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Antimicrobial Effects of Herbal Extracts on *Streptococcus mutans* and Normal Oral Streptococci

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Streptococcus mutans **is associated with dental caries. A cariogenic biofilm, in particular, has been studied extensively for its role in the formation of dental caries. Herbal extracts such as *Cudrania tricuspidata*, *Sophora flavescens*, *Ginkgo biloba*, and *Betula Schmidtii* have been used as a folk remedy for treating diseases. The purpose of this study was to evaluate and compare the antibacterial activity of herbal extracts against normal oral streptococci, planktonic and biofilm of *S. mutans*. *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *S. mutans* were cultivated with brain heart infusion broth and susceptibility assay for the herbal extracts was performed according to the protocol of Clinical and Laboratory Standard Institute. Also, *S. mutans* biofilm was formed on a polystyrene 12-well plate and 8-well chamber glass slip using BHI broth containing 2% sucrose and 1% mannose after conditioning the plate and the glass slip with unstimulated saliva. The biofilm was treated with the herbal extracts in various concentrations and inoculated on Mitis-Salivarius bacitracin agar plate for enumeration of viable *S. mutans* by counting colony forming units. Planktonic *S. mutans* showed susceptibility to all of the extracts and *S. mutans* biofilm exhibited the highest level of sensitivity for the extracts of *S. flavescens*. The normal oral streptococci exhibited a weak susceptibility in comparison to *S. mutans*. *S. oralis*, however, was resistant to all of the extracts. In conclusion, the extract of *S. flavescens* may be a potential candidate for prevention and management of dental caries.**

Keywords: antibacterial activity, herbal extracts, *S. mutans* biofilm

Introduction

Streptococcus mutans is associated with dental caries (Loesche, 1986). *S. mutans*, being an aciduric bacteria, consistently produces acid within the acidic environment of oral biofilm (Hamilton and Buckley, 1991). Also, glucan produced by *S. mutans* from sucrose using glucosyltransferases (Monchois *et al.*, 1999) play a key role in the development of oral biofilm. Water–soluble or –insoluble glucans synthesized by glucosyltransferases B, C, and D (Aoki *et al.*, 1986; Hanada and Kuramitsu, 1988, 1989) contribute to biofilm formation by providing binding sites for bacterial colonization on tooth surface (Lee *et al.*, 2012). Therefore, *S. mutans* is considered to be the most important contributor to the formation of a cariogenic biofilm and induction of dental caries.

Herbal extracts have frequently been used in traditional medicine for treating various diseases. Herbal extracts also exhibit an antibacterial activity for oral pathogens. *Cudrania tricuspidata*, *Sophora flavescens, Ginkgo biloba*, and *Betula schmidtii* have been reported to exhibit a strong antibacterial activity for *S. mutans* (Baek, 2007). *C. tricuspidata* is a deciduous tree frequently used to treat inflammation and tumor (Lee *et al.*, 1996). Glycoprotein isolated from *C. tricuspidata* inhibited inducible nitric oxide (iNO) and cyclooxygenase-2 (COX-2) expression in lipopolysaccharide-stimulated RAW 264.7 cells (Joo and Lim, 2009). The extracts of *S. flavescens* and *G. biloba* are known as anti-inflammatory molecules used in treatment of wound (Cheung *et al.*, 2002; Kotakadi *et al.*, 2008; Han and Guo, 2012) and have also been illustrated for their antibacterial activity (Cheung *et al.*, 2002; Sati and Joshi, 2011; Kim *et al.*, 2013). The extracts of *G. biloba* are especially used in cardiovascular disorders and Alzheimer's diseases for their protective effects on such diseases (Zhang *et al.*, 2011; Vellas *et al.*, 2012). The efficacy of *B. schmidtii* for oral bacteria is scarcely known. Investigation for antibacterial activity of these herbal extracts against *S. mutans* biofilm and normal oral streptococci has yet been performed. Normal oral streptococci such as *S. gordonii*, *S. salivarius*, *S. sanguinis*, and *S. oralis* play an important role in the maintenance of oral hygiene by suppressing the colonization of cariogenic and periodontal pathogens (Costerton, 1999; Haffajee and Socransky, 2006). Therefore, the best example of antibacterial agents for management of oral diseases will be to kill oral pathogens or inhibit their growth without affecting normal oral streptococci.

The purpose of this study was to investigate the antibacterial activity of four herbal extracts against *S. mutans* and normal oral streptococci. Furthermore, the bactericidal effect on biofilm or planktonic *S. mutans*, and normal oral streptococci was compared.

Materials and Methods

Bacterial species and cultivation

Streptococcus mutans ATCC 25175 was purchased from American Type Culture Collection (ATCC), and cultivated

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with Brain heart infusion broth (BHI; BD Bioscience, USA). *Streptococcus gordonii* ATCC 10558, *Streptococcus oralis* ATCC 9811, *Streptococcus salivarius* ATCC 13419, and *Streptococcus sanguinis* ATCC 10556 were cultivated with BHI broth. For biofilm formation, *S. mutans* was cultured in 8-well chamber glass slip or 12-well polystyrene plate using BHI broth supplemented with 2% sucrose and 1% mannose according to methods of described by Lee *et al.* (2012).

Preparation of herbal extracts

Herbal extracts with methyl-alcohol were purchased from the Korean plant extract bank (International Biological Material Research Center, Korea). The extracts were *Cudrania tricuspidata*, *Sophora flavescens*, *Ginkgo biloba*, and *Betula Schmidtii* from. The extracts were dissolved with dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) to a final concentration of 1 mg/ml.

Antibacterial activity of herbal extracts against *S. mutans* and normal oral streptococci

The susceptibility assay of *S. mutans* and normal oral streptococci for the herbal extracts was performed according to the methods of Clinical Laboratory Standard Institute (CLSI, 2012). Briefly, the bacteria was cultured in Typicase Soy broth (TSB; BD Bioscience) overnight before the testing day, and bacterial number was counted with a bacterial counting chamber (Hausser Science, USA). The bacterial concentration was adjusted to 2.5×10^7 cells/ml using fresh TSB after harvesting by centrifugation. Fresh TSB (180 µl) was dispensed into each well in 96-well plate (SPL Lifescience, Korea), and the extracts were inoculated into 12th well. The extracts were diluted with micropipette over a serial two-fold dilution range (final concentration range of 0.98–250 µg/ml). The bacterial suspensions (20 µl; 5×10^6 cells) were inoculated into the extracts contained in 96-well plate and incubated at 37°C in aerobic atmosphere overnight. The bacterial growth was measured with a spectrophotometer at 660 nm. The minimum inhibitory concentration for the bacteria was defined as the lowest concentration of the extract which completely inhibited bacterial growth.

Preparation of polystyrene and glass chamber for biofilm

Unstimulated saliva from 10 healthy donors was centrifuged at 7,000×g for 10 min at 4°C, and mixed with an equal volume of phosphate buffered saline (PBS). The mixture was dispensed into each well of 12-well plate (SPL Lifescience) or 8-well chamber glass slip (BD Falcon^{TM}, USA) and completely dried in dry oven at 40°C followed by UV sterilization. These procedures were repeated 5 times.

Formation and observation of *S. mutans* biofilm

S. mutans was incubated using BHI broth at 37°C overnight and then inoculated into fresh BHI broth supplemented with 2% sucrose and 1% mannose. For formation and observation of *S. mutans* biofilm, *S. mutans* suspension was inoculated into the prepared 12-well plate and 8-well chamber glass slip and incubated at 37°C in aerobic condition for 72 h. The medium was changed every day with fresh BHI containing 2% sucrose and 1% mannose. The biofilm was washed three times with PBS to remove planktonic *S. mutans* and treated with herbal extracts in various concentrations or in various times. After removing the herbal extract, the biofilm was washed three times with PBS and then mixed with 1 ml of BHI broth. The biofilm was physically disrupted with a small cell scraper (Corning Co., USA) and transferred into 1.5 ml tube. After homogenization using vortex for 1 min, the bacterial suspensions were serially diluted from 10 to 10^7 with BHI broth and 50 µl of suspension from each dilute was inoculated onto Mitis-salivarius bacitracin agar plate (BD Bioscience). The plates were incubated at 37°C for 36 h and colonies of *S. mutans* were counted. In order to analyze the extract-treated biofilm using imaging, the biofilm was washed three times with PBS after treating with the extracts and stained with bacterial live/dead staining kit (Invitrogen, USA). The biofilms were analyzed by LSM 700 confocal laser scanning microscope (CLSM; Carl-Zeiss, Germany). The depth and bacterial survival of biofilm were measured using z-stack scans of five randomly chosen locations from 0 to 30 µm per sample.

Statistical analysis

The statistical analysis of total data was analyzed with Kruskal-Wallis test and the significant difference between non-treated and extract-treated bacteria was analyzed with Mann-Whitney test using SPSS 10 (SPSS Inc., USA). *P* values less than 0.05 were considered statistically significant and expressed with an asterisk (*).

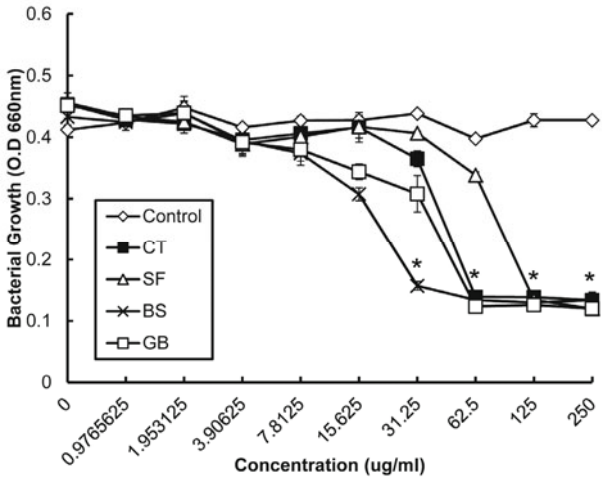

Fig. 1. Susceptibility assay of *S. mutans* for herbal extracts. *S. mutans* was cultured in BHI broth and inoculated into TSB. After cultivating overnight, susceptibility test of *S. mutans* for herbal extracts was performed according to the protocol of CLSI. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. * Significance compared to untreated control bacteria ($P<0.05$). CT, extract of *C. tricuspidata*; SF, extract of *S. flavescens*; GB, extract of *G. biloba*; BS, extract of *B. schmidtii.*

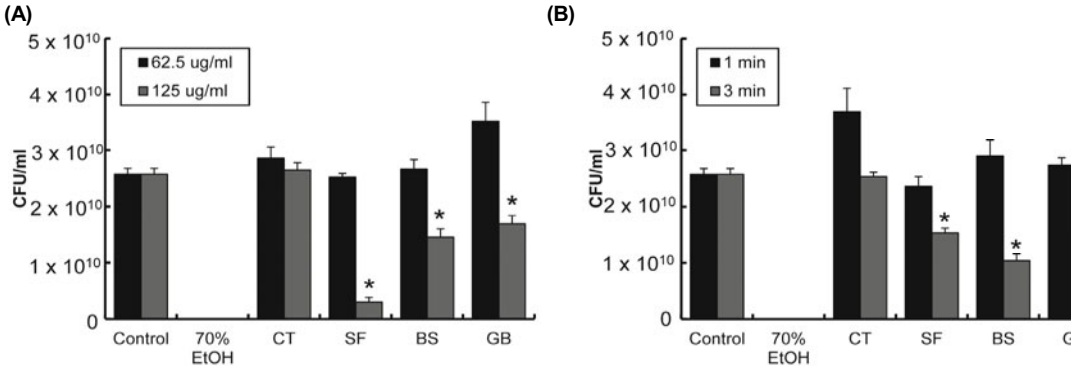

Fig. 2. Anti-biofilm activity of herbal extracts. *S. mutans* was cultured in BHI broth and biofilm was formed for 72 h using fresh BHI broth including sucrose and mannose. *S. mutans* biofilm was treated with the extracts in the indicated concentrations for 1 min (A) or at 62.5 μg/ml for various times (B) and then inoculated on MSB agar plate after disruption of the biofilm. The plate was incubated for 36 h, and colonies were counted. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. * Significance compared to untreated control bacteria (P<0.05). CT, extract of *C. tricuspidata*; SF, extract of *S. flavescens*; GB, extract of *G. biloba*; BS, extract of *B. schmidtii.*

Results

Susceptibility assay of *S. mutans* against herbal extracts

The susceptibility test of *S. mutans* for the herbal extracts was investigated. The extracts of *C. tricuspidata*, *S. flavescens*, and *G. biloba* inhibited the growth of *S. mutans* at the concentration of 62.5 μg/ml. *B. schmidtii* reduced the growth of *S. mutans* at the concentration of 31.25 μg/ml (Fig. 1). Although the concentrations of herbal extracts for antibacterial efficacy was slightly different from the previous studies (Baek, 2007), the extracts exhibited a bactericidal effect on planktonic *S. mutans*.

Antibacterial activity of herbal extracts for *S. mutans* biofilm

Since dental caries is associated with oral biofilm rather than planktonic bacteria, the effect of herbal extracts on *S. mutans* biofilm was investigated. After the formation of *S. mutans* biofilm on a 12-well polystyrene plate, the biofilm was treated with two different concentrations of herbal extracts and then inoculated on MSB agar plates. The extract of *S. flavescens* (125 μg/ml) showed the highest level of antibacterial activity for *S. mutans* biofilm, and the extracts of *G. biloba* and *B. schmidtii* also significant antibacterial activity but less than *S. flavescens* extract. However, the extract of *C. tricuspidata* had negligible effect. Furthermore, no antibacterial activity was shown at the concentration of 62.5 μg/ml for all of the extracts (Fig. 2A). In time kinetic test of herbal extracts at 62.5 μg/ml, only the extracts of *S. flavescens*, *G. biloba*, and *B. schmidtii* showed a bactericidal effect in 3 min (Fig. 2B). In order to investigate whether reduction of CFU was resulted from bactericidal effect or removing biofilm of the herbal extracts, biofilms were analyzed by confocal laser microscope after bacterial live/dead staining. As shown in Fig. 3, *S. flavescens* exhibited removal and bactericidal effect on *S. mutans* biofilm, and *B. schmidtti* and

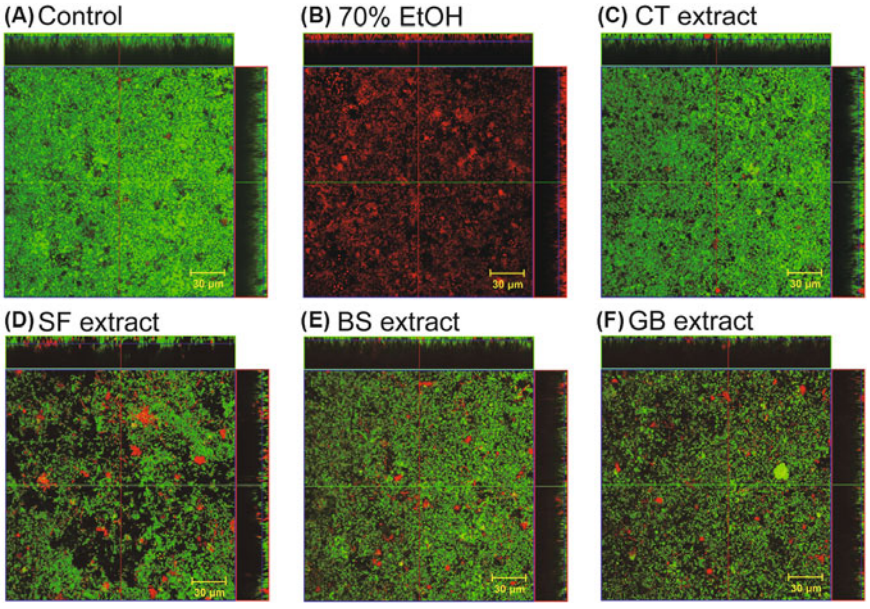

Fig. 3. Image of *S. mutans* biofilm using confocal laser microscope. *S. mutans* was cultivated on saliva-conditioned 8-well chamber glass slip for 72 h, and then treated with the extracts (125 μg/ml) for 1 min. The biofilm was analyzed by confocal laser microscope after staining with bacterial live/dead staining kit. Green and red colors indicated live and dead bacteria, respectively.

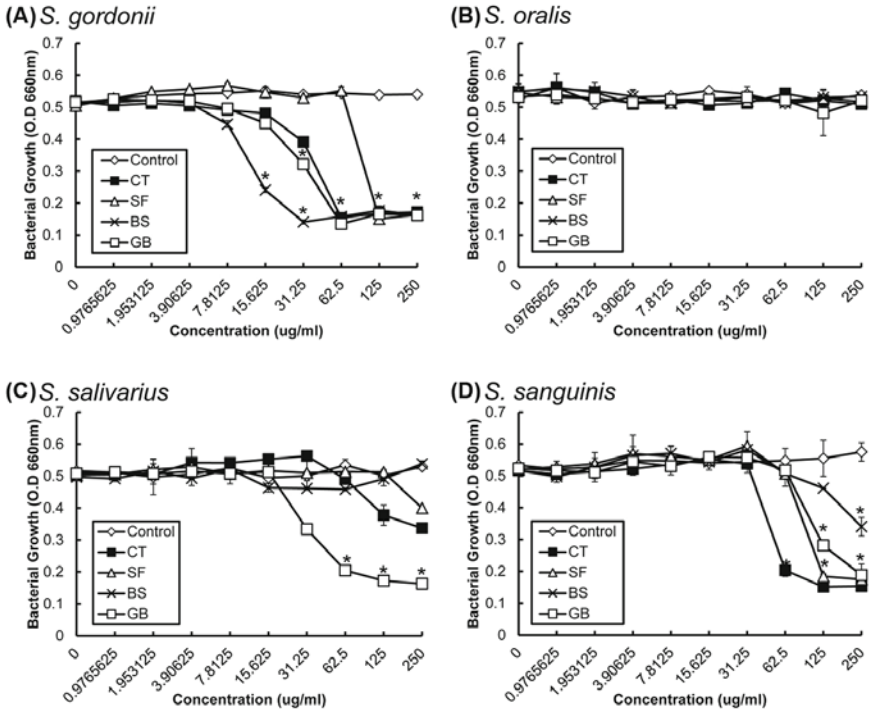

Fig. 4. Susceptibility assay of normal oral streptococci for herbal extracts. *S. gordonii*, *S. oralis*, *S. salivarius*, and *S. sanguinis* were cultivated in TSB overnight and susceptibility test for herbal extracts was carried out according to the protocol of Clinical and Laboratory Standard Institute. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. * Significance compared to untreated control bacteria ($P<0.05$). CT, extract of *C. tricuspidata*; SF, extract of *S. flavescens*; GB, extract of *G. biloba*; BS, extract of *B. schmidtii.*

G. biloba showed only bactericidal effect.

Susceptibility assay of normal oral streptococci for herbal extracts

In order to investigate the antibacterial activity of the herbal extracts for normal oral streptococci, *S. gordonii*, *S. salivarius*, *S. sanguinis*, and *S. oralis* were treated with the extracts in various concentrations according to the CLSI's protocol. The susceptibility of *S. gordonii*, *S. salivarius*, and *S. sanguinis* to the herbal extracts were different. As shown in Fig. 4, *S. gordonii*, *S. salivarius*, and *S. sanguinis* showed susceptibility to *B. schmidtii*, *G. biloba*, and *C. tricuspidata* extracts at 15.625 μg/ml, 62.5 μg/ml, and 62.5 μg/ml concentrations, respectively. However, *S. oralis* was resistant to all of the herbal extracts tested in this study (Fig. 4B).

Discussion

S. mutans play an important role in the induction of dental caries (Loesche, 1986). In particular, *S. mutans* biofilm is directly associated with dental caries formation rather than its planktonic counterpart (Lee *et al*., 2012). *S. mutans* synthesize glucan as exopolysaccharide from sucrose using glucosyltransferases (Monchois *et al*., 1999). Thus, cariogenic biofilm, an oral biofilm containing *S. mutans*, is composed of oral bacteria, exopolysaccharide, bacterial debris and so on (Marsh and Bradshaw, 1995). Exopolysaccharide has been reported to occupy up to 50% of oral biofilm and has various functions such as a barrier for antibacterial agents and basement of biofilm architecture (Socransky and Haffajee, 2002). *S. mutans* biofilm exhibit a different susceptibility to the antibacterial agents compared to the planktonic *S. mutans* (Pasquantonio *et al*., 2008; Sendamangalam *et al*., 2011). In this study, planktonic *S. mutans* as well as *S. mutans* biofilm were investigated and compared for susceptibility to herbal extracts in two conditions. Normal oral streptococci, such as *S. gordonii*, *S. oralis*, *S. salivarius*, and *S. sanguinis*, was also investigated for susceptibility to the herbal extracts and the candidate extract for prevention or management of dental caries was elucidated.

The susceptibility test of herbal extracts for antibacterial activity against oral pathogens was initially performed according to the protocol of Clinical Laboratory Standard Institute with reference to the results of Baek (Baek, 2007). Planktonic *S. mutans* showed susceptibility to the extracts of *B. schmidtii*, *G. biloba*, *C. tricuspidata*, and *S. flavescens*. The extract of *B. schmidtii* showed the highest antibacterial activity for planktonic *S. mutans*. Kim *et al*. (2013) showed that sophoraflavanone G, which is a fraction of *S. flavescens* extract, has a bactericidal effect on *S. mutans* and *S. sobrinus* at a concentration up to 100 μg/ml. This is in accordance with the results of our study.

Next, the bactericidal effect of the herbal extracts was analyzed with *S. mutans* biofilm. *S. mutans* biofilm was formed according to the methods illustrated by Lee *et al.* (2012). Briefly, polystyrene plates were conditioned using unstimulated saliva and then inoculated with *S. mutans* followed by incubation for 72 h. As a positive control, 70% ethyl alcohol was used which completely killed *S. mutans* in biofilm. *S. mutans* biofilm showed the highest level of sensitivity for *S. flavescens* extract followed by *B. schmidtii* and *G. biloba* extracts. However, *C. tricuspidata extract* had no influence on *S. mutans* biofilm. While *B. schmidtii* extract had the strongest bactericidal effect on planktonic *S. mutans*, *S. flavescens* extract showed the highest level of antibacterial activity for *S. mutans* biofilm. The biofilm is composed of

exopolysaccharide, bacterial DNA, and debris, which act as a filtration on antibacterial agents like chromatography (Gilbert and Allisaon, 1999; Socransky and Haffajee, 2002). Thus, the effect of antibacterial agent on the bacteria in biofilm differs by its penetration. Also, the bactericidal concentration of antibacterial agents for biofilm may be up to 100–1,000 fold higher than planktonic bacteria (Costerton, 1999). However, the results of this study showed that the bactericidal concentration of herbal extracts had no difference between biofilm and planktonic *S. mutans*. It can be assumed that the herbal extracts may not be affected by the components within biofilm.

An ideal antimicrobial agent for clinical application should only affect oral pathogens without disturbing normal oral streptococci (Sbordone and Bortolaia, 2003). Therefore, susceptibility assay of normal oral streptococci for the herbal extracts was investigated. Susceptibility of *S. gordonii*, *S. oralis*, *S. salivarius*, and *S. sanguinis* for herbal extracts showed a different trend. *S. gordonii*, *S. salivarius*, and *S. sanguinis* showed the highest sensitive for the extracts of *B. schmidtii*, *G. biloba*, and *C. tricuspidata*, respectively. *S. oralis* was resistant to all of the extracts tested in this study. In comparison to *S. mutans*, *S. gordonii* showed a similar sensitivity to the extracts while the other species did not. Oral biofilm forms in a particular sequence as follows; first, early colonizers such as *S. gordonii*, *S. oralis*, *S. salivarius*, and *S. sanguinis* attach to the tooth surface, and then *S. mutans* bind to these early colonizers. For this reason, an ideal agent for caries prevention should possess an antibacterial activity against *S. mutans* without the same effect for normal oral streptococci or the early colonizers. *C. tricuspidata* extract only affected *S. sanguinis* at 62.5 μg/ml. Essential oil of *C. tricuspidata* showed antibacterial effect against *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli* in the range of 250–1,000 μg/ml (Bajpai, 2013). The bactericidal effect of *C. tricuspidata* on oral bacteria was first illustrated in this study. *B. schmidtii* extract had antibacterial activity for *S. gordonii* and *S. mutans* but had no effect on *S. salivarius* and *S. sanguinis*. Baek (2007) examined the antibacterial activity of eight extracts from wild plant, including *B. schmidtii*, against oral bacteria and found only *Alnus* species and *Schizandra chinensis* to be effective. *B. schmidtii* was reported to have no effect on oral bacteria which is contrary to the results of our study. Further investigation is necessary to fully explain such differences.

In conclusion, among various herbal extracts, the extract of *S. flavescens* has the greatest potential for prevention of *S. mutans* biofilm and weak or no bactericidal effect on normal oral streptococci. Further investigation is required to isolate the fraction of *S. flavescens* responsible with antibacterial activity and also the effect of such herbal extracts on human oral cells.

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