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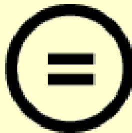

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A Dissertation for the Degree of Doctor of Philosophy

Anti-bacterial and remineralization effects of *Galla Chinensis in vitro*

오배자 추출물의 항균효과와 재광화효과

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Anti-bacterial and remineralization effects of
Galla Chinensis in vitro

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Abstract

Anti-bacterial and remineralization effects of *Galla Chinensis in vitro*

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Galla Chinensis has been used in traditional medicine for years. It inhibits the adherence of planktonic oral bacteria as well as inhibiting acid production by cariogenic bacteria. 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 cariogenic bacteria. Also, experiments have not been performed to investigate the co-operative effects of calcium and the GCE on enhancing the remineralization underneath the biofilm model. Thus, this study aimed to evaluate the antimicrobial activity of various concentrations of GCE on *S.*

mutans and other oral streptococci related to dental caries and to investigate the effects of GCE with calcium on enhancing remineralization *in vitro.*

For the anti-bacterial effect experiment, biofilm formed on glass surfaces were treated with GCE at different concentrations at different exposure time. For the remineralization experiment, *S. mutans* biofilm was formed on bovine enamel specimens over a 72 h period and treated with the following compounds for 10 min: 1.0 mol calcium (CA), a 4,000 ppm aqueous solutions of *G. Chinensis* extract (GCE) and 4,000 ppm aqueous solutions of GCE with 1.0 mol CA. The enamel specimens were analyzed for enamel surface microhardness after remineralization.

In bacterial growth at different GCE concentrations of bacteria over time, bacterial growth was inhibited as the concentration of GCE increased. 1.0 mg/ml GCE had similar bactericidal effects against *S. mutans* and *S. oralis* biofilms to that of 2.0 mg/ml CHX, and also showed incomplete septa was also observed in the outline of the cell wall, disruption of the cell membrane. This study also found that natural *G. Chinensis* has a significant effect on enhancing the remineralization of enamel lesion, and it had combined synergic effects with calcium in improving remineralization. This suggests that GCE might be a useful agent for preventing dental caries.

Keywords: Antibacterial effects, Biofilms, *Galla Chinensis*, *Streptococcus mutans*, Remineralization

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List of Abbreviations

ANOVA	One-way analysis of variance
CA	Calcium
CFU	Colony forming units
CLSI	Clinical Laboratory Standard Institute
CHX	Chlorhexidine
DMSO	Dimethyl sulfoxide
G. Chinensis	*Galla Chinensis*
GCE	*Galla Chinensis* Extract
MIC	Minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
S. mutans	*Streptococcus mutans*
S. sanguinis	*Streptococcus sanguinis*
S. oralis	*Streptococcus oralis*
SEM	Scanning electron microscopy
SPSS	Statistical packages for social science
TEM	Transmission electron microscopy
TSB	Tryptic soy broth
QLF	Quantitative light-induced fluorescence
VHN	Vickers hardness number

1. Introduction

1.1 Backgrounds

Dental caries is the most common infectious disease. It is a multifactorial disease caused by the interactions of bacteria, food, and saliva on the teeth and is progressive, eventually leading to tooth destruction (Hamada *et al.*, 1984). *Streptococcus mutans* (*S. mutans*) has been reported as a primary cariogenic pathogen associated with dental caries (Loesche, 1986). *S. mutans* forms glucan from sucrose using various glucosyltransferases and attaches to the tooth surface where it develops an oral biofilm that produces acid and induces dental caries (Monchois *et al.*, 1999). Of the microorganisms found in the mouth, *Streptococcus sanguinis* (*S. sanguinis*) and *Streptococcus oralis* (*S. oralis*) are also considered major causes of dental caries.

Enamel is highly mineralized and is the strongest biological hard tissue in the human body (Cheng *et al.*, 2009). In contrast to other tissues, dental enamel cannot heal itself and must be re-hardening by a physiochemical process involving in organic constituents from saliva or solutions (Zero, 1999). Accordingly, high levels of mineral supplements, such as calcium, fluoride, and phosphates, have preventive effects on enamel mineral loss. However, some of these substances may have side effects in long-term use. For these reasons, there is growing interest in finding new compounds for long-term use (Phan & Marquis, 2006).

Among them are several natural extracts that show the ability to have a better effect on balance tooth de-/remineralization of dental enamel (Jeon *et al.*, 2011; Palombo, 2011).

Various chemical plaque control methods to reduce cariogenic biofilms have been suggested. Synthetic chemical antimicrobial agents are typically suggested. An example is chlorhexidine (CHX), which is widely used and has an excellent antibacterial effect, but there are side effects with long-term use, such as tooth discoloration, promotion of bacterial colonization and desquamation of the oral mucosa (Scheie, 1989). Therefore, there has been increasing interest in the substances extracted from natural products that can inhibit the adherence of cariogenic bacteria and, thus, the formation of bacterial plaque on the teeth, without side effects. The need for affordable, effective, and nontoxic alternatives has led to the search for compounds from natural sources, such as plants, which may overcome the high incidence of oral disease.

Herbal extracts are often used in traditional medicine for treating various diseases. In addition, they exhibit antibacterial activity against oral pathogens. *Galla Chinensis* (*G. Chinensis*), a natural product, has been widely used in traditional Chinese herbal medicine for thousands of years (Zhang *et al.*, 2016). It is primarily composed of hydrolyzable tannins (e.g., gallotannin and gallic acid). This type of tannin is structurally different from the condensed tannins, as seen from tea polyphenols. Polyphenols take part in

antioxidant reactions and structural interactions with proteins (Cheynier, 2005). Polyphenol compounds also inhibit the glucosyltransferase activity of *S. mutans* (Tagashira *et al.*, 1997; Furiga *et al.*, 2008). These actions are relevant for adaptation to the oral environment since protein is a component of dental plaque and the above enzyme is responsible for plaque metabolism. These compounds have received much attention recently and could be valuable resources in the search for new bioactive anti-caries compounds (Huang *et al.*, 2005). Previous studies have indicated that *G. Chinensis* had an ability to inhibiting cariogenic bacteria (Huang *et al.*, 2003; Xie *et al.*, 2005), enamel demineralization, and enhancing remineralization (Cheng & ten Cate, 2010; Liu *et al.*, 2003).

It is necessary to evaluate the effect of their chemical compounds on promoting remineralization of dental enamel. A previous study reported those co-operative effects of fluoride and the chemical compounds of *G. Chinensis* on enhancing remineralization of dental enamel (Lei *et al.*, 2008), however, experiments have not been performed to investigate the co-operative effects of calcium and the *G. Chinensis* on enhancing the remineralization underneath a biofilm model. Moreover, some experiment also performed the potential rehardening effect of *G. Chinensis* under pH-cyclic conditions, but since this does not reflect the complex environment in the mouth, we performed this study to assess the effect of *G. Chinensis* on enamel by reproducing the

oral ecological environment as much as possible using biofilm model. So far, no biofilm model has persuasively addressed the effectiveness of caries-preventive agents such as traditional herb on remineralization of dental hard tissue. Thus, it is of interest to study remineralization underneath a biofilm. In addition, the biofilm study on the antimicrobial effect of *G. Chinensis* against several cariogenic bacteria is very limited. Also, Normal oral Streptococci, such as *S. sanguinis* and *S. oralis*, play an important role in maintaining oral hygiene by inhibiting the colonization of cariogenic and periodontal bacteria (Costerton, 1999; Haffajee & Socransky, 2006). These bacteria are sensitive to the exposure time to, and the concentration of, the inhibitory substance. Therefore, studies should be conducted to better understand their properties, efficacy, and safety, as related to exposure time and concentration, to prevent adverse effects from overuse when using the substance as an antibiotic agent (Nascimento *et al.*, 2000). However, the biofilm studies on the antimicrobial effects of *G. Chinensis* at various exposure times and concentrations are very limited for several cariogenic bacteria. Therefore, it is a significant area of study due to the need for antibacterial agents for oral disease management that can reduce oral pathogens without affecting normal oral flora. If optimal concentrations of *G. Chinensis* extract (GCE) are found, further studies may lead to the use of oral health products containing GCE as active antimicrobial agents. Thus, this study aimed to evaluate the antimicrobial activity of various

concentrations of GCE on *S. mutans* and other oral streptococci related to dental caries and to determine the optimum concentration. And, the remineralization effect of GCE with calcium on enhancing remineralization, and also the antibacterial effect of *G. Chinensis* underneath *S. mutans* biofilm was evaluated by examining the bactericidal activity, acidogenesis, and morphology *in vitro.*

1.2 Research purposes

The purpose of this study was to investigated the antibacterial activity of GCE against a dental cariogenic microorganism such as *S. mutans, S. oralis, and S. sanguinis* biofilm, determined the optimum concentration of GCE *in vitro,* and investigated the effects of *G. Chinensis* with calcium on enhancing remineralization of dental enamel after enamel erosion using pH-cycling and biofilm model *in vitro.*

2. Literature Review

2.1 Research Trends on Natural Products

Natural products are living organisms such as animals and plants living on land, and in the ocean, secondary metabolites present in trace amounts in living organisms, and organism derived cells or tissue culture products. It exists in all organisms as physiologically active substances that directly or indirectly affect the living body (Samuelson, 1999). In addition, it refers to secondary metabolites distributed only in certain organisms, such as alkaloids, terpenoids, flavonoids, and substances involved in primary metabolism necessary for living, and they exist only in specific plants (Hanson, 2003). Recently, as side effects of artificially created drugs are becoming a problem, attention is increasing to physiologically active substances, which are secondary metabolites of specific components or natural products, from medicinal plants and herbal medicines. For this reason, there is a study with a focus on natural products considerably developed, and is an interest in the physiologically active substance is contained in the plant material growth proceeds, many studies on this (Jung *et al*., 2007). Physiologically active substances are high-value substances that exhibit remarkable activity in minimal amounts. Numerous kinds are now being used for humanity, and new materials are being developed (Bakle, 1972; Cushman *et al*., 1977).

As the industrial civilization is highly developed, some of the

artificial syntheses are becoming more and more restricted due to safety issues, and as consumer's desire for safety and health increases, it is a trend to restrict the use of synthetic products. As a result, the field of use of natural products is getting wider.

Researchers are underway to select various plant resources including herbs containing a large number of functional substances effective for anti-cancer, anti-allergy, anti-obesity, antioxidant and antibacterial, and to develop materials using them as raw materials for medicines, food additives or cosmetics (Ali *et al.*, 2005; Kim *et al.*, 2008).

Many studies on the antimicrobial activity of natural substances against some pathogenic microorganisms have been conducted. A grapefruit seed extract (von Woedtke, 1999), *Curcuma Xanthorrhiza* extract (Kim *et al.*, 2008) are a naturally antibacterial material that can weaken the function of the physiologically active enzyme in microorganism cells and destroy the cell wall function. Another previous study, the polypheonid compound from cranberry juice is found to have the same effect (Duarte *et al.*, 2006). Also, a study reported that *salvia miltiorrhiza* extract showed antimicrobial activity against *S. mutans* (Kwang & Baek, 2003).

Studies on *G. Chinensis* have been reported on antimicrobial activities (Xie *et al.*, 2005; Tian *et al.*, 2009; Cheng *et al.*, 2011), remineralization effects (Chu *et al.*, 2007; Cheng *et al.*, 2008; Zou *et al.*, 2008; Huang *et al.*, 2010), anti-diarrheal effect (Chen *et al.*, 2006). However, little studies have been reported on the effect on

the antibacterial activity by the concentration of the extract of *G. Chinensis.*

2.2 *Galla Chinensis*

Galla Chinensis (G. Chinensis), also known as Chinese gall, is one of the traditional natural, non-toxic herbs for the past 2,000 years. It is a stabbing insect house of Melaphis Chinensis Bell and Eriosomatidae on the leaves of *Rhus Javanica* Lenné or other Anacardiaceae. Its shape is uneven, irregularly divided into 2 to 4 pockets or cracked. The outer surface is grayish-brown with short hairs. It is 3-7 cm long, 2-5 cm wide, 2 mm thick, hard, brittle, easily broken, and with a horn-like shiny section. Its inside is empty, but dead worms and secretions remain. It is tasteless and astringent. *G. Chinensis* is distributed in most parts of China and Korea. The galls are usually picked in fall. 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 (Cheynier, 2005).

G. Chinensis has been used for antibiotic, antiviral (Djakpo & Yao, 2015), anti-caries (Cheng & ten Cate, 2010), anti-oxidative (Cheng *et al.*, 2008), anti-cariogenic (Chu *et al.*, 2007), anti-diarrhea (Fejerskov and Kidd, 2004), antibacterial (Liu *et al.*, 2003), anti-inflammatory effect (Li *et al.*, 2002) and anti-thrombin (Wongkhantee *et al.*, 2006) effects. Previous studies have indicated that *G. Chinensis* can enhance remineralization and promote

mineral deposition in the lesion body (Zou *et al.*, 2008). Furthermore, it has been demonstrated to help rehardening of artificial carious lesions (Ehlen *et al.*, 2008).

Fig. 2-1. General features of *Galla Chinensis*

2.3 Research trends on the antibacterial activity of *G. Chinensis*

Several studies have reported on the antibacterial activity of *G. Chinensis* (Huang *et al.*, 2003; Liu *et al.*, 2003; Xie *et al.*, 2008; Tian *et al.*, 2009; Cheng *et al.*, 2011; Huang *et al.*, 2017). Huang *et al.* investigated the effects of various traditional Chinese medicines on the formation of acquired pellicle of *Streptococcus mutans* and reported that *G. Chinensis* is the most effective interfering agent (Huang *et al.*, 2003). In the study by Lee *et al.* investigating the effect of *G. Chinensis* on the pathogens isolated from oral and KB human oral epidermoid carcinoma cells, it was observed *G. Chinensis* could induce apoptosis in the oral and KB human oral epidermoid carcinoma cells through Caspase-3 activation and anticancer effects (Lee *et al.*, 2003). In a study of Xie *et al.* which tested the anti-bacterial effects on GCE, sucrose solution and sodium fluoride solution, GCE and fluoride may inhibit the cariogenicity of the oral biofilm (Xie *et al.*, 2008). In a previous study evaluating the antioxidant and antimicrobial activity of gallatanin extracted from five different solvents of *G. Chinensis,* the extracts with weaker polarity contained gallotannins with higher molecular weight and had stronger antioxidant and antibacterial activities (Tian *et al.*, 2009). Another study from Cheng *et al.*, it was to investigate the effects of GCE at different stages of

salivary microsome biofilm formation. The results showed that bioactive components in GCE reduce or inhibit both growth and lactic acid formation in biofilms (Cheng *et al*., 2011). Huang *et al.* reported that ethanol extract of GCE showed a better effect on inhibiting the acid formation and biofilm formation as a result of a variety of different isolation methods (Huang *et al.,* 2017).

2.4 Research trends on the remineralization effect of *G. Chinensis*

The ability of *G. Chinensis* to cause enamel remineralization has been widely used by many researchers *in vitro* studies (Liu *et al.*, 2003; Liu *et al.*, 2003; Chu *et al.*, 2007; Zou *et al.*, 2008; Cheng *et al.*, 2009; Cheng & ten Cate, 2010). Chu *et al.*, and Zou *et al.* In the study by Chu *et al.* and Zoe *et al.*, after extracting GCE by several methods, and divided into different groups and it was confirmed that all the GCE groups had a better antimicrobial effect than the control group (Chu *et al.*, 2007; Zou *et al.*, 2008). Other many previous studies also proved the remineralization effect of GCE through *in vitro* research. In a study by Cheng *et al.* in which bovine enamel was used, the chemical compound of GCE could regulate the de-/remineralization balance through influencing the morphology and structure of enamel crystals, and the mechanisms appear to be different for GCE and gallic acid (Cheng *et al.*, 2009). Similar results were obtained in many previous studies in which similar experiments were conducted using bovine enamel (Liu *et al.*, 2003; Liu *et al.*, 2003; Cheng & ten Cate, 2010).

An animal study has been tested. In a study by Zhang *et al.*, it experimented to investigate the effect of *G. Chinensis* chemical compounds on enamel caries remineralization in rats, it showed that *G. Chinensis* compounds remineralize enamel caries lesions in

most molars on rat (Zhang *et al.,* 2016).

Some studies have confirmed the remineralization effect by combining *G. Chinensis* with other materials. In a study by Lei *et al.*, who investigated the effect of combining GCE and fluoride on remineralization of initial enamel lesion, it showed that they had combined effects with fluoride on enhancing remineralization (Lei *et al.*, 2008). As a result similar to the previous study, Huang *et al.* investigated the effect of combining nano-hydroxyapatite and *G. Chinensis* on remineralization of initial enamel lesion; it showed a significant synergistic effect of combined GCE and nano-HA treatment on promoting the remineralization of initial enamel lesion (Huang *et al.*, 2010).

3. Material & Method

3.1 Anti-bacterial activity of GCE against cariogenic bacteria in Biofilm model

3.1.1 GCE samples and test compounds preparation

GCE was prepared as reported in a previous study (Xie *et al.*, 2008). *G. Chinensis* was produced in Gyeongbuk province of the Republic of Korea. It (1 kg) was dried in an oven (WiseVen® WON, Witeg, Germany) at 60° C for 3 days and ground to a fine powder that was extracted in 600 ml of distilled water. The mixture was stirred at 60° C for 10 hours and then filtered. The extraction was repeated twice with distilled water under the same conditions. The final extract was then dissolved in 500 ml ethanol (100%) at 60° C for 2 days at an agitated speed of 150 rpm by using shaking incubator (Biofree, Seoul, Korea). Then, the remaining extract was lyophilized to render powder of GCE by using freeze dryer (Ilshinbiobase Co. Ltd., Seoul, Korea) (yield of 160 g). Following evaporation of the ethanol, 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml GCE suspensions were prepared for this study. A solution of 2.0 mg/ml CHX (Sigma, USA) was used as the positive control, and 1% dimethyl sulfoxide (DMSO) was used as the negative control.

3.1.2 Bacterial species, cultivation and formation of biofilm

Streptococcus mutans KCOM 1054, *Streptococcus oralis* KCOM 1401, and *Streptococcus sanguinis* KCOM 1070 were obtained from the Korean Collection for Oral Microbiology (KCOM, Gwangju, Korea) and cultivated with Tryptic Soy Broth (TSB, Difco, Detroit, Mich., USA). Each organism was stored as a freeze-dried culture, inoculated into a liquid medium supplemented with 10% lactose in TSB and cultured in a 37° C incubator for 24 hours. For biofilm formation, a sterile 12 mm diameter slide was placed on a 24-well plate. Then, the bacteria were cultured at 37° C for 24 hours, inoculated at a density of 1×10^{-7} colony forming units per milliliter (CFU/ml) and incubated at 37° C for 48 hours.

3.1.3 Determination of minimum inhibitory concentration

The Minimum Inhibitory Concentration (MIC) was determined by microdilution according to the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 2000). The bacteria used in this study (*S. mutans, S. oralis, S. sanguinis*) were cultured in a 37° C incubator for 24 hours in TSB medium, diluted to 1×10^{-7} CFU/ml and dispensed into 96-well plates. The GCE was added to the bacterial

culture at concentrations of 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml. DMSO was used as the negative control for the experiment, and 2.0 mg/ml CHX was used as the positive control. The inoculated 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 has repeated a minimum of five times and averaged.

3.1.4 Antibacterial activity of GCE against *S. mutans* and normal oral streptococci

The GCE susceptibility assay of *S. mutans* and normal oral streptococci was performed according to the methods of the Clinical Laboratory Standard Institute (CLSI, 2012). Briefly, the bacteria were cultured in TSB broth for 24 hours before testing, and bacterial colony number was counted. Following harvest by centrifugation, the bacterial concentration was adjusted to 2.5×10^7 cells/ml using fresh TSB. The GCE was diluted with a micropipette to concentrations 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 glasses on a 24-well plate and incubated at 37° C in an 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 under the same conditions as the experimental group after inoculation with pure TSB medium. Each experiment was repeated five times.

3.1.5 Acidogenicity of *S. mutans* and normal oral streptococci biofilms

The acid production levels from the *S. mutans* and normal oral streptococci biofilms treated with 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml were determined by measuring pH (Koo *et al.*, 2006). The pH was measured using a pH electrode (Orion ROSS^{TM}, 8102 BNUWP, Beverly, MA, USA) connected to a pH meter (Orion Star^{TM}, Beverly, MA, USA). After a 3, 6, 9, 12, and 24 hours treatment with the test compounds, the pH of the media was measured each time point. These assays were repeated at least five times.

3.1.6 Morphological changes analysis

The *S. mutans* and normal oral streptococci biofilms on the sterile 12 mm diameter slide glass on a 24-well plate were treated with 1.0 mg/ml GCE, 1% DMSO or 2.0 mg/ml CHX for 1 hour at

37° C. Following removal of the culture medium, the biofilms were washed 3 times with 0.1 M PBS. Samples used for Transmission electron microscopy (TEM) measurement were fixed for 60 min at RT with Karnovsky's glutaraldehyde, and samples for SEM measurement are fixed in 4% paraformaldehyde at RT for 60 min. Scanning electron microscopy (SEM) S-4700 (Hitachi, Tokyo, Japan) was used to examine the changes in the *S. mutans* morphology. TEM JEM 1011 (JEOL, Tokyo, Japan) was used to examine the intracellular changes in *S. mutans* and normal oral streptococci. The *S. mutans* and normal streptococci were fixed and dehydrated on the slide surface. The fixed cells were subsequently embedded, and small blocks of bacteria were cut with an ultra-microtome (Leica, Wein, Austria).

3.1.7 Statistical analysis

The statistical analysis of the data was transformed using the natural logarithm, normalized with the 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 analyses.

3.2 Remineralization effect of GCE and calcium of enamel in *S. mutans* Biofilm model

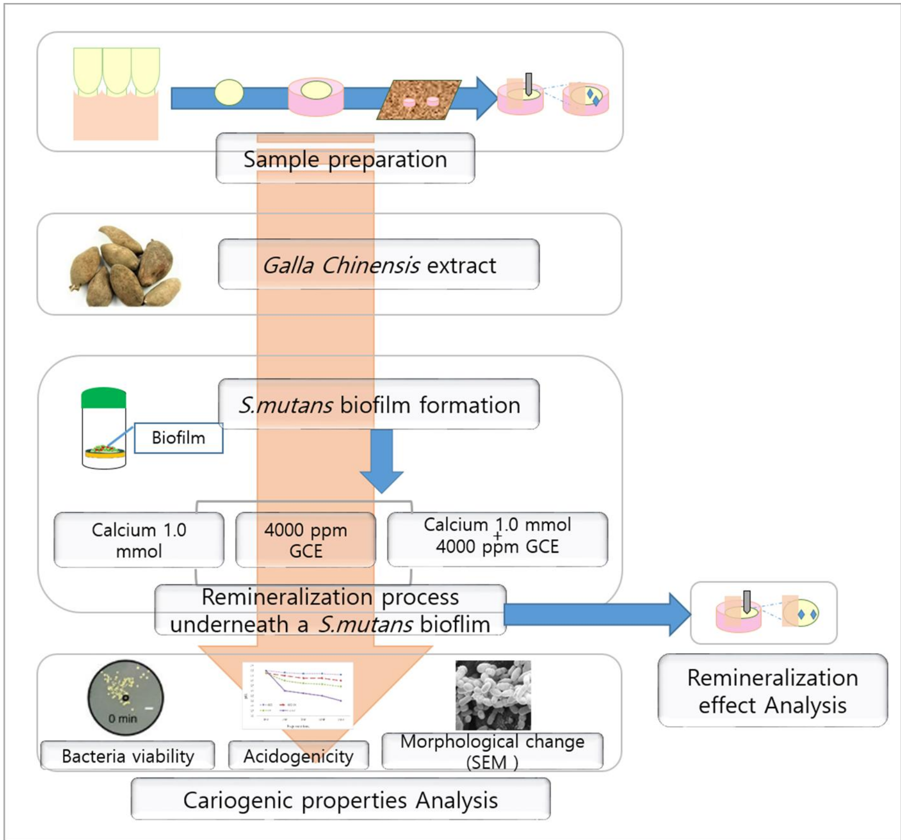

Fig. 3-1. Flowchart of the *in vitro* experimental study design.

3.2.1 Preparation of enamel specimens

Sound bovine incisors without cracks, infection or any lesions under Quantitative Light-Induced Fluorescence (QLF, QLF Pro®, Inspektor Research System BV, Amsterdam, Netherlands) were selected in this study. Cylindrical cores 5 mm in diameter were punched out at the top of the bovine enamel surface. Samples were placed in 1.2 x 1.0 x 0.8 cm molds and mounted in acrylic resin. The specimens were ground flat and polished using wetted silicon carbide paper (600-2,000 grid). Specimens were rinsed thoroughly with distilled water and stored in a 100% relative humidity before use. A total of 84 specimens were used in the experiment. Only those specimens which enamel surface hardness raged from 300-330 vickers hardness number (VHN) were selected. The selected specimens were treated with a pH 5.0 solution containing 0.2% Carbopol (#980, Noveon Inc, Cleveland, USA) with 0.1 M lactic acid containing 50% calcium hydroxide phosphate for 72 hours to form initial artificial caries enamel. The VHN of demineralized specimens was measured, and 84 specimens were having the surface hardness of the initial dental enamel with an average VHN of 35-55 were selected. For each group, GCE, GCE+CA, and CA groups were assigned to 24 and control to 12.

3.2.2 Bacteria strain, media, growth conditions

Streptococcus mutans ATCC 25175 was provided from Korean Collection for Oral Microorganisms at Seoul National University and cultivated with a TSB at 37° C and 5% CO_2. The *S. mutans* genome sequence was determined using a shotgun high-throughput sequencing approaches as described. The detailed methods are published as supporting information on the PNAS web site (www.pnas.org) (Chu *et al.*, 2007). Biofilms of *S. mutans* were formed on bovine specimens in a 50 ml tube. Each specimen was transferred daily to fresh medium over a 3-day period (Koo *et al.*, 2003). The *S. mutans* biofilms on each specimen contained approximately 2 x 10^7 colony forming units per milliliter before experiment start.

3.2.3 Remineralization process

After exposing the biofilm to each solution (1.0 M calcium, a 4,000 ppm aqueous solution of GCE and a 4,000 ppm aqueous solution of GCE containing 1.0 M calcium) for 10 min, and then they were placed in a 50 ml tube containing a sterile saline solution. The specimens in the tube were ultra-sonicated at 50 W (Branson Sonic, Danbury, Conn., USA) using 3 X 10 sec pulses with

2 X 5 sec intervals before measuring.

3.2.4 Measurement of bacterial viability

Using sterile bovine specimens that did not process anything, after exposing the biofilms to the solutions (1.0 M calcium, 4,000 ppm GCE and a 4,000 ppm aqueous solution of GCE containing 1.0 M calcium) for 1, 5, 10 min and 1 hour, they were placed in 50 ml tube containing the sterile saline solution. The bovine specimens in the 50 ml tube were ultra-sonicated using 3 x 10 sec pulses with 2 x 5 sec intervals (Koo *et al.*, 2002; Koo *et al.*, 2003). The suspension was diluted serially from 10^{-1} to 10^{-6}, and plated on tryptone soy agar. The plates were incubated in 5% CO_2 at 37° C for 48 h, and the colony forming units (CFU) were determined by counting the number of colonies.

3.2.5 Measurement of acid production

The level of acid production from the *S. mutans* biofilms treated with the compounds was determined by measuring the pH (Koo *et al.*, 2006). The pH was measured using a pH electrode connected to a pH meter. After a 5, 10 min, and 1-hour treatment with the test compounds, the pH of the media was measured each time

point. These assays were repeated at least three times.

3.2.6 Morphological changes analysis

SEM S-4700 was used to examine the changes in the *S. mutans* morphology. The bovine enamel specimens were fixed in 4% paraformaldehyde in 0.1 M PBS for 1 hour at room temperature. The fixed samples were then washed 2 times with PBS and distilled water, and sputter-coated with platinum and observed by SEM.

3.2.7 Assessment of remineralization effect

The surface microhardness of enamel specimens was assessed using a Vickers microhardness tester (Shimadzu, HMV-2, Kyoto, Japan) at the beginning of the experiment, and after being immersed in a mineral and natural supplement. Indentations were measured for 10 s using diamonds at 9.807 N with a magnification of 40 X. The average microhardness was calculated.

3.2.8 Statistical analysis

The differences between the groups and antibacterial effects were analyzed using one-way analysis of variance (ANOVA) and followed by a Tukey's post hoc honestly significant differences (HSD) test using the studentized range. The level of statistical significance was $\alpha \leq 0.05$. The SPSS (Statistical Packages for Social Science, Ver. 19.0, Chicago, IL, USA) statistical program was used for all statistical analyzes.

4. Results

4.1 Antibacterial activity of GCE

4.1.1 Antibacterial activity of GCE on cariogenic bacteria biofilm

This study was performed with three cariogenic bacteria, *S. mutans, S, sanguinis* and *S. oralis*. The MIC of GCE for all three cariogenic bacteria was 0.1 mg/ml. *S. mutans, S, sanguinis* and *S. oralis* showed 84%, 81% and 87% bacterial reduction at a GCE concentration of 0.1 mg/ml, respectively. In addition, all bacterial groups showed a statistically significant decrease with CHX 2.0 mg/ml and GCE 1.0 mg/ml concentrations (Table 4-1).

The bacterial growth inhibitory effects on these bacteria were measured at 0.1, 0.2, 0.4, 0.8, and 1.0 mg/ml concentrations over time and the absorbance using 600 nm. It was found that there was a statistically significant difference in bacterial growth inhibition effect depending on the concentration of GCE ($p < 0.05$). For bacterial growth at different GCE concentrations over time, bacterial growth was increasingly inhibited as the concentration of GCE increased. In particular, the groups treated with 1.0 mg/ml GCE and 2.0 mg/ml CHX had significantly lower numbers of surviving *S. mutans* and *S. oralis* CFU than the negative control group and other GCE concentration groups at all-time points (3, 6, 9, 12 and 24 hours) (Table 4-2 and Fig. 4-1). Also, the bacterial growth inhibition rates at 12 and 24 hours for the GCE 1 mg/ml group, which had a high bacterial growth inhibitory effect, were

86% and 89% at 12 hours and 88% and 89% at 24 hours for *S. mutans* and *S. oralis*, respectively. This result is similar to that exhibited by CHX 2.0 mg/ml in all bacterial groups, which showed a 90% bacterial growth inhibition rate (data was not shown). These results indicate that 1.0 mg/ml GCE and 2.0 mg/ml CHX have similar bactericidal effects against *S. mutans* and *S. oralis* biofilms.

Table 4-1. MIC induced by GCE in different concentration groups.

Treatment group		*S. mutans**	*S. sanguinis**	*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 ± 0.12[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 ± 0.07[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 ± 0.08[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 ± 0.03[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: The CFU of the control group was taken as 100, and the ratio of the GCE of each concentration.
The different superscripts in the same column indicate statistically significant difference from each group ($p < 0.05$).
*Statistically significant by repeated measured ANOVA at the a=0.05 level Post hoc Tukey's HSD.

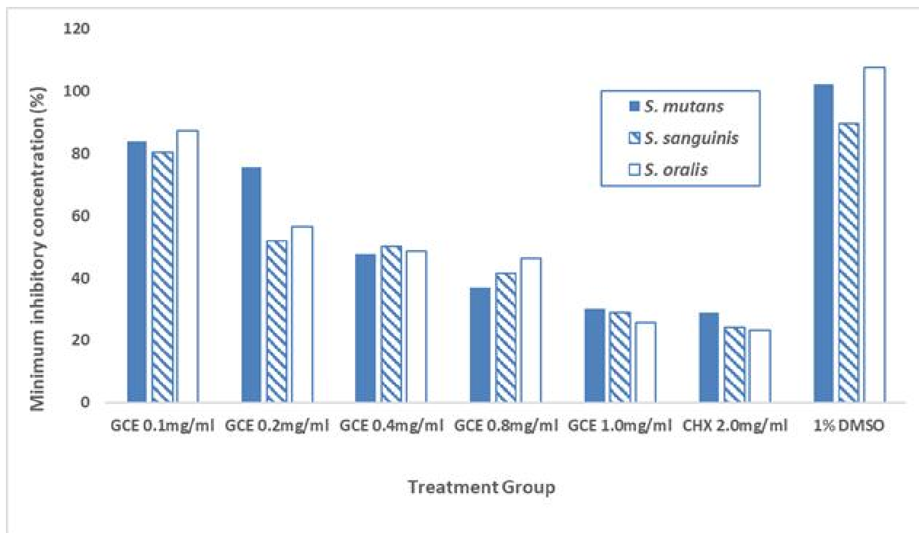

Fig. 4-1. Graph showing MIC of GCE against normal streptococci strain at different concentrations.

Table 4-2. Antibacterial effects of the GCE against normal oral streptococci biofilms.

Treatment group	Time (hour)														
	3			6			9			12			24		
	M	SD	*F*	M	SD	*F*	M	SD	*F*	M	SD	*F*	M	SD	*F*
S. mutans															
GCE 0.1 mg/ml	0.95^{a}	0.01	576.82*	1.40^{a}	0.01	1347.41*	1.47^{a}	0.01	1792.65*	2.23^{a}	0.04	130.72*	2.36^{a}	0.01	638.98*
GCE 0.2 mg/ml	0.69^{b}	0.01		1.26^{b}	0.04		1.79^{b}	0.04		2.32^{a}	0.21		2.47^{a}	0.06	
GCE 0.4 mg/ml	0.65^{b}	0.01		1.14^{b}	0.01		1.51^{b}	0.04		2.00^{b}	0.07		2.73^{b}	0.21	
GCE 0.8 mg/ml	0.63^{b}	0.02		0.95^{c}	0.02		1.24^{c}	0.02		1.65^{c}	0.43		2.61^{b}	0.14	
GCE 1.0 mg/ml	0.50^{c}	0.01		0.54^{d}	0.01		0.56^{d}	0.06		0.55^{d}	0.04		0.46^{c}	0.08	
CHX 2.0 mg/ml	0.48^{c}	0.01		0.51^{d}	0.01		0.53^{d}	0.03		0.51^{d}	0.02		0.51^{c}	0.02	
1% DMSO	0.73^{d}	0.03		1.43^{a}	0.05		2.30^{e}	0.03		2.64^{e}	0.02		2.62^{b}	0.01	
S. sanguinis															
GCE 0.1 mg/ml	1.30^{a}	0.01	5918.09*	2.28^{a}	0.01	7793.86*	3.02^{a}	0.03	5442.02*	2.94^{a}	0.02	3334.86*	2.98^{a}	0.01	385.16*
GCE 0.2 mg/ml	1.10^{b}	0.01		2.08^{b}	0.01		2.78^{b}	0.02		2.84^{a,b}	0.03		2.69^{a,b}	0.02	
GCE 0.4 mg/ml	0.98^{c}	0.01		1.89^{c}	0.02		2.34^{c}	0.02		2.67^{a,b}	0.07		2.74^{a,b}	0.15	
GCE 0.8 mg/ml	0.73^{d}	0.01		1.13^{d}	0.02		1.48^{d}	0.04		1.63^{c}	0.03		2.44^{a,b}	0.08	
GCE 1.0 mg/ml	0.59^{e}	0.03		0.57^{e}	0.04		0.94^{e}	0.03		1.10^{d}	0.01		1.91^{c}	0.14	
CHX 2.0 mg/ml	0.57^{e}	0.01		0.54^{e}	0.01		0.56^{f}	0.03		0.56^{e}	0.03		0.60^{d}	0.04	
1% DMSO	1.14^{b}	0.01		2.40^{a}	0.01		3.04^{a}	0.03		3.02^{a,b}	0.05		3.00^{a,b}	0.07	

S. oralis															
GCE 0.1 mg/ml	1.18^{a}	0.04	2214.74*	1.26^{a}	0.01	5396.94*	1.29^{a}	0.01	18874.66*	1.33^{a}	0.10	729.33*	1.74^{a}	0.53	102.89*
GCE 0.2 mg/ml	1.16^{b}	0.01		2.54^{b}	0.02		2.67^{b}	0.01		2.62^{b}	0.02		2.52^{b}	0.01	
GCE 0.4 mg/ml	0.87^{c}	0.01		2.08^{c}	0.02		2.57^{b}	0.03		2.58^{b}	0.04		2.41^{b}	0.04	
GCE 0.8 mg/ml	0.53^{d}	0.02		0.85^{d}	0.04		1.11^{a}	0.03		1.63^{c}	0.20		2.53^{b}	0.31	
GCE 1.0 mg/ml	0.47^{d}	0.01		0.51^{e}	0.04		0.54^{c}	0.01		0.52^{d}	0.01		0.50^{c}	0.05	
CHX 2.0 mg/ml	0.51^{d}	0.02		0.54^{e}	0.04		0.48^{c}	0.01		0.48^{d}	0.01		0.48^{c}	0.01	
1% DMSO	1.17^{a}	0.01		2.67^{f}	0.02		2.65^{b}	0.01		2.67^{b}	0.05		2.74^{b}	0.11	

Data are presented as log_{10} colony-forming units (CFU)/disc.
M= Ln(Log_{10} CFU), SD= standard deviation.
The different superscripts in the same column indicate statistically significant difference from each group ($p < 0.05$).
Post hoc Tukey's HSD, $*p < 0.05$.

Table 4-3. General liner model included time and treatment groups.

Source	df	Mean Square	F	Sig.	Partial Eta Square
Treatment groups	6	15.12	5694.59	< 0.001	0.995
Time	4	10.11	3806.47	< 0.001	0.989
Treatment groups Time	24	0.89	337.88	< 0.001	0.979

Note. R Squared = 0.997, (Adjusted R Squared = 0.996).

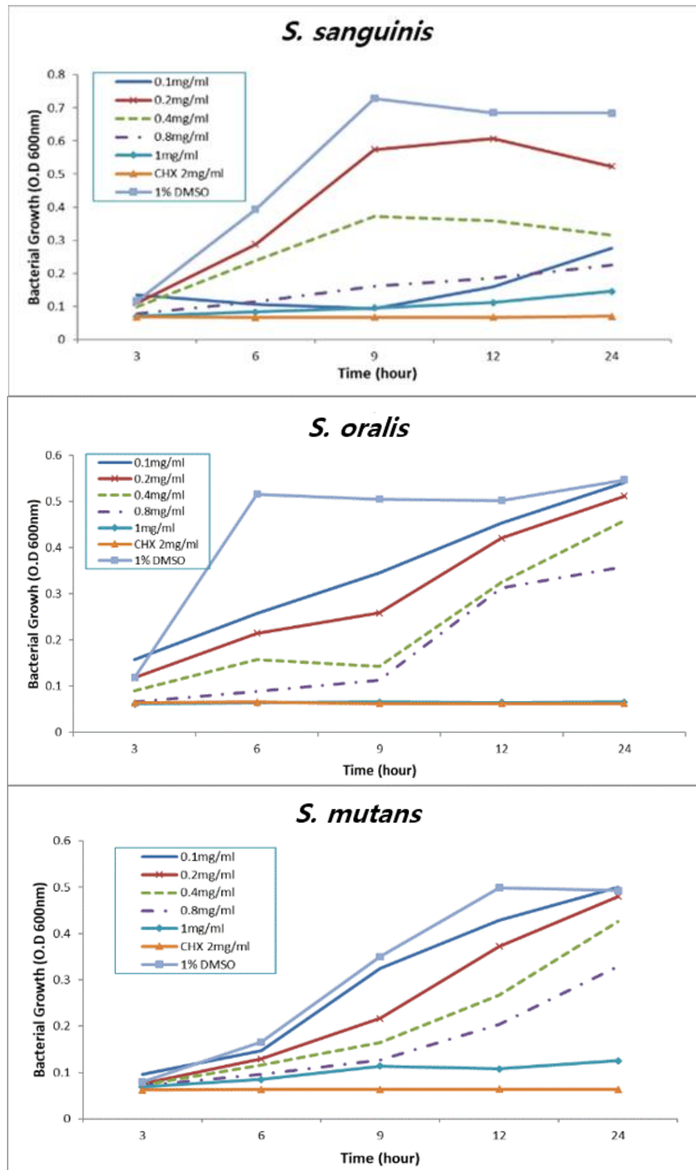

Fig. 4-2. The bacterial growth curve of normal Streptococci biofilms by GCE concentration. After the biofilms had been exposed to the test solutions for 3, 6, 9, 12, and 24 hours, the number of colonies was counted to determine the CFU. There was a significant difference over time at all concentrations.

4.1.2 Effects of GCE on acid production inhibition

The acid production levels from the *S. mutans* and normal oral streptococci biofilms treated with 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml were determined by measuring pH (Fig. 2). After a 3, 6, 9, 12, and 24 hours treatment with the test compounds, the pH of the media was measured each time point. There was a significant difference in pH over time at all concentrations ($p < 0.05$). In the 1% DMSO group, the pH was 5.80 at 12 hour point, 5.71 at 24 hour point, while the GCE 1.0 mg/ml, and CHX 2.0 mg/ml groups' pH was 7.10 and 7.21 at 12 hour point, 7.10 and 7.21 at 24 hour point in *S. mutans* group, respectively. These changes in acidity are also similar to those in *S. sanguinis* and *S. oralis* groups ($p < 0.05$) (Table 4-4, Fig. 4-3).

Table 4-4. Acidogenicity of *S. mutans, S. sanguinis*, and *S. oralis* biofilms

Treatment group	Time (hour)														
	3			6			9			12			24		
	M	SD	*F*	M	SD	*F*	M	SD	*F*	M	SD	*F*	M	SD	*F*
S. mutans															
GCE 0.1 mg/ml	7.15^{a}	0.07	6.07*	$6.85^{a,b}$	0.08	21.65*	$6.62^{a,b}$	0.08	87.66*	6.31^{a}	0.13	64.56*	6.33^{a}	0.09	103.43*
GCE 0.2 mg/ml	7.43^{b}	0.07		$6.89^{a,b}$	0.09		$6.50^{a,b}$	0.10		6.40^{a}	0.08		6.34^{a}	0.13	
GCE 0.4 mg/ml	$7.34^{a,b}$	0.10		$6.99^{b,c}$	0.13		$6.60^{b,c}$	0.07		6.50^{a}	0.09		6.40^{a}	0.07	
GCE 0.8 mg/ml	$7.33^{a,b}$	0.08		$7.11^{c,d}$	0.08		$7.01^{c,d}$	0.06		6.89^{b}	0.09		6.78^{b}	0.06	
GCE 1.0 mg/ml	7.41^{b}	0.08		7.23^{d}	0.09		7.11^{d}	0.08		7.10^{c}	0.10		7.10^{c}	0.13	
CHX 2.0 mg/ml	$7.24^{a,b}$	0.08		7.22^{d}	0.08		7.24^{d}	0.07		7.21^{c}	0.08		7.21^{c}	0.08	
1% DMSO	$7.33^{a,b}$	0.07		6.67^{a}	0.07		6.28^{a}	0.06		5.80^{d}	0.14		5.71^{d}	0.07	
S. sanguinis															
GCE 0.1 mg/ml	7.45^{a}	0.03	98.91*	7.32^{a}	0.02	56.04*	7.11^{a}	0.05	78.66*	6.65^{a}	0.08	212.62*	6.65^{a}	0.08	305.83*
GCE 0.2 mg/ml	7.36^{b}	0.03		7.1^{b}	0.06		7.01^{a}	0.03		6.51^{a}	0.10		6.11^{b}	0.08	
GCE 0.4 mg/ml	7.35^{b}	0.01		6.87^{c}	0.07		6.54^{b}	0.08		6.23^{b}	0.09		6.22^{b}	0.05	
GCE 0.8 mg/ml	7.15^{c}	0.01		6.6^{c}	0.02		6.77^{b}	0.03		6.66^{a}	0.08		6.58^{a}	0.07	
GCE 1.0 mg/ml	7.32^{b}	0.02		$7.15^{a,b}$	0.08		7.11^{a}	0.10		7.09^{c}	0.06		6.99^{c}	0.07	
CHX 2.0 mg/ml	7.25^{d}	0.03		$7.24^{a,b}$	0.04		7.23^{a}	0.08		7.12^{c}	0.07		7.05^{c}	0.05	
1% DMSO	7.43^{a}	0.02		6.85^{c}	0.06		6.21^{c}	0.13		5.33^{d}	0.09		5.26^{d}	0.08	

S. oralis															
GCE 0.1 mg/ml	7.25^{a}	0.07	3.45*	7.13^{a}	0.08	42.21*	6.97^{a}	0.05	68.11*	6.67^{a}	0.05	148.36*	6.61^{a}	0.05	87.7*
GCE 0.2 mg/ml	7.34^{a}	0.08		7.14^{a}	0.07		6.99^{a}	0.15		$6.81^{a,b}$	0.09		6.78^{a}	0.15	
GCE 0.4 mg/ml	7.35^{a}	0.05		7.21^{a}	0.08		7.01^{a}	0.09		$6.89^{a,b}$	0.08		6.88^{a}	0.09	
GCE 0.8 mg/ml	7.27^{a}	0.08		7.21^{a}	0.13		7.12^{a}	0.08		$6.91^{a,b}$	0.13		6.89^{a}	0.12	
GCE 1.0 mg/ml	7.22^{a}	0.12		7.31^{a}	0.12		7.21^{a}	0.09		7.12^{c}	0.10		$6.98^{a,b}$	0.09	
CHX 2.0 mg/ml	7.36^{a}	0.09		7.27^{a}	0.05		7.25^{a}	0.09		7.25^{c}	0.06		7.33^{b}	0.10	
1% DMSO	7.12^{a}	0.11		6.34^{b}	0.08		6.11^{b}	0.05		5.45^{d}	0.10		5.31^{c}	0.16	

Data are presented as the mean pH of the *S. mutans*, *S. sanguinis*, and *S. oralis* bioflim.
M = mean pH, SD= standard deviation.
The different superscripts in the same column indicate statistically significant difference from each group ($p < 0.05$).
Post hoc Tukey's HSD, $^{*}p < 0.05$.

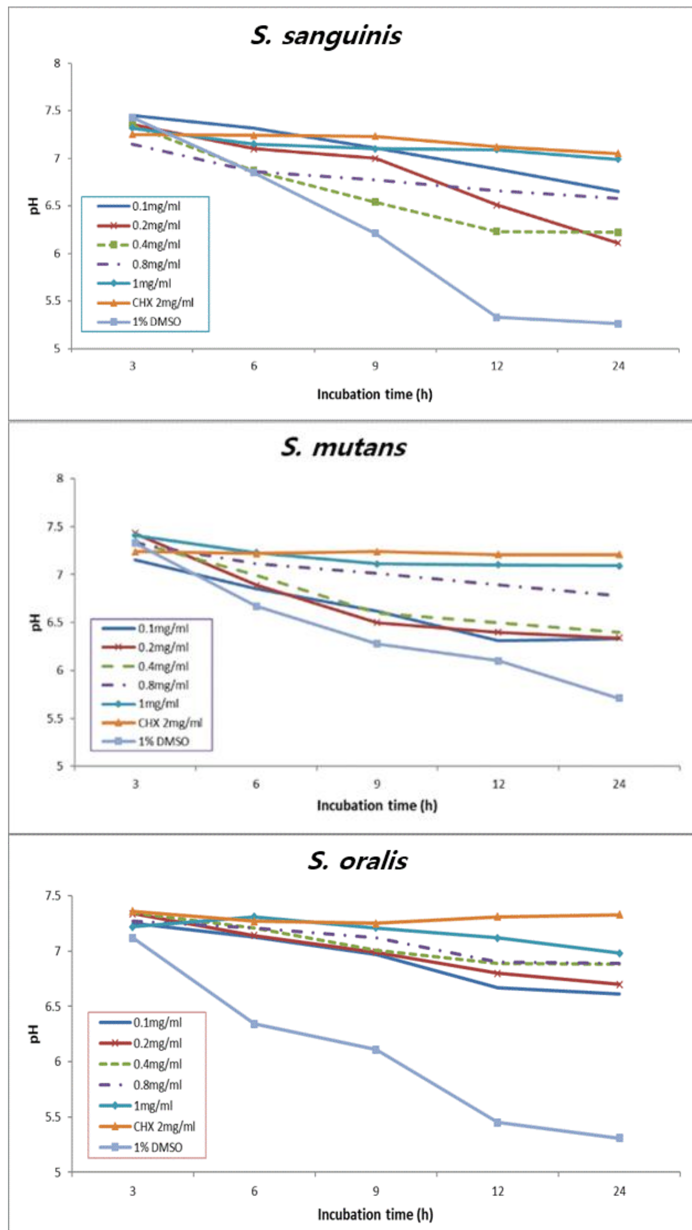

Fig. 4-3. The pH response of normal streptococci biofilms was determined by measuring the pH of media.

4.1.3 Morphological changes by SEM and TEM

The effects of GCE were examined by observing the morphological changes of *S. mutans, S. oralis, and S. sanguinis* by SEM and TEM images after 1 hour of treatment. Once the *S. mutans* biofilms had been exposed to 1% DMSO for 1 hour as the negative control, the SEM and TEM showed a clear outline of the cell wall and a peptidoglycan layer (Fig. 3A, 3D). However, most of the peptidoglycan layers of *S. mutans* in the CHX group had disappeared (Fig. 3B, 3E). In a 1.0 mg/ml GCE group showed incomplete septa that were observed in the outline of the cell wall as a disruption of the cell membrane (Fig. 3C, 3F). In addition, there was a slight exudation of the intracellular contents in both the 1.0 mg/ml GCE and 2.0 mg/ml CHX groups (Fig. 4-4B, 3C, 3E, 3F).

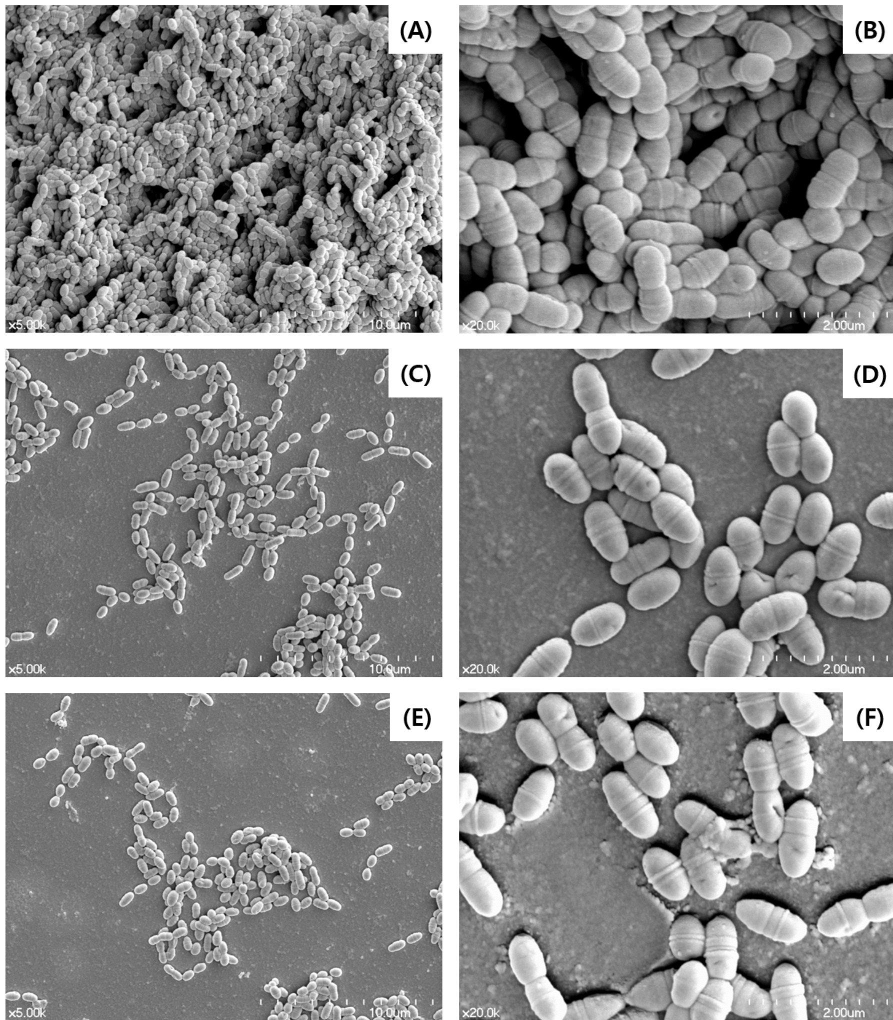

Fig. 4-4. SEM images of *S. mutans* biofilm after 1 hour treatment. (A), (B) 1% DMSO group, (C), (D) 2 mg/ml CHX, and (E), (F) 1.0 mg/ml GCE. The group containing GCE and CHX could reduce the biofilm growing on the disks. (A), (C), and (D) is an image measured at 5,000 magnification, and (B), (D), and (F) is measured at 20,000 magnification.

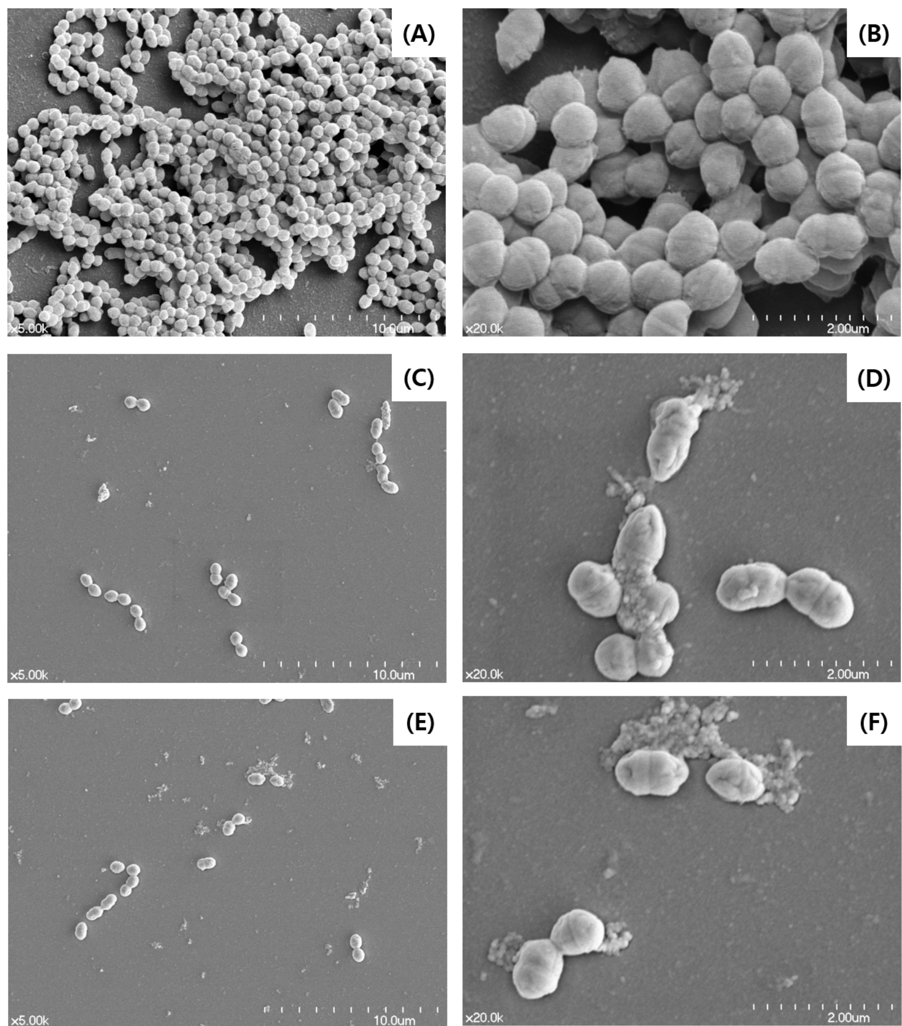

Fig. 4-5. SEM images of *S. oralis* biofilm after 1 hour treatment. (A), (B) 1% DMSO group, (C), (D) 2 mg/ml CHX, and (E), (F) 1.0 mg/ml GCE. The group containing GCE and CHX could reduce the biofilm growing on the disks. (A), (C), and (D) is an image measured at 5,000 magnification, and (B), (D), and (F) is measured at 20,000 magnification.

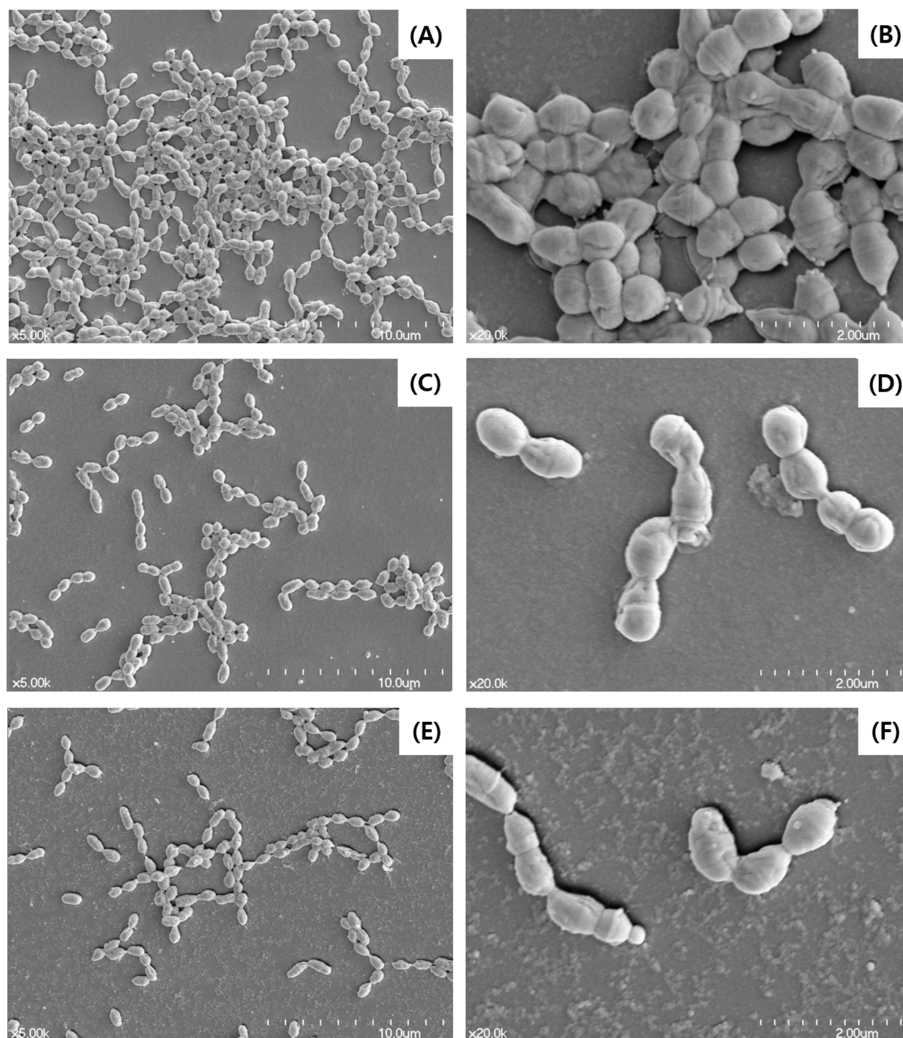

Fig. 4-6. SEM images of *S. sanguinis* biofilm after 1 hour treatment. (A), (B) 1% DMSO group, (C), (D) 2 mg/ml CHX, and (E), (F) 1.0 mg/ml GCE. The group containing GCE and CHX could reduce the biofilm growing on the disks. (A), (C), and (D) is an image measured at 5,000 magnification, and (B), (D), and (F) is measured at 20,000 magnification.

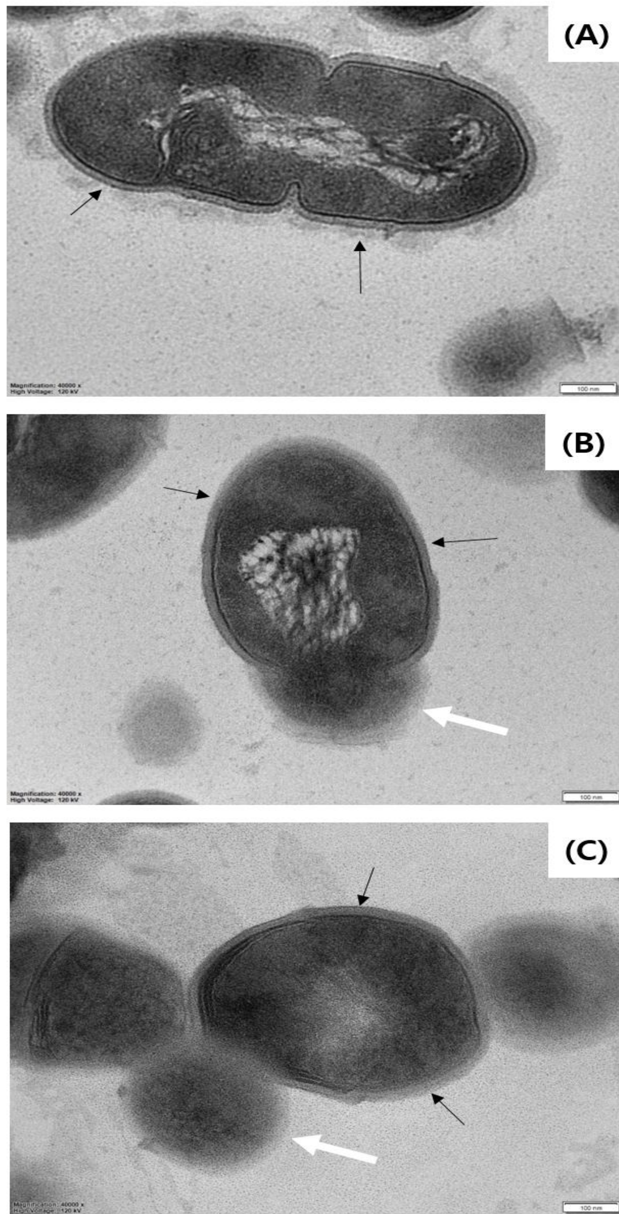

Fig. 4-7. TEM micrographs images of the *S. mutans* biofilm after 1 hour treatment. The black arrows indicate the wall of *S. mutans,* and the white arrows indicate the intracellular contents from the bacteria. The scale bar is 100nm. (A) 1% DMSO group, (B) 2 mg/ml CHX, and (C) 1.0 mg/ml GCE.

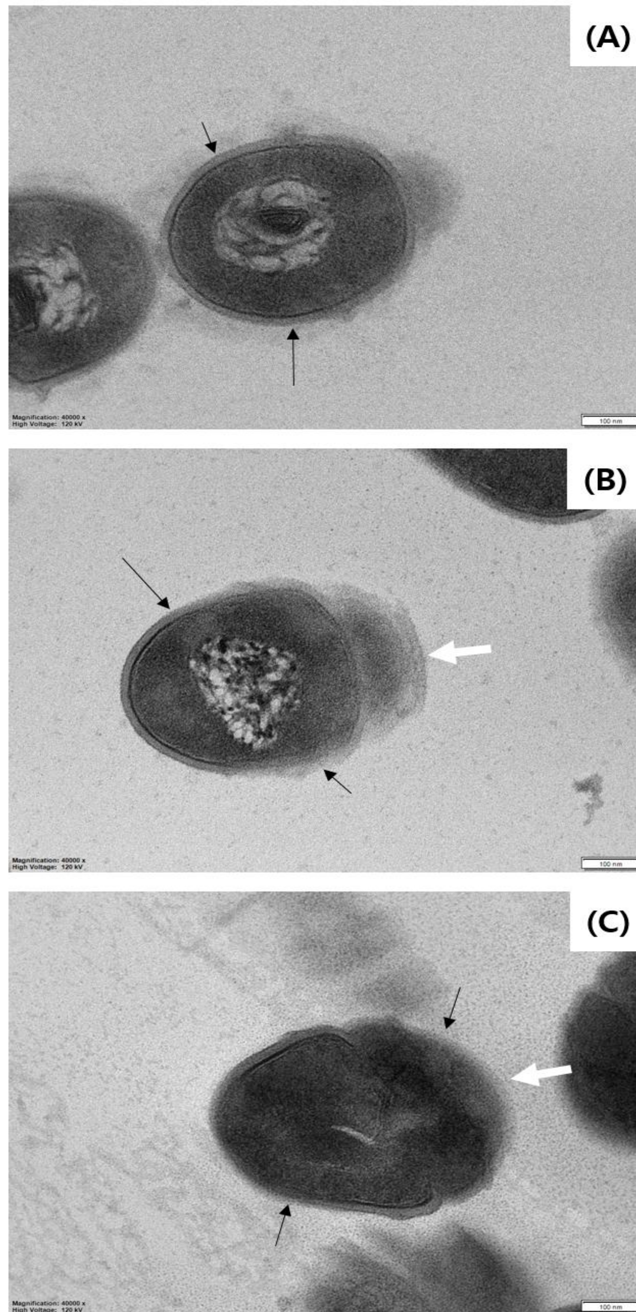

Fig. 4-8. TEM images of *S. oralis* biofilm after 1 hour treatment. (A) 1% DMSO group, (B) 2 mg/ml CHX, and (C) 1.0 mg/ml GCE.

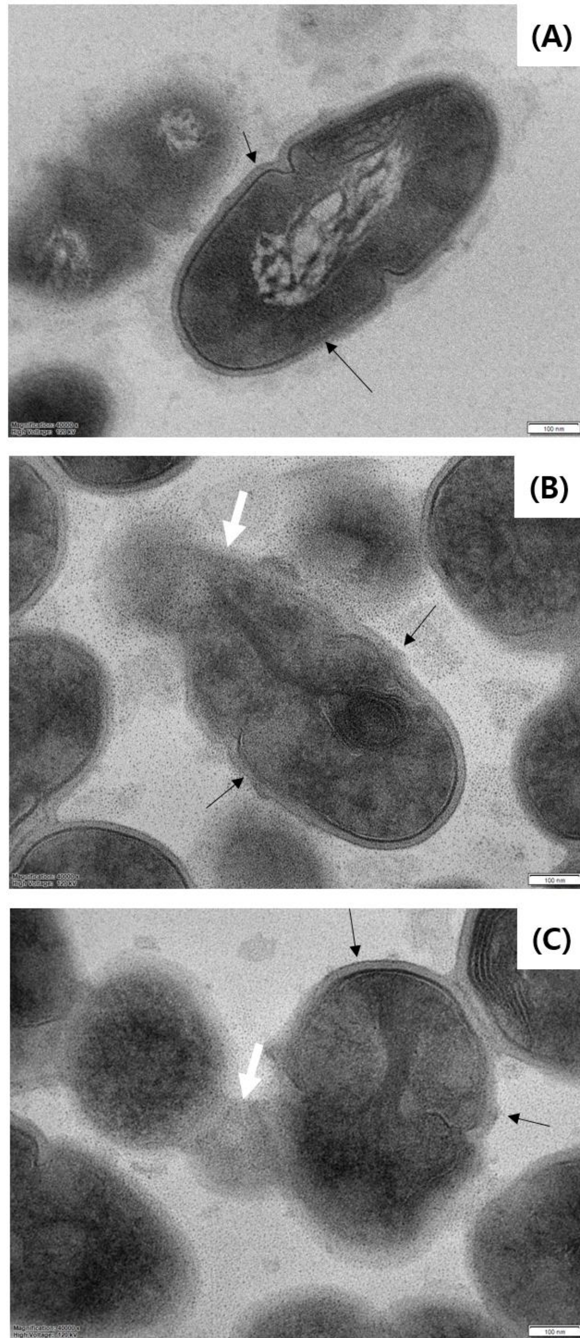

Fig. 4-9. TEM images of *S. sanguinis* biofilm after 1 hour treatment. (A) 1% DMSO group, (B) 2 mg/ml CHX, and (C) 1.0 mg/ml GCE.

4.2 Remineralization effect of GCE and calcium of enamel

4.2.1 Enamel microhardness changes after the experimental procedure

Table 4-5 shows the mean enamel surface hardness after exposure to the remineralization solutions (GCE, GCE+CA, and CA). The GCE+CA groups showed the most enhanced remineralization; 44.2 △VHN; the lowest remineralization effect was found in the CA group; 27.1 △VHN. As a result of one-way ANOVA analysis, the CA group were significantly different from GCE group and GCE+CA group ($p < 0.05$).

Table 4-5. Comparison of the surface microhardness of different groups after remineralization

Condition	N	VHN	VHN	∆VHN	p
		Baseline	After artificial caries formation	After remineralization	
GCE	24	308.8 ± 5.1	48.70 ± 5.00	41.8 ± 3.9[a]	0.009*
GCE+ CA	24	308.9 ± 6.3	46.85 ± 4.95	44.2 ± 3.9[a]	
CA	24	303.2 ± 6.3	42.72 ± 5.19	27.1 ± 5.4[b]	
Control	12	307.3 ± 7.3	44.85 ± 4.85	0.5 ± 3.9[c]	

Values are mean ± SD.
VHN= Vickers hardness test.
∆VHN= After remineralization VHN - After artificial caries formation VHN.
CA, immersed in 1.0 mol $CaCl_2$ for 10 min; GCE, immersed in 4,000 ppm GCE for 10 min; CA+GCE, immersed in 1.0 mol $CaCl_2$ and 4,000 ppm GCE for 10 min; Control, no treatment.
*Statistically significant by repeated measured ANOVA at the a=0.05 level.
a-c The different lower case letters indicate statistically significant differences between same groups by Tukey's HSD post-hoc test at $p < 0.05$.

4.2.2 Antibacterial activity of GCE for *S. mutans* biofilm

After the formation of *S. mutans* biofilm on bovine enamel species, the biofilm was treated with three different solutions. The GCE and GCE+CA groups showed significantly lower numbers of surviving *S. mutans* CFU than those of the control groups ($p < 0.05$). The GCE showed the highest level of antibacterial activity for *S. mutans* biofilm, and the GCE+CA also significant antibacterial activity but less than GCE. The GCE+CA exhibited similar bactericidal activity to GCE. The GCE and GCE+CA groups for 5 min showed 91.0% and 87.5% fewer CFU, respectively than the control group. The GCE group for 10 min showed greater bactericidal activity (94.6%) than that of the GCE+CA and CA group exposed at the same time. However, the CA group (39.3%) showed antibacterial activity but not as much as GCE or GCE+CA (Table 4-6).

Table 4-6. Antibacterial effects of the GCE against *S. mutans* biofilms

Condition	Exposure time		
	CFU (x10^8)		
	0 min	5 min	10 min
GCE	48.8±10.6	$5.2±1.4^a$	$3.1±0.9^a$
GCE+CA	49.5±6.7	$7.8±0.2^a$	$6.5±2.2^a$
CA	51.2±8.8	$34.1±7.8^b$	$30.1±8.4^b$
Control	50.5±9.7	$56.5±9.7^c$	$60.5±10.8^c$

The different superscripts in the same column indicate statistically significant difference from each group ($p < 0.05$).
The data shown are the Mean±SD.

4.2.3 Inhibition of acid production

The pH of culture medium was recorded during 1 hour after each solution treatment to determine the effect of GCE on acid production. The pH patterns of the GCE were significantly different from the control group after 1 min ($p < 0.05$). Both GCE and GCE+CA groups maintained a pH of approximately 7.0 for 1 hour whereas the pH of the control group decreased rapidly from pH 7.3 to pH 6.1 after 1 hour (Fig. 4-10).

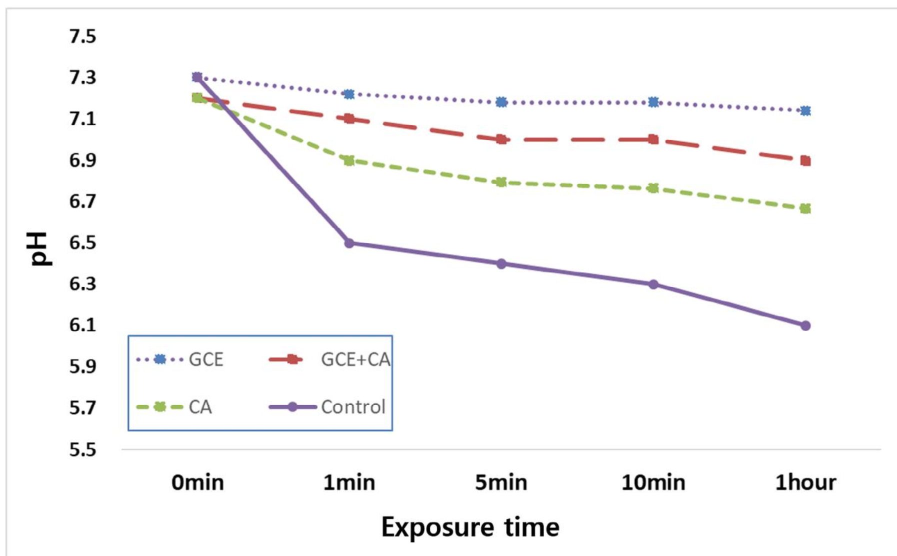

Fig. 4-10. Acidogenicity of *S. mutans* biofilms was determined by measuring the pH of media.

4.2.4 Morphological changes in *S. mutans* biofilms

The mechanism responsible for the antimicrobial activity of GCE was investigated by observing the morphological changes of *S. mutans* by SEM after treating the biofilm with the treatment solution for 10 min. SEM showed less morphological and intracellular content in the GCE and GCE+CA groups for 10 min compared to the control group. Also, GCE and GCE+CA groups showed irregular cell wall structure and showed fewer cells in the chain than the typical long chains observed in the control group (Fig. 4-11).

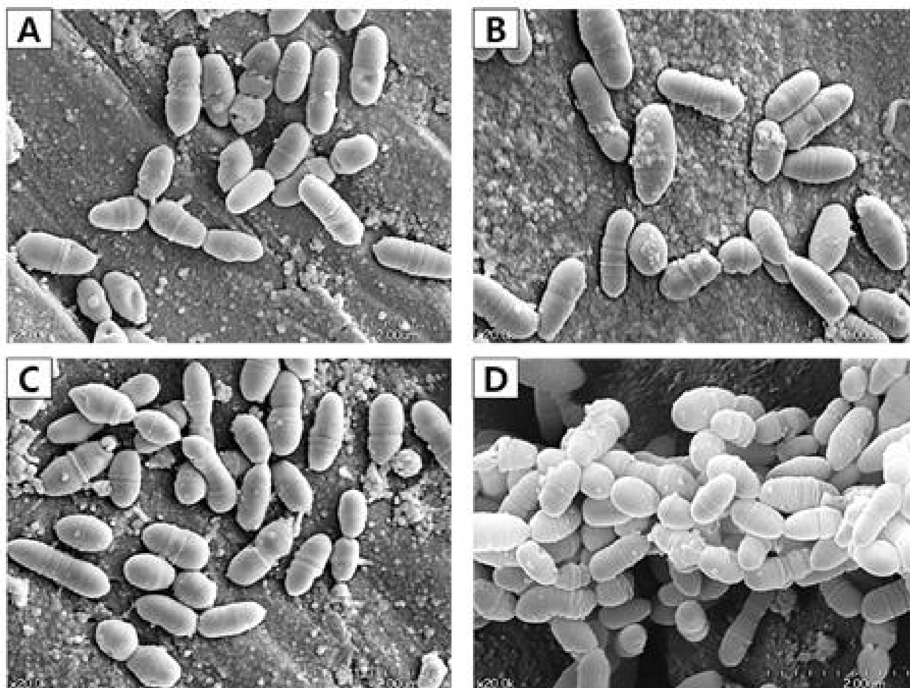

Fig. 4-11. SEM images of the *S. mutans* biofilm after 10 min treatment. (A) GCE, (B) GCE+CA, (C) CA, and (D) Control.

5. Discussion

Dental plaque is a representative example of a biofilm, which plays an essential role in the pathogenesis of dental caries. Dental caries is one of the most well-known biofilm-related diseases that originate from certain bacteria, primarily, *S. mutans*. *S. mutans* plays an important role in metabolizing sucrose to lactic acid, which induces demineralization of the tooth enamel (Loeche, 1986). It initiates the cariogenic process by biofilm formation (Gamboa *et al.*, 2004). A biofilm is a multicellular aggregation of microorganisms attached to the surface of the tooth and deposited as a weak layer. To prevent dental caries, it is crucial to reduce the number of bacteria in the mouth and to inhibit the formation of biofilm. These reasons, biofilm research is used to assess the antibacterial effects of antibiotics, accurately. Synthetic compounds such as chlorhexidine have been used as an antimicrobial agent to inhibit the growth of bacteria and reduce the adhesion of biofilm to prevent dental caries. However, excessive use leads to side effects of synthetic compounds including alteration of the oral cavity, the bacterial tolerance, taste disorders, dry mouth, and tooth discoloration (Flötra *et al.*, 1971; Flötra, 1973). The search for natural anti-plaque agents with safe efficacy and potent activity has focused on reducing the use of synthetic antimicrobials in daily oral care products (Xie *et al.*, 2008).

Natural products have been used for the development of dental caries prevention reagents. However, the complexity of biological samples remains a major obstacle to revealing the effect on

constituents of interest (Huang *et al.*, 2017). *G. Chinensis* has been used in traditional medicine for years. It inhibited the adherence of planktonic oral bacteria as well as inhibiting acid production by cariogenic bacteria. It has been widely studied as a reconstruct tooth enamel following enamel mineral loss and discussed as an effective preventative caries agent due to its unique potential remineralization effects (Grobler & Horst, 1982; Li & Tang, 2006). Also, previous studies on the safety of *G. Chinensis* showed that *G. Chinensis* did not cause toxicity to the cells (Lee *et al.*, 2003). Although a previous study (Kim *et al.*, 2018) investigated the antimicrobial effect of *G. Chinensis* against *S. mutans*, little is known about the relevant conditions regarding GCE exposure time and concentration or about the effect of GCE on the structural and functional activity of various cariogenic bacteria. Also, some studies on antibacterial activity on biofilm model have been conducted, none of the studies investigate the effect of chemical compounds of *G. Chinensis* on the remineralization and antibacterial effects underneath a biofilm model. It was, therefore, necessary to determine the optimal concentration by discovering the anti-caries effect at various concentrations and time on biofilms of various cariogenic bacteria. Its anti-caries effect at various concentrations and times in biofilm conditions of various cariogenic bacteria. Along with this, the present study also investigated the effect of *G. Chinensis* and the combined effect of *G. Chinensis* with calcium on the remineralization and antibacterial effects of enamel underneath

S. mutans biofilm *in vitro*.

The pharmacological effects of herbal medicines and edible plants differ from each other in extraction methods and specific solvents used. In general, solvent selection is the most important variable, since the purity of the extracts depends on the extraction solvent used, such as ethanol, methanol, hexane, and dichloromethane. In this study, we used ethanol as the solvent by previous studies that showed the most significant effect when extracting *G. Chinensis* (Huang *et al.*, 2017). To determine the effect of each concentration of GCE on the growth of bacteria over time, we measured the number of bacteria according to time by biofilm with different concentrations of extract. Our data showed that the GCE has an inhibitory effect when exposed to multispecies oral biofilm at all concentrations. These results were comparable to those obtained in the previous studies (Xie *et al.*, 2008), but since the earlier studies only used a single concentration, it was not possible to determine exactly what the GCE MIC was, and which concentration showed effective growth inhibition. The MIC test determines the lowest concentration at which growth is inhibited by antimicrobial agents. It is used to assess the performance of all other susceptibility testing methods because it is considered the 'gold standard' for determining the susceptibility of microorganisms to antimicrobial agents (Andrews, 2001). Therefore, when searching for new antimicrobial agents, the MIC test is necessary to prevent the side effects resulting from

overuse. In our study, a more detailed analysis of the varying concentrations showed that less than 90% of all bacteria were present at 0.1 mg/ml of GCE, indicating that bacterial growth was inhibited by exposure to a small amount of GCE.

Additionally, when each strain was exposed to 1.0 mg/ml GCE, there was an antibacterial effect comparable to CHX, continuously over long-term exposure. In particular, both the MIC and the bacterial growth curve showed statistically similar effects in the CHX and 1.0 mg/ml GCE groups. Acidogenicity is one of the major physiological factors associated with dental caries (Pecharki *et al.*, 2005). *S. mutans* produce fermentation products such as lactate. Acidogenicity of *S. mutans* changes the ecological environment and reduce plaque pH in the plaque flora (Ajdíc *et al.*, 2002). A pH below 5.4 on the plaque will cause the enamel demineralization (Oatmen, 2011). Therefore, the change of pH was used as an indicator for determining the potential of anti-cariogenicity. Recently, the measurement of the acid production of bacteria has been reported to be measured in the supernatant of the medium, but up to now, acidity measurements of bacterial biofilms have measured the acidity of the medium in many studies (Ajdíc *et al.*, 2002; Kim JE *et al.*, 2008; Yu JH *et al.*, 2018). Acid production was significantly decreased when exposed to GCE compared with 1% DMSO in this study. Especially, the biofilms exposed to 1.0 mg/ml GCE and 2.0 mg/ml CHX in three bacterial groups maintained a constant pH of about 7. This result is comparable to the

antibacterial effect of CHX. It shows that GCE plays a role in inhibiting the production of additional acid from cariogenic bacteria like CHX.

SEM and TEM revealed the effect of GCE on biofilm integrity. SEM and TEM images showed changes in the morphology of bacteria. The bacteria in the control group (1% DMSO) biofilm were dense on the surface, whereas the bacteria in GCE and CHX groups were sparse on the surface, and had longer adherent chains, on the other hands, the bacterial in GCE and CHX groups were sparse on the surface and had short scattered chains in SEM image (Fig. 3A, 3B, 3C). In the TEM images, cariogenic bacteria biofilm exposed to GCE and CHX showed peptidoglycan layer damage and leakage of the intracellular contents as compared with the control group (Fig. 3D, 3E, 3F). Previous studies reported that grapefruit seed extract (Miele, 1988), *Curcuma Xanthorrhiza* extract (Kim *et al.*, 2008) are 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 polyphenol compound from cranberry juice has the same effect (Duarte *et al.*, 2006). The results here are also consistent with the previous study of GCE (Xie *et al.*, 2008). The TEM image data in our study showed that the biofilm structure was also clearly affected by exposing GCE to bacterial biofilm. Studies examining the effect of GCE on these bacterial structures with images such as TEM have been rare thus far. The evidence

for the inhibitory effect of GCE on bacterial adhesion is unclear, though polyphenols can form complexes with proteins and polysaccharides (Haslam, 1996). GCE (data not presented) has been shown to contain significant quantities of monomeric and polymeric polyphenols along with other components. GEC polyphenols can interact with bacterial membrane proteins through hydrogen bonding with hydroxyl groups. This reaction can change the permeability of the membrane, causing cell destruction and inhibiting cell proliferation (Burt, 2004). In addition, polyphenols can penetrate bacterial cells and disrupt proton power, electron flow, active transport, and cell contents, thereby reducing lactic acid production (Huang *et al*., 2017). Based on these findings, GCE may inhibit bacterial growth by destroying the bacterial cellular structure.

Our data showed that the GCE exhibits an apparent remineralization effect on bovine enamel. In the present study, surface hardness change was assessed with microhardness measurement. Since the enamel surface is not uniform to remineralization, we tried to measure it at the same spot, and it was repeatedly measured three times when measurement after experiment process, for reduce errors during the measurement process. The measurement of surface enamel hardness using microhardness determination is judged as a suitable tool to investigate the surface softening of enamel (Curzon & Hefferren, 2001). The one finding in this study was that enamel remineralized

with GCE and GCE+CA showed more deposited than those of the CA group. Their mineralization effect of GCE was previously observed by Chu *et al.* (2007), Kang *et al.* (2008). It showed a thick layer was formed on the surface of the enamel in the GCE group. The reason for this is not apparent, but maybe these results indicate that GCE has more ion channels to the lesion body, so makes minerals to deposit more. In addition, when GCE was combined with calcium, a higher remineralization effect was seen compared to the calcium group (Table 4-6). It indicated that the combined use of calcium and GCE has a synergistic effect in improving remineralization on enamel underneath biofilm model. The results of this study corresponded well with those of an earlier study that reported that a chemical compound in GCE might act as a calcium ion carrier, supplying the caries lesion with calcium ions from the remineralization solution (Tian *et al.*, 2009). Cheng & ten Cate (2010) also proposed that some component of GCE might combine with the enamel crystals of a surface layer and inhibit the demineralization of enamel. This may be mainly due to their different mechanisms of action for remineralization. Based on all these results, it can be shown that GCE can directly affect remineralization of the enamel surface and can affect the calcium deposition on the demineralized enamel surface in the combined group during the remineralization process.

There were significantly fewer CFUs of *S. mutans* in the group exposed to GCE and GCE+CA than in the group exposed to CA

and control group ($p < 0.05$). In addition, the biofilm exposed to GCE and GCE+CA maintained a constant pH around 7. This result shows that GCE can stop the additional acid production of *S. mutans*. This is similar to the results of previous studies on the antibacterial effect of CHX, the most effective antibacterial agent (Kim *et al*., 2008). From the above results, GCE has potential as an antimicrobial agent against *S. mutans* instead of CHX, which has many side effects when used for a long time. According to the SEM images in this study, GCE might have destroyed *S. mutans* chain. *S. mutans* exposed to GCE also showed morphology change compared with the control group. The antimicrobial activity of *G. Chinensis* on common oral bacteria has been confirmed, as its main component, gallotannins, was found to be bactericidal for *S. mutans* strains (Wu-Yuan *et al*., 1998). GCE may additionally function by adjusting biofilm structure, composition, and glucosyltransferase activity besides directly inhibiting both bacteria growth and lactic acid formation. Also, GCE has been proven to limit acidic accumulation from carbohydrate metabolism and reduce the proportion of cariogenic bacteria in the biofilm and inhibit demineralization. These results are similar to those of previous studies of natural materials. A grapefruit seed extract is a naturally antibacterial material. It can weaken the function of the physiologically active enzyme in microorganism cells and also destroy the cell wall function (Camargo *et al*., 2006). And also, Kim *et al*. (2008) studied about *Curcuma Xanthorrhiza* extract, it has

strong bactericidal acidity, inhibitory effects on acidogenesis, and alters the microstructure of *S. mutans* biofilm.

Oral tissue cells are more resistant to compounds *in vivo* than *in vitro* because oral tissue cells are continuously supplied with nutrients through blood flow *in vivo*, resulting in improved regenerative capacity. Alternatively, since oral bacteria form a biofilm, it is necessary to introduce a higher concentration of antimicrobial agent than the concentrations generally required for antimicrobial activity against airborne bacteria. Nevertheless, antibiotics such as penicillin, vancomycin, and tetracycline, which are used to inhibit bacteria that form dental biofilms, can lead to tolerance when they are used too frequently. In addition, this study performed using bovine enamel instead of human enamel specimens. However, bovine enamel specimens instead of human enamel specimens were primarily chosen due to several reasons. Human enamel is often difficult to obtain in sufficient quantity and with adequate quality, due to extensive caries lesions or other defects. And also, it can cause significant variations in the outcome measures due to the source and age of the collected human teeth (Yassen *et al.*, 2011). Bovine teeth have been used instead of human teeth, as in other investigations (Cheng & ten Cate, 2010; Huang *et al.*, 2010). Camargo *et al.* (2006) revealed no significant difference between bovine and human teeth in the pH measurement. That is why we used bovine teeth in this study. This study confirmed the bacterial growth effect of representative

causative bacteria of dental caries in various GCE concentrations. However, since this is an *in vitro* study, future studies investigating the methods of testing the efficacy and stability of GCE should be *in vivo*, and studies using biofilm models are needed. Further, future studies using randomized clinical trials will be required to determine clinical relevance.

6. Conclusions

This study aimed to evaluate the antimicrobial activity of various concentrations of GCE on *S. mutans* and other oral streptococci related to dental caries and to determine the optimum concentration and to investigate the effects of *G. Chinensis* with calcium on enhancing remineralization, and also the antibacterial effect of *G. Chinensis* underneath *S. mutans* biofilm was evaluated by examining the bactericidal activity, acidogenesis, and morphology *in vitro*.

There was a statistically significant difference in bacterial growth inhibition depending on the concentration of GCE. Bacterial growth over time was 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 as that of 2.0 mg/ml CHX. The 1.0 mg/ml GCE group showed incomplete septa that were observed as a disruption of the cell membrane in the outline of the cell wall. In addition, there was a slight exudation of the intracellular contents in both the 1.0 mg/ml GCE and 2.0 mg/ml CHX groups. GCE+CA group showed the highest efficacy in enhancing remineralization. The GCE group showed the highest level of antibacterial activity for *S. mutan*s biofilm, and the GCE+CA group also significant antibacterial activity but less than GCE group ($p < 0.05$). Both the GCE and GCE+CA groups maintained a pH of approximately 7.0 for 1-hour treatment whereas the pH of the control group decreased rapidly from pH 7.3 to pH 6.1.

These results demonstrate that GCE inhibits the growth of *S. mutans*, *S. sanguinis*, and *S. oralis* with increasing time and concentrations. Additionally, it alters the microstructure of *S. mutans* biofilm. In particular, when GCE concentration was 1.0 mg/ml, there was a statistically significant effect comparable with 2.0 mg/ml CHX. This study also found that natural *G. Chinensis* has a significant impact on enhancing the remineralization of enamel lesion, and it had combined synergic effects with calcium in improving remineralization. This study result suggests that GCE might be a useful anti-bacterial agent for preventing dental caries.

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국문초록

오배자 추출물의 항균효과와 재광화효과

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김 은 정

오배자는 붉나무 잎에 오배자 진딧물이 자상을 주어 생기는 벌레집으로서, 항균작용, 항산화 및 재광화 작용을 하는 것으로 보고되었다. 새로운 물질을 항생제로 사용할 때 남용으로 인한 악영향을 방지하기 위하여 물질의 노출과 관련하여 그 특성, 효능 및 안전성을 보다 잘 이해하기 위한 연구가 수행되어야 한다. 이전 연구에서 불소와 오배자의 화합물이 법랑질의 재광화를 향상시키는 영향에 대하여 보고한 바는 있으나, 칼슘과 오배자의 화합물이 치아의 법랑질 재광화에 미치는 영향에 대해 발표된 연구는 아직 없었다.

이에 이번 연구에서는 항균, 재광화 효과가 있다고 알려진 오배자 추출물의 항균활성을 *Streptococcus mutans* 와 기타 치아 우식을 유발하는 균을 이용하여 항균효과를 평가하고, 최적 농도를 조사하였으며, 오배자 추출물과 칼슘 혼합 물질의 법랑질 재광화 효과를 확인하

고자 하였다.

항균 작용의 효과 실험을 위하여, 오배자 추출물과 클로르헥시딘(양성대조군), dimethyl sulfoxide(음성대조군)을 사용하였다. *S. mutans, S. sanguinis, S. oralis* 로 형성된 바이오 필름을 다양한 오배자 추출물의 농도와 노출 시간으로 처리한 후, 살균 활성, 산 발생, 최소 억제 농도 및 세균 형태 변화를 평가하였다. 재광화 효과 실험을 위하여, 1.0 mol 칼슘(CA), 4,000 ppm 오배자추출물(GCE), 4,000 ppm 오배자추출물과 1.0 mol 칼슘 혼합액(GCE+CA)으로 처리한 후, 법랑질 표면 경도 등을 측정하여 다음의 결론을 얻었다.

1. 오배자의 농도가 증가함에 따라 시간에 따른 박테리아 성장은 억제되었다.
2. 1.0 mg/ml 오배자의 농도는 *S. mutans* 와 *S. oralis* 바이오 필름에 대해 2.0 mg/ml 클로르헥시딘과 유사한 항균효과를 보였다.
3. TEM 관찰 결과, 1.0 mg/ml 오배자 군은 불완전 충격을 보여 세포벽 윤곽에서 세포막의 파괴가 관찰되었고, 1.0 mg/ml GCE 실험군과 2.0 mg/ml CHX 대조군에서 세포 내 내용물의 삼출이 보였다.
4. GCE 와 CA 혼합군에서 재광화 효과가 가장 높았다 ($p < 0.05$).

결론적으로, 오배자가 법랑질의 재광화를 향상시키고 칼슘과 상승효과가 있으며, 산 생성을 억제하고 치아우식세균의 미세구조를 변화시키는 것을 보여, 항우식제로서 사용 가능성을 가늠해 볼 수 있었다.

주요어: 오배자, 재광화효과, 항균효과

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