibacterial effects of natural *G. Chinensis* extract on *S. mutans*, *S. sanguinis*, and *S. oralis* biofilms were evaluated in vitro.
Methods: Biofilms formed on glass surfaces were treated with different concentrations of GCE at different exposure times. The effects were assessed by examining the bactericidal activity, acidogenesis, minimum inhibitory concentration, and morphology.
Results: There was a statistically significant difference in the bacterial growth inhibition depending on the concentration of the GCE, with bacterial growth being inhibited as the concentration of GCE increased. A concentration of 1.0 mg/ml GCE had similar bactericidal effects against *S. mutans* and *S. oralis* biofilms to those produced by 2.0 mg/ml CHX. In the 1.0 mg/ml GCE group, incomplete septa were also observed in the outline of the cell wall, together with disruption of the cell membrane. In addition, there was also a slight exudation of the intracellular content from the bacteria in the 1.0 mg/ml GCE and 2 mg/ml CHX groups.
Conclusions: These results indicate that GCE inhibits the growth of *S. mutans*, *S. sanguinis*, and *S. oralis* with increasing concentrations. It alters the microstructure of *S. mutans* biofilms. These results suggest that GCE might be a useful anti-bacterial agent for preventing dental caries.

Key Words: Antibacterial effects, Biofilms, *Galla Chinensis*, *Streptococcus mutans*

Introduction

Dental caries is the most common infectious disease. It is a multifactorial disease caused by the interactions of bacteria, food, and saliva in the teeth, and is a progressively infectious disease with tooth destruction[1]. *Streptococcus mutans* (*S. mutans*) has been reported as a primary cariogenic pathogen associated with dental caries[2]. *S. mutans* are formed glucan from sucrose using various kinds of glucosyltransferases and attaching to the surface of tooth, developing of oral biofilm and producing acid to induce dental caries[3]. Among the various microorganisms in the mouth, such as *S. mutans*, *Streptococ-*

cus sanguinis (*S. sanguinis*) and Strpetococcus oralis (*S. oralis*) is also considered to be a major cause of dental caries.

Various chemical plaque control methods to reduce cariogenic biofilm have been suggested. Synthetic chemical antimicrobial agents are typical such as chlorhexidine (CHX), which is widely used and has excellent antibacterial effect, but have side effects in long-term use, such as tooth coloring, promoting bacterial colonization, and esquamation of oral mucosa[4]. Therefore, there has been increasing interest in the substances extracted from natural products that can inhibit the adherence of cariogenic bacteria and the formation of bacterial plaque in the teeth without side effects. Because of the need for affordable, effective, and nontoxic alternatives has led to the search for compounds from natural resources such as plants, which may overcome the high incidence of oral disease.

Herbal extracts are often used in traditional medicine for treating a various disease. In addition, it represents antibacterial activity against oral pathogens. *Galla chinensis* (*G. Chinensis*), natural products, has been widely used in traditional Chinese herbal medicine for more than thousands of years[5]. It is mainly composed of hydrolyzable tannins (e.g. gallotannin and gallic acid). This tannin is structurally different from the condensed tannin, as can be seen from tea polyphenols. Polyphenols include antioxidant reactions and structural interactions with proteins[6]. Besides, polyphenol compounds inhibit the glycosyltransferace activity of *S. mutans*[7,8]. This kind of action are relevant for adapt to the oral environment because the protein is a component of the dental plaque and the enzyme is responsible for plaque metabolism. It has been received much attention recently, could be a valuable resource in the search for new bioactive compounds for anti-caries[9]. Normal oral streptococci such as *S. sanguinis*, *S. oralis* play an important role in the maintaining oral hygiene by inhibiting the colonization of cariogenic and periodontal bacteria[10,11]. These bacteria are sensitive to the exposure time and concentration of the substance. Therefore, studies should be conducted to better understand their properties, efficacy and safety to exposure time and concentration to prevent adverse effects from overuse when using the substance as an antibiotic agent[12]. However, the biofilm studies on the antimicrobial effects of *G. Chinensis* at various exposure times and concentrations are very limited for several cariogenic bacteria. So, it is very important study to test this because the necessary requirement of antibacterial agents for oral disease management can be reduce the oral pathogens without affecting normal oral flora.

If an optimal concentrations of *G. Chinensis* extract are found, further studies may result in the use of other oral health products containing *G. Chinensis* extract as effective antimicrobial agents. Thus, this study aimed to evaluate the antimicrobial activity of various concentrations of *G. Chinensis* extract on *S. mutans*, *S. sanguinis* and *S. oralis* related to dental caries, and to investigate the optimum concentration.

Materials and Methods

1. *G. Chinensis* extract (GCE) samples and test compounds preparation

GCE was prepared as reported by previous study[13]. *G. Chinensis* produced in the Gyeongbuk province of the Republic of Korea. It was dried at 60℃ for 3 days in oven. Then ground to a fine powder that was added to 600 ml of distilled water to extract. The mixture was stirred at 60℃ for 10 hours and filtered. The extract was extracted twice with distilled water under the same conditions, then dissolved in ethanol (100%) 500 ml at 60℃ for 2 days and an agitated it at speed of 150 rpm. After evaporation of the ethanol. 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml GCE was used in this study. 2.0 mg/ml CHX (Sigma, USA) was used as the positive control, 1% dimethyl sulfoxide (DMSO) was used as a negative control.

2. Bacterial species, cultivation and formation of biofilm

Streptococcus mutans KCOM 1054, *Streptococcus oralis* KCOM 1401, and *Streptococcus sanguinis* KCOM 1070 was obtained from the Korean Collection for Oral Microbiology (KCOM, Gwangju, Korea), and cultivated with Trypton soy broth (TSB, Difco, Detroit, Mich., USA). Each bacterium stored in freeze-dried culture were inoculated into a liquid medium supplemented with 10% lactose in Trypton soy broth and cultured in a 37℃ incubator for 24 hours. For biofilm formation, place a sterile 12 mm diameter slide glass on a 24-well plate. Then, inoculate the bacteria cultured at 37℃ for 24 hours each with $1\times10-7$ colony forming units per milliliter (CFU/ml). Then, incubate at 37℃ for 48 hours.

3. Determination of minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was analyzed by micro dilution according to the NCCLS standard[14]. The cells were cultured in a 37℃ incubator for 24 hours using TSB medium (*S. mutans*, *S. oralis*, *S. sanguinis*) used in this study, diluted to $1\times10-7$ CFU/ml and dispensed into 96-well plates. The GCE was added to the bacterial culture to be 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml. DMSO was used as the negative control of

the experiment, and 2.0 mg/ml CHX was used as the positive control. Bacterial culture in 96-well plate was incubated for 24 hours and MIC was measured. The colonies formed after incubation in the incubator were counted and measured. Each reaction was repeated at least five times and averaged.

4. Antibacterial activity of GCE against *S. mutans*, *S. sanguinis* and *S. oralis* oral streptococci

The susceptibility assay of *S. mutans*, *S. sanguinis* and *S. oralis* for the GCE was performed according to the methods of Clinical Laboratory Standard Institute[15]. Briefly, the bacteria were cultured in TSB broth for 24 hours before the testing day, and bacterial number was counted. The bacterial concentration was adjusted to 2.5×10^7 cells/ml using fresh TSB after harvesting by centrifugation. The GCE was diluted with a micropipette to a concentration of 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml. The bacterial suspensions were inoculated into the extracts contained in 12 mm diameter slide glass on a 24-well plate and incubated at 37℃ in aerobic atmosphere. The bacterial growth was measured at 3, 6, 9, 12, and 24 hours after culture using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 600 nm. The control group was cultured in the same methods as in the experimental group after inoculation with the pure TSB medium. Every experiment was repeated five times.

5. Morphological changes analysis

The *S. mutans*, *S. sanguinis* and *S. oralis* biofilms on the sterile 12 mm diameter slide glass on a 24-well plate were treated with 1.0 mg/ml GCE, 1% DMSO, 2.0 mg/ml CHX for 1 hour at 37℃. After removing the culture solution, wash 3 times with 0.1M PBS. Samples for TEM measurement are fixed at 60 min RT with Karnovsky's glutraldehyde. Transmission electron microscopy (TEM) JEM 1011 (JEOL, Tokyo, Japan) was used to examine the intracellular changes in *S. mutans*, *S. sanguinis* and *S. oralis*. The *S. mutans*, *S. sanguinis* and *S. oralis* were fixed and dehydrated on the slide surface. The fixed cells were embedded and small blocks of bacteria were cut with ultra-microtome (Leica, Wein, Austria).

6. Statistical analysis

The statistical analysis of total data values was logarithmically transformed using natural logarithm, and normalized through

Table 1. MIC induced by GCE at different concentrations

Treatment group		*S. mutans**	*S. sanguis**	*S. oralis**
GCE 0.1 mg/ml	M±SD	2.43 ± 0.04^{a}	2.58 ± 0.03^{a}	2.60 ± 0.02^{a}
	Proportion (%)	84.04	80.51	87.18
GCE 0.2 mg/ml	M±SD	2.32 ± 0.04^{a}	$2.14\pm0.12^{a,b}$	2.16 ± 0.04^{b}
	Proportion (%)	75.75	52.05	56.5
GCE 0.4 mg/ml	M±SD	1.95 ± 0.14^{b}	$2.10\pm0.07^{a,b}$	2.41 ± 0.06^{b}
	Proportion (%)	47.83	50.21	48.68
GCE 0.8 mg/ml	M±SD	1.69 ± 0.08^{b}	$2.08\pm0.08^{a,b}$	1.95 ± 0.05^{c}
	Proportion (%)	36.96	41.51	46.18
GCE 1.0 mg/ml	M±SD	1.48 ± 0.10^{c}	1.54 ± 0.12^{c}	1.35 ± 0.08^{d}
	Proportion (%)	30.13	28.92	25.68
CHX 2.0 mg/ml	M±SD	1.40 ± 0.30^{c}	1.35 ± 0.06^{c}	1.25 ± 0.05^{d}
	Proportion (%)	28.81	24.1	23.23
1% DMSO	M±SD	2.69 ± 0.05^{a}	$2.69\pm0.03^{a,b}$	2.74 ± 0.01^{a}
	Proportion (%)	102.35	89.72	107.59

Data are presented as $\log_{10}$ colony-forming units (CFU)/disc and percentages.
M, Ln (Log_{10} CFU); SD, standard deviation; Proportion, If the OD value is less than 90-95% of the control 100%, the bacteria is affected and has not grown.
^{a,b,c,d}The different superscripts in the same column indicate a statistically significant difference from each group ($P<0.05$).
Post hoc Tukey's HSD, *$P<0.05$.

Table 2. General linear model including time and treatment groups

Source	df	Mean square	F	Sig.	Partial eta square
Treatment groups	6	15.12	5694.59	<.001	0.995
Time	4	10.11	3806.47	<.001	0.989
Treatment groups×Time	24	0.89	337.88	<.001	0.979

R Squared=.997 (Adjusted R Squared=.996).

Shapiro-wilk normalization test and analyzed using General Linear model, one-way ANOVA and Tukey's post hoc analysis. *P* values less than 0.05 were considered statistically significant. The SPSS (Statistical Packages for Social Science, Ver. 21.0, Chicago, IL, USA) statistical program was used for all statistical analyzes.

Results

1. Antibacterial activity of GCE for cariogenic bacteria biofilm

This study was performed with three cariogenic bacteria, such as *S. mutans*, *S. sanguinis* and *S. oralis*. The MIC of GCE for all three cariogenic bacteria was 0.1 mg/ml. All three bacteria showed 84%, 81% and 87% of bacteria reduction at GCE concentration of 0.1 mg/ml, respectively. In addition, all bacteria groups showed a statistically significant decrease with CHX 2.0 mg/ml at GCE 1.0 mg/ml concentration (Table 1).

To measure the bacterial growth inhibitory effects of *S. mutans*, *S. sanguinis*, and *S. oralis*, which are different from the concentration of GCE, the concentration of 0.1, 0.2, 0.4, 0.8, and 1.0 mg/ml was sampled over time and absorbance was measured at 600 nm. As a result, there was a statistically significant difference in bacterial growth inhibition effect depending on the concentration of the GCE ($P<0.05$). In bacterial growth at different GCE concentrations of bacteria over time, bacterial growth was inhibited as the concentration of GCE increased. Especially, the groups treated with 1.0 mg/ml GCE and 2.0 mg/ml CHX was significantly lower number of surviving *S. mutans* and *S. oralis* CFU than those of the negative control groups and other GCE concentration groups at all-time points (3, 6, 9, 12 and 24 hours) (Table 2 and Fig. 1). In addition, bacterial growth inhibition rates at 12 and 24 hours at GCE 1 mg/ml, which had a high bacterial growth inhibitory effect, were 86% and 89% at 12 hours and 88% and 89% at 24 hours in *S. mutans* and *S. oralis*,

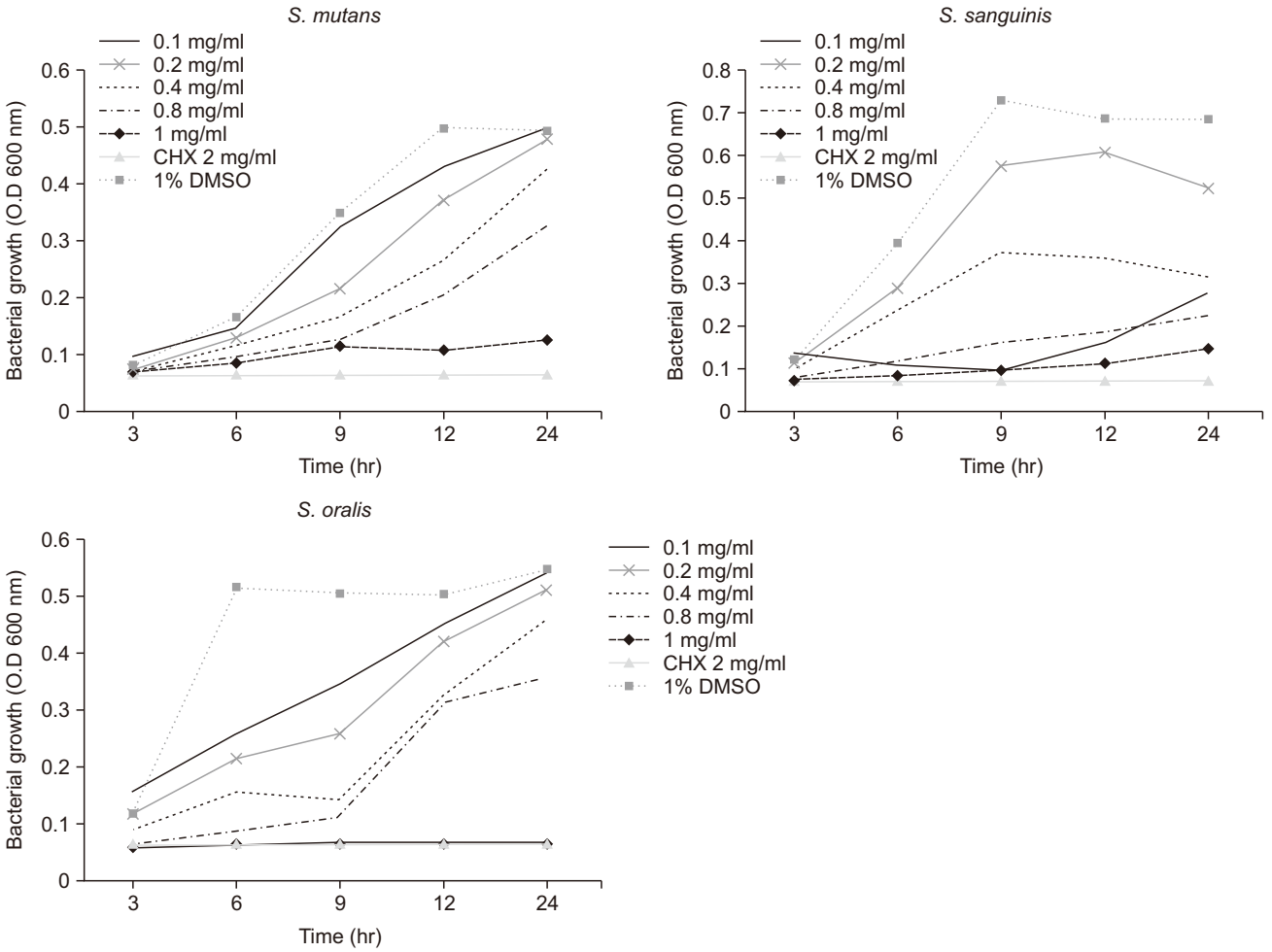

Fig. 1. The bacterial growth curve of *S. mutans*, *S. sanguinis* and *S. oralis* biofilms by GCE concentration. After the biofilms had been exposed to the test solutions for 3, 6, 9, 12, and 24 hours, the number of colonies was counted to determine the CFU. There was a significant difference over time at all concentrations.

respectively. This is very similar bacterial growth inhibition that when CHX 2.0 mg/ml in all bacteria group, it showed 90% bacterial growth inhibition rate (data was not shown). These results show that 1.0 mg/ml GCE has similar bactericidal effects against *S. mutans* and *S. oralis* biofilms to that of 2.0 mg/ml CHX.

2. Morphological changes in *S. mutans* biofilm

The effects of GCE were examined by observing the morphological changes of *S. mutans* by TEM images after 1-hour treatment. After the *S. mutans* biofilms had been exposed to 1% DMSO for 1 h as the negative control, the TEM showed a clear outline of the *S. mutans* cell wall and a peptidoglycan layer (Fig. 2A). However, the most of the peptidoglycan layers of *S. mutans* in the CHX group had disappeared (Fig. 2B). In the 1.0 mg/ml GCE group showed incomplete septa was also observed in the outline of the cell wall, disruption of cell membrane (Fig. 2C). In addition, there was also a slight exudation of the intracellular a contents from the bacteria in both 1.0 mg/ml GCE group and 2.0 mg/ml CHX group (Fig. 2B, 2C).

Discussion

Dental caries is one of the most well-known biofilm related diseases that originate from certain bacteria, especially, *S. mutans*. *S. mutans* plays an important role in metabolizing sucrose to lactic acid and induces demineralization of the tooth enamel[2]. It initiates the cariogenic process by biofilm formation[16]. A biofilm is multicellular aggregation of microorganism attached to the surface and deposited as a thick layer. To prevent dental caries, it is important to reduce the number of bacteria in the mouth and to inhibit the formation of biofilm. Therefore, a biofilm research is used to accurately assess the antibacterial effects of antibiotics.

Natural products have been widely used for the development of dental caries prevention reagents. However, the great complex biological samples still remain a major obstacle to revealing the effect of the true constituents[17]. *G. Chinensis* has been used in a traditional medicine for years. It inhibited the adherence of planktonic oral bacteria as well as inhibiting acid production by cariogenic bacteria. Although previous study[5] have investigated the antimicrobial effect of *G. Chinensis* against *S. mutans*, however, little is known about the relevant conditions of GCE exposure time and concentration and the effect of GCE on the structural and functional activity of various cariogenic bacteria. It was therefore necessary to discover the optimal concentration by discovering the anti-caries effect at various concentrations and times in biofilm conditions of various cariogenic bacteria.

The pharmacological effects of herbal medicines and edible plants differ from each other in extraction methods and in specific solvents. In general, selection of a solvent is the most important because the substances to be extracted depend on the extraction solvent such as ethanol, methanol, hexane, and dichloromethane. In this study, we used ethanol as an extraction solvent on the basis of previous studies that showed the greatest effect when *G. Chinensis* was extracted[17]. To determine the effect of each concentration of GCE on the growth of bacteria by time, we measured the number of bacteria according to time by biofilm with different concentration of extract. Our data showed that the GCE has an inhibitory effect when exposed to multispecies oral biofilm at all concentration. These results were very similar to those of the previous studies[13], but since the previous studies only used single concentration, it was not possible to know exactly how the GCE MIC concentration was, and which showed effective growth inhibition. The MIC test

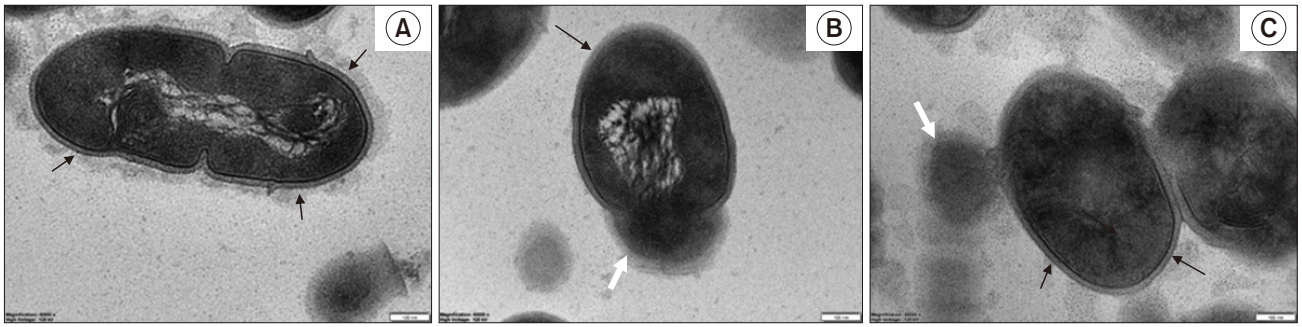

Fig. 2. TEM images of the *S. mutans* biofilm after 1 hour of treatment. The black arrows indicate the wall of *S. mutans*, and the white arrows indicate the intracellular contents. The scale bar is 100 nm. (A) 1% DMSO, (B) 2 mg/ml CHX, and (C) 1.0 mg/ml GCE. After the *S. mutans* biofilms had been exposed to 1% DMSO for 1 h as the negative control, the TEM showed a clear outline of the *S. mutans* cell wall and a peptidoglycan layer. However, most of the peptidoglycan layers of *S. mutans* in the CHX group had disappeared. In the 1.0 mg/ml GCE group, incomplete septa were also observed in the outline of the cell wall. In addition, there was a slight exudation of the intracellular contents in the 1.0 mg/ml GCE and 2 mg/ml CHX groups.

demonstrates the lowest level of growth inhibiting antimicrobial agents. It is used to determine the performance of all other susceptibility testing methods because it is considered the 'gold standard' for determining the susceptibility of microorganisms to antimicrobial agents[18]. Therefore, when searching for antimicrobial agents for new substances, the MIC test is necessary to prevent the side effects of overuse. In our study, more detailed analysis on the concentrations showed that less than 90% of all bacteria were present at 0.1 mg/ml of GCE, indicating that the bacteria did not grow when exposed to small amount of GCE. Also, when each strain was exposed to 1.0 mg/ml GCE, it showed a similar antibacterial effect to CHX continuously over a long-term exposure time. In particular, both the MIC and the bacterial growth curve showed statistically similar effects with CHX in the 1.0 mg/ml GCE group.

TEM revealed the effect of GCE on biofilm integrity. TEM images showed changes in the morphology of bacteria. The bacteria in control group (1% DMSO) biofilm were dense on the surface, whereas, the bacterial in GCE and CHX groups were sparse on the surface. And also, cariogenic bacteria biofilm exposed to GCE and CHX showed damaged peptidoglycan layer and leakage of the intracellular contents compared to the control group (Fig. 2). Previous studies reported that the grapefruit seed extract[19], and the *Curcuma Xanthorrhiza* extract[20] are the natural antibacterial agents capable of degrading the functions of physiologically active enzymes and destroying cell wall functions in microbial cells. Another study showed that the polypheonid compound from cranberry juice has the same effect[21]. The results here are also consistent with the previous study of GCE[13]. The TEM image data in our study showed that the biofilm structure was also clearly affected by exposing GCE in bacteria biofilm. Studies examining the effect of GCE on these bacterial structures with images such as TEM have been rare so far. The evidence for the inhibitory effect of GCE on bacterial adhesion is unclear, polyphenols can form complexes with proteins and polysaccharides[22]. GCE (data not presented) revealed that it contains significant quantities of monomeric and polymeric polyphenols and some other components. The polyphenols of GCE can interact with bacterial membrane proteins through hydrogen bonding through their hydroxyl groups. This can change the permeability of the membrane, causing cell destruction and inhibit cell proliferation[23]. In addition, polyphenols can penetrate bacterial cells and disrupt proton power, electron flow, active transport, and cell contents, thereby reducing lactic acid production[17]. Based on these findings, GCE may inhibit the bacterial growth by destroying bacterial structure.

Oral tissue cells are more resistant to compounds in vivo than in vitro because the oral tissue cells are continuously supplied with nutrients through the blood in vivo, resulting in better regenerative capacity. On the other hand, since oral bacteria form biofilm, it is necessary to introduce a higher concentration of antimicrobial agent than the concentration that normally shows antimicrobial activity against airborne bacteria. Nevertheless, antibiotics such as penicillin, vancomycin, and tetracycline, which are used to inhibit bacteria that form the dental biofilm, can cause tolerance when they are used too frequently. This study confirmed the bacterial growth effect of representative causative bacteria of dental caries in various GCE concentrations and exposure time. However, since this is an in vitro study, future studies investigating the methods of testing the efficacy and stability of GCE in vivo, and studies using biofilm models are needed.

Conclusions

These results found that GCE inhibits the growth of *S. mutans*, *S. sanguinis*, and *S. oralis* with increasing time and concentrations. Also, it alters the microstructure of a *S. mutans* biofilm. Especially, when GCE was 1.0 mg/ml, it showed statistically significant effect with 2.0 mg/ml chlorhexidine. This results suggests that GCE might be a useful anti-bacterial agent for preventing dental caries.

In this context, GCE, which is a natural extract, can be an effective preventive measure of dental caries that doesn't cause anti-biotic tolerance. Our study will enable GCE to be used effectively to prevent dental caries through various concentrations of GCE.

ORCID

Eun-Jeong Kim, https://orcid.org/0000-0002-6316-7807

References

1. Hamada S, Koga T, Ooshima T. Virulence factors of *Streptococcus mutans* and dental caries prevention. J Dent Res 1984;63:407-411.
2. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986;50:353-380.
3. Monchois V, Willemot RM, Monsan P. Glucansucrases: mechanism of action and structure-function relationships. FEMS Microbiol Rev 1999;23:131-151.
4. Scheie AA. Modes of action currently known chemical antiplaque agents other than chlorhexidine. J Dent Res 1989;68:1609-1616.
5. Zhang TT, Guo HJ, Liu XJ, Chu JP, Zhou XD. *Galla chinensis* compounds remineralize enamel caries lesions in a rat model. Caries

Res 2016;50:159-165.
6. Cheynier V. Polyphenols in foods are more complex than often thought. Am J Clin Nutr 2005;81:223S-229S.
7. Tagashira M, Uchiyama K, Yoshimura T, Shirota M, Uemitsu N. Inhibition by hop bract polyphenols of cellular adherence and water-insoluble glucan synthesis of *mutans* streptococci. Biosci Biotechnol Biochem 1997;61:332-335.
8. Furiga A, Lonvaud-Funel A, Dorignac G, Badet C. In vitro anti-bacterial and anti-adherence effects of natural polyphenolic compunds on oral bacteria. J Appl Microbiol 2008;105:1470-1476.
9. Huang S, Gao S, Cheng L, Yu H. Combined effects of nano-hydroxyapatite and *Galla Chinensis* on remineralization of initial enamel lesion in vitro. J Dent 2010;38:811-819.
10. Costerton JW. Introduction to biofilm. Int J Antimicrob Agents 1999;11:217-221.
11. Haffajee AD, Socransky SS. Introduction to microbial aspects of periodontal biofilm communities, development and treatment. Periodontol 2000 2006;42:7-12.
12. Nascimento GG, Locatelli J, Freitas PC, Silva GL. Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Braz J Microbiol 2000;31:247-256.
13. Xie Q, Li J, Zhou X. Anticaries effect of compounds extracted from *Galla Chinensis* in a multispecies biofilm model. Oral Microbiol Immunol 2008;23:459-465.
14. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved Standard M7-A5. NCCLS, Wayne, PA, USA.
15. Clinical and Laboratory Standards Institute. Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard M11-A8. 8th ed. PA, USA:CLSI;2012.
16. Gamboa F, Estupinan M, Galindo A. Presence of *streptococcus mutans* in saliva and its relationship with dental caries: antimicrobial susceptibility of the isolates. Universitas Scientiarum 2004;9:23-27.
17. Huang X, Deng M, Liu M, Cheng L, Exterkate RAM, Li J, et al. Comparison of composition and anticaries effect of *Galla Chinensis* extracts with different isolation methods. Open Dent J 2017;11:447-459.
18. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 2001;48:5-16.
19. Miele WH. Efficacy of grapefruit seed extract against Salmonella typhi, Escherichia coli, and Staphylococcus aureus, Microbiological food analysis report reviewed and approved by Southern Testing and Research Laboratories. NC:Wilson Inc;1988:1-5.
20. Kim JE, Kim HE, Hwang JK, Lee HJ, Kwon HK, Kim BI. Antibacterial characteristics of *Curcuma xanthorrhiza* extract on *Streptococcus mutans* biofilm. J Microbiol 2008;46:228-232.
21. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, et al. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. FEMS Microbiol Lett 2006;257:50-56.
22. Haslam E. Natural polypheonols (vegetable tannins) as drugs: possible modes of action. J Nat Prod 1996;59:205-215.
23. Burt S. Essential oils: Their antibacterial properties and potential applications in foods - a review. Int J Food Microbiol 2004;94:223-253.