Journal of Medicinal Plants Research Vol. 5(25), pp. 6061-6066, 9 November, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals
DOI: 10.5897/JMPR11.1008

Full Length Research Paper

Tribulus terrestris inhibits caries-inducing properties of *Streptococcus mutans*

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Accepted 9 September, 2011

In the present study, we examined the inhibitory effects of the ethanol extract of *Tribulus terrestris* on the growth, acid production, adhesion, and water-insoluble glucan synthesis of *Streptococcus mutans*. The growth and acid production of *S. mutans* were significantly inhibited in the presence of the ethanol extract of *T. terrestris* (0.1 to 0.5 mg/ml). The ethanol extract of *T. terrestris* (0.1 to 0.5 mg/ml) also significantly lowered the adherence of *S. mutans* in a dose-dependent manner. In the water-insoluble glucan synthesis assay, 0.025 to 0.5 mg/ml of the ethanol extract of *T. terrestris* significantly inhibited the formation of water-insoluble glucans. These results suggest that *T. terrestris* may inhibit the caries-inducing properties of *S. mutans*. Further studies are necessary to clarify the active constituents of *T. terrestris* responsible for such biomolecular activities.

Key words: *Tribulus terrestris*, acid production, adhesion, water-insoluble glucan, *Streptococcus mutans.*

INTRODUCTION

Dental caries is a bacterial disease affecting dental hard tissues (Namba et al., 1982). Oral cariogenic bacteria produce organic acids, which result in the decay of teeth. *Streptococcus mutans* is an important oral pathogen that causes dental caries (Marlise et al., 2010; Wiater et al., 1999). *S. mutans* secretes glucosyltransferase (GTFase) around the cell or on its surface, and this activates the synthesis of glucans on the tooth surface via the use of sucrose contained in food as substrate (Matsumoto-Nakano et al., 2011; Matsumoto et al., 1998). Glucans, together with oral bacteria, form dental plaques on the surface of the teeth. In dental plaques, the oral bacteria produce organic acids, such as lactic acid, which demineralize the enamel surface and destroy the enamel and dentin (Matsumoto-Nakano et al., 2011; Ooshima et al., 2000). Several plaque control agents have been used to prevent dental plaques and caries. Fluoride is one of such agents (Guha-Chowdhury et al., 1995), but it is cytotoxic if used at concentrations over 80 ppm (Jeng et al., 1998). Other agents such as mouth rinsing solutions also have been developed to prevent dental plaques and caries (Otten et al., 2011; Pan et al., 1999; Marsh, 1993), but dental caries still remains the major cause of tooth loss. This evidence suggests that current plaque control agents are not sufficiently effective. Therefore, we need to develop new agents that are sufficiently effective to control dental plaques and to prevent dental caries.

Recently, some natural products have shown potential as candidates for developing new plaque control agents to prevent dental caries (Matsumoto-Nakano et al., 2011; Marsh, 1992; Matsumoto et al., 1999; Namba et al., 1982; Shouji et al., 2000; Wennstrom and Lindhe, 1985). *Tribulus terrestris* L. is a member of Zygophyllaceae and is mainly harvested in Korea and China (Bensky et al., 1993). *T. terrestris* fruits consist of 5 mericarps, radially arranged and 7 to 12 mm in diameter. Each mericarp is hatchet-shaped and 3 to 6 mm long. The dorsal surface is yellowish-green and prominent, with longitudinal ribs and numerous spinelets, and bears a pair of long symmetrical

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spines and a pair of short spines; the lateral surfaces are grayish-white and rough, with reticular striations. *T. terrestris* has a hard texture and bitter taste and it is odorless and pungent (The Pharmacopoeia Commission of PRC, 1997). In traditional Chinese medicine, *T. terrestris* is used for treating toothache, dental caries, periodontal disease, eye diseases, cutaneous pruritus, edema, inflammation and tracheitis. It is also used for promoting blood circulation to remove blood stasis (Lee, 1986). However, scientific evidence about the effect of *T. terrestris* on dental caries is scarce.

In this study, we clearly demonstrated that *T. terrestris* has anticariogenic properties such as the inhibition of bacterial growth, acid production, bacterial adherence and water-insoluble glucan synthesis of *S. mutans*.

MATERIALS AND METHODS

Materials

Brain heart infusion (BHI) broth, mitis salivarius agar (MSA), and phenol red broth were purchased from Difco Laboratories (Detroit, MI, USA). Glucose, sodium azide, dimethyl sulfoxide (DMSO), and bacitracin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). *S. mutans* ATCC 25175 was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Hydroxyapatite beads were purchased from Bio-Rad (diameter, 80 μm; Bio-Rad, Hercules, CA, USA). Other reagents were of analytical grade.

Plant material and extraction

The fruits of *T. terrestris* were obtained from the oriental drug store, Hongin Dang (Iksan, South Korea). The identity of the fruits was confirmed by Dr. Bong-Seop Kil at the Department of Natural Science, Wonkwang University. A voucher specimen (number 06-05-13) has been deposited at the Herbarium of the Department of Oral Biochemistry, School of Dentistry, Wonkwang University. Dried *T. terrestris* fruit was chopped into small pieces and extracted with ethanol.

The powder (100 g) was soaked separately in 1 L of 100% ethanol for 72 h at room temperature. The extracted solution was filtered and evaporated under reduced pressure to yield the ethanol extract (1.11 g). Later, the extracts were thoroughly dried for complete removal of solvents. The dry extract was then dissolved in DMSO to obtain the desired stock solutions of the extract. The final concentration of DMSO was adjusted to 0.1% (v/v) in the culture system, and this did not interfere with the testing system. The control group was treated with media containing 0.1% DMSO.

Bacterial growth inhibition

Bacterial growth was determined by using a modification of the methods described previously (Choi et al., 2001; Matsumoto et al., 1999). The growth of *S. mutans* ATCC 25175 was examined at 37°C in 0.95 ml of BHI broth containing 1% glucose and various concentrations of *T. terrestris* extract. These tubes were inoculated with 0.1 ml of an overnight culture grown in the BHI broth and incubated at 37°C for 24 h. The optical density (OD) of cells was measured spectrophotometrically at 550 nm. Three replicates were made for each concentration of the test extract. NaF (1%) was used as a positive control.

Acid production

To examine the effect of *T. terrestris* on acid production of *S. mutans* ATCC 25175, we used the method described by Matsumoto et al. (1999) with a slight modification. The filter-sterilized *T. terrestris* was added to 0.95 ml of phenol red broth containing 1% glucose, which was then inoculated with 0.05 ml of the seed culture of *S. mutans* ATCC 25175. The cultures were incubated at 37°C for 24 h, and the pH of the cultures was determined using a pH meter (Corning Inc., Corning, NY, USA). NaF (1%) was used as a positive control. Three replicates were made for each concentration of the test extract.

Bacterial adherence

The assay of bacterial adherence was based on a method described previously (Fukushima et al., 1981). *S. mutans* ATCC 25175 was grown for 24 h at 37°C in BHI. Hydroxyapatite beads (30 μg) were coated with clarified human saliva (Hay et al., 1971) for 1 h at room temperature. The saliva-coated hydroxyapatite beads (S-HAs) were washed 3 times with distilled water and immersed in either bacterial suspensions (1×10^7 colony-forming units [CFU]/ml) or suspensions containing *T. terrestris.*

After incubation of the S-Has with bacteria for 90 min at 37°C, under gentle agitation, the S-HAs were washed and transferred to a tube containing potassium phosphate buffer (KPB; pH 7.0). Bacteria adsorbed on the S-HAs were dispersed using a sonicator at 50 W for 30 s (Fisher Scientific, Springfield, NJ, USA), diluted, and spread on an MSA plate containing bacitracin (3.2 mg/ml). After incubation for 48 h at 37°C, the bacterial colonies on the MSA plate were counted. Three replicates were made for each concentration of the test extracts and CFU was calculated.

Inhibition of water-insoluble glucan synthesis

The assay and preparation of crude glucosyltransferase (GTFase) were based on methods described previously (Koo et al., 2000; Wiater et al., 1999). The cell-free enzymes were precipitated from 800 ml of the culture supernatant by adding solid ammonium sulfate to 70% saturation. The mixture was stirred at 4°C for 1 h and allowed to stand for 1 h in the cold. The precipitate was collected by centrifugation (12,000 × g for 20 min), dissolved in a small volume of 0.01 M KPB (pH 6.0), and then dialyzed against 0.01 M KPB (pH 6.0) at 4°C for 24 h by dialysis tube (molecular weight cut off; 3,500, Spectrum, Houston, TX, USA). The liquid supernatant volume was made up to 28 ml with 0.1 M KPB (pH 6.0).

The crude enzymatic preparation was stored at –80°C and used for the synthesis of water-insoluble glucans. A reaction mixture consisting of 0.25 ml of crude enzyme and 0.25 ml of the ethanol extract of *T. terrestris* (0, 0.025, 0.05, 0.1, 0.2, 0.3, and 0.5 mg/ml; final concentration) in 0.4 M KPB (pH 6.0) containing 0.04% sodium azide and 0.25 ml of 0.4 M sucrose were incubated at 37°C for 18 h. After incubation, the fluid was removed, and the contents of the tube were washed with sterile water. Total amounts of water-insoluble glucans were measured by the phenol-sulfuric acid method (Dubois et al., 1956). Three replicates were made for each concentration of the test extract.

Phytochemical screening

Phytochemical testing of the extract was performed as previously described (Houghton and Raman, 1998; Woo, 2001). Mayer's reagent was used for alkaloids, ferric chloride reagent for phenolics, Molisch test for glycosides, Biuret reagent for peptides, Mg-HCl reagent for flavonoids, Liebermann–Burchard reagent for steroids,

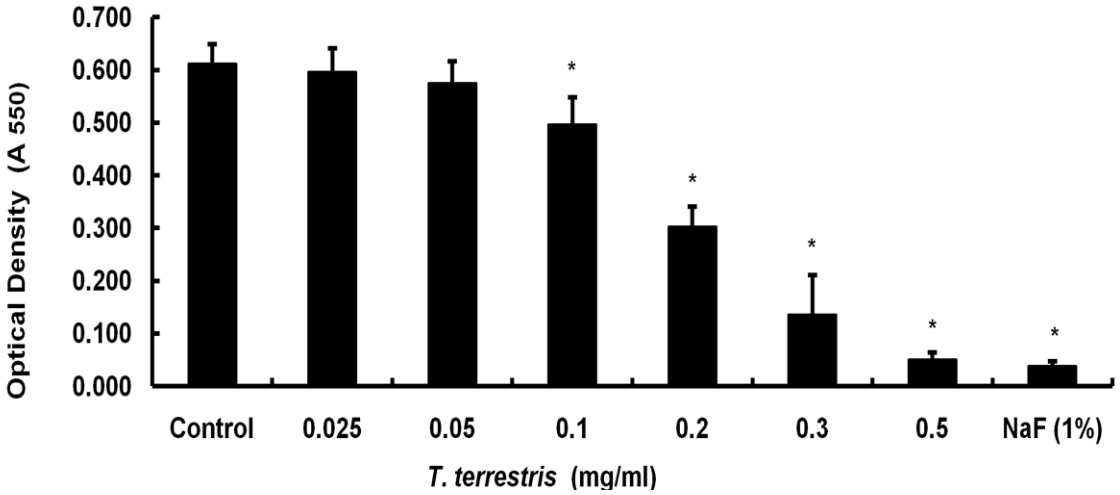

Figure 1. Effect of the ethanol extracts of *Tribulus terrestris* on the growth of *Streptococcus mutans.* The bacteria were inoculated in brain heart infusion (BHI) broth with each indicated concentration of *T. terrestris* and anaerobically incubated for 24 h at 37°C. The optical density at A_{550} was measured using a spectrophotometer. $^{*}P < 0.05$ was statistically significant, as determined by the Student's *t*-test for the mean values different from the control group.

Table 1. Effect of the ethanol extract of *Tribulus terrestris* on acid production of *Streptococcus mutans.*

Concentration (mg/ml)	Ethanol extract
Control	5.40 ± 0.200
0.025	5.77 ± 0.416
0.05	5.73 ± 0.379
0.10	6.13 ± 0.208*
0.20	6.93 ± 0.153*
0.30	7.00 ± 0.200*
0.50	7.13 ± 0.041*
NaF (1%)	7.13 ± 0.041*

S. mutans was inoculated in brain heart infusion (BHI) broth with each indicated concentration of *T. terrestris* and incubated for 24 h at 37°C. $^{*}P < 0.05$ was statistically significant, as determined by the Student's *t*- test for the mean values different from the control group.

and silver nitrate reagent for organic acids.

Statistical analysis

All experiments were carried out in triplicate. Data were analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL). The data were expressed as mean ± S.D. Differences between means of the experimental and control groups were evaluated by Student's *t*-test.

RESULTS

This study was performed to investigate the anti-cariogenic properties of *T. terrestris. S. mutans* is an important cariogenic bacterium. The bacteria were exposed to 0.025, 0.05, 0.1, 0.2, 0.3 and 0.5 mg/ml of the ethanol extract of *T. terrestris*. The extract exhibited antibacterial activity against *S. mutans* in a dose-dependent manner. The groups treated with the extractat concentrations higher than 0.1 mg/ml showed significant inhibition of growth of *S. mutans* ($P < 0.05$) compared to the control group (Figure 1). The positive control (1% NaF) also showed antibacterial activity. Table 1 shows the effect of the ethanol extract of *T. terrestris* on acid production of *S. mutans.* The groups treated with the ethanol extract (0.1 to 0.5 mg/ml) showed significant decrease in pH compared to the control group. Positive control (1% NaF) also inhibited the decrease of pH. These results suggest that organic acid production in *S. mutans* may be inhibited by the ethanol extract of *T. terrestris*. The bacterial adherence assay was performed to examine whether the adherence of *S. mutans to* S-HAs is inhibited by the extract of *T. terrestris*.

Result in Figure 2 shows that the adherence of

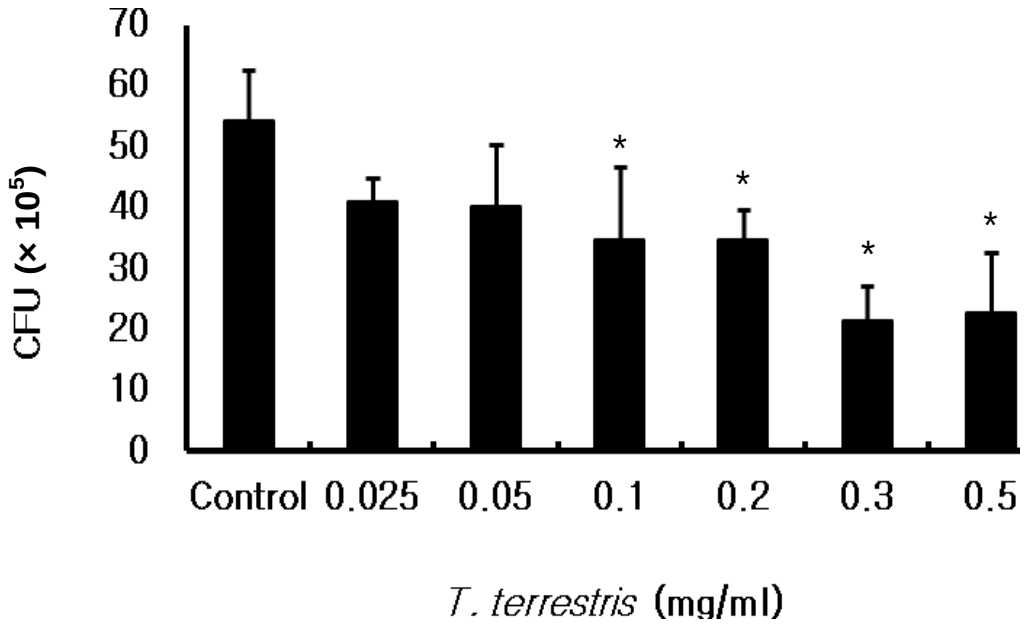

Figure 2. Effect of *Tribulus terrestris* on the adherence of *Streptococcus mutans* to the saliva-coated hydroxyapatite (S-HA) beads. The colony-forming unit (CFU) of *S. mutans* on the saliva-coated hydroxyapatite beads at various concentrations of the ethanol extract of *T. terrestris* was determined. $^{*}P < 0.05$ was statistically significant, as determined by the Student's *t*- test for the mean values different from the control group.

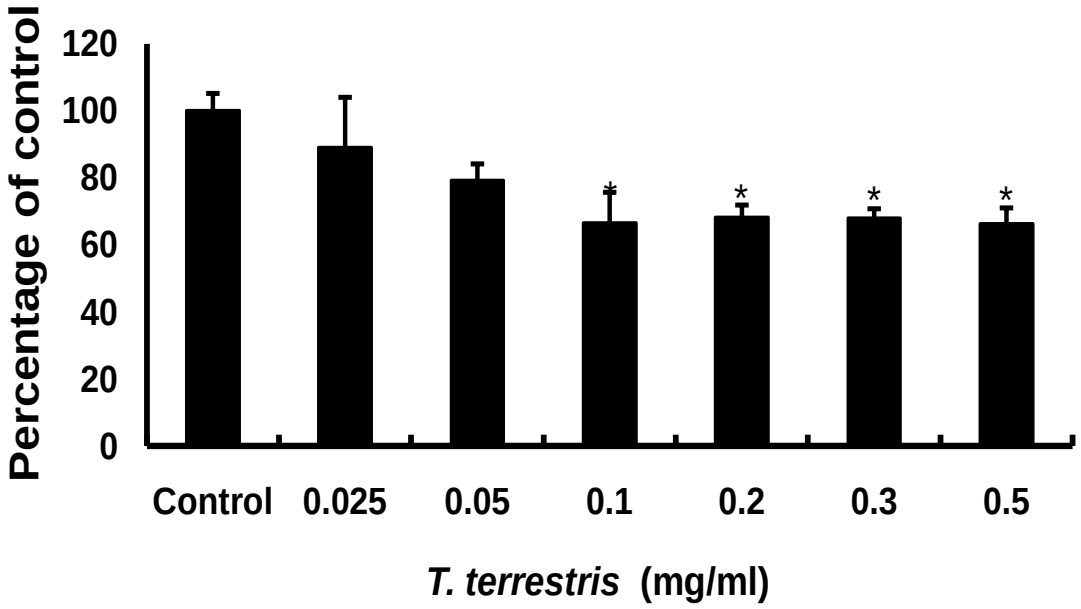

Figure 3. Effect of the ethanol extract of *Tribulus terrestris* on the water-insoluble glucan production by glucosyltransferase. $^{*}P < 0.05$ was statistically significant, as determined by the Student's *t*-test for the mean values different from the control group.

S. mutans to S-HAs was significantly inhibited by the ethanol extract (0.1 to 0.5 mg/ml). We also examined whether the ethanol extract of *T. terrestris* inhibited the synthesis of water-insoluble glucans by crude GTFase. Result in Figure 3 shows that the ethanol extract of *T. terrestris* inhibited the synthesis of water-insoluble

Table 2. Phytochemical analysis of *Tribulus terrestris.*

Plant constituent	Ethanol extract
Alkaloids	-
Phenolic compound	++
Glycosides	++
Peptides	+
Flavonoids	++
Steroids, terpenoids	+++
Organic acids	++

glucans. The synthesis of water-insoluble glucans was significantly suppressed by higher doses of the ethanol extract of *T. terrestris* (0.1 to 0.5 mg/ml). Table 2 shows the results of the phytochemical tests for the ethanol extract. Preliminary phytochemical analysis showed high levels of steroids and terpenoids, medium levels of phenolic compounds, flavonoids, and organic acids, low levels of peptides, and the absence of alkaloids.

DISCUSSION

T. terrestris is known to have therapeutic effects on oral diseases such as halitosis, dental caries, and periodontal disease (Lee, 1986; Kim et al., 1991). In this study, we examined whether the ethanol extract of *T. terrestris* has any effects on the growth, acid production, adherence, and water-insoluble glucan synthesis of *S. mutans*. The growth of *S. mutans* was inhibited by the ethanol extract of *T. terrestris* in a dose-dependent manner. *S. mutans* plays the most important role in the formation of dental plaques and caries. Knowledge of the fact that *T. terrestris* inhibits the growth of *S. mutans* can be the reason why the native population uses *T. terrestris* for the treatment of dental diseases.

S. mutans grows in plaques, metabolizes food sugars in the mouth, and then releases organic acids. Tooth surfaces are demineralized by these organic acids, thereby initiating dental caries (Matsumoto-Nakano et al., 2011). In our investigation, we found that the ethanol extract of *T. terrestris* reduced the acid production of *S. mutans*. Adherence of *S. mutans* to the tooth surface is an important step in the formation of dental plaques (Matsumoto et al., 1999; Akira et al., 2007). Previous studies have shown that the inhibition of the adherence of *S. mutans* to the surface of the tooth prevents plaque formation; therefore, we investigated the inhibitory effect of the ethanol extract of *T. terrestris* on the adhesion of *S. mutans* to S-HAs, which have been used as an experimental model system. In the treatment groups, the adherence of *S. mutans* on S-HA beads was significantly reduced at all concentrations (0.025, 0.05, 0.1, 0.2, 0.3, and 0.5 mg/ml), compared to control. Hydrophobicity is one of the most important factors in the adhesion mechanism of *S. mutans* to the tooth surface (Ayumi et al., 2008). Westergren and Olsson (1983) showed that mutant strains of *S. mutans*, that is, *S. sanguis*, and *S. salivarius* that have lost their cell surface hydrophobicity could not adhere to S-HA beads. Therefore, further studies are required to determine whether *T. terrestris* may reduce the cell surface hydrophobicity of *S. mutans.*

S. mutans promotes plaque formation by synthesizing water-insoluble glucans (Matsumoto-Nakano et al., 2011; Koo et al*.,* 2000). On the surface of a saliva-coated tooth, GTFase can absorb in an active form and synthesize water-insoluble glucans from sucrose (Koo et al*.,* 2000). Because the synthesis of water-insoluble glucans is one of the most important virulence properties of *S. mutans* that contributes to the development of mature dental plaques, we also examined whether the extract of *T. terrestris* may inhibit the synthesis of water-insoluble glucans via the activity of crude GTFase. In the water-insoluble glucan synthesis assay, 0.1 to 0.5 mg/ml of the ethanol extract of *T. terrestris* significantly inhibited the formation of water-insoluble glucans. To the best of our knowledge, this is the first time that the effect of the ethanol extract of *T. terrestris* on the synthesis of water-insoluble glucans has been evaluated. Previous reports have suggested that the fruits of *T. terrestris* contain kaempferol, kaempferol 3-glucoside, kaempferol 3-rutinoside, tribuloside, harman, harmine, tribulusimide D, terrestriamide and quercetin 3-glucoside (Byun et al., 2010; Bensky et al., 1992). In this study, we found high levels of steroids and terpenoids, medium levels of phenolic compounds, flavonoids, and organic acids, and low levels of peptides in *T. terrestris*. The anticariogenic activity of *T. terrestris* observed in this study can be explained on the basis of these compounds.

ACKNOWLEDGEMENT

This paper was supported by Wonkwang University in 2009.

REFERENCES

Akira S, Takashi K, Hisataka K (2007). Reduction of *Streptococcus mutans* Adherence and Dental Biofilm Formation by surface Treatment with Phosphorylated Polyethylent Glycol. Antimicrob. Agents Chemother., 51(10): 3634-3641.

Ayumi YO, Emiko S, Tomoko K (2008). Inhibitory Effect of Cranberry Polyphenol on Cariogenic Bacteria. Bull. Tokyo Dent. Coll., 49(3): 107-112.

Bensky D, Gamble A, Kaptchun T (1993). Chinese Herbal Medicine Materia Medica. Eastland Press, Seattle, USA, P. 425.

Byun E, Jeong GS, An RB, Min TS, Kim YC (2010). Tribuli fructus constituents protect against tacrine-induced cytotoxicity in HepG2 cells. Arch. Pharm. Res., 33(1): 67-70.

Choi BK, Kim KY, Yoo YJ, Oh SJ, Choi JH, Kim CY (2001). *In vitro* antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. Int. J. Antimicrob. Agents, 18: 553-557.

Dubois M, Gilles A, Hamilton JK, Rebers PA, Smith F (1956). Colorimetric method for determination of sugars and related substance. Anal Chem., 28: 350-356.
Fukushima K, Motoda R, Ikeda T (1981). Effects of exogenous insoluble glucan primer on insoluble glucan synthesis by *Streptococcus mutans*. J. Dent. Res., 60: 1707-1712.
Guha-Chowdhury N, Iwami Y, Yamada T, Pearce EI (1995). The effect of fluorhydroxyapatite-derived fluoride on acid production by streptococci. J. Dent. Res., 74: 1618-1624.
Hay DI, Gibbons RJ, Spinell DM (1971). Characteristics of some high molecular weight constituents with bacterial aggregating activity from whole saliva and dental plaque. Caries Res., 5: 111-123.
Houghton PJ, Raman A (1998). Laboratory handbook for the fractionation of natural extracts. Chapman & Hall, London, UK, pp. 154-185.
Jeng JH, Hsieh CC, Lan WH, Chang MC, Lin SK, Hahn LJ, Kuo MY (1998). Cytotoxicity of sodium fluoride on human oral mucosal fibroblasts and its mechanisms. Cell Biol. Toxicol., 14: 383-389.
Kim RM, Jeon SE, Choi Y (1991). EuiBangRuChui (6). Yeokang Publications, Seoul, Korea, P. 385.
Koo H, Vacca SAM, Bowen WH, Rosalen PL, Cury JA, Park YK (2000). Effects of *Apis mellifera* propolis on the activities of streptococcal glucosyltransferases in solution and adsorbed onto saliva-coated hydroxyapatite. Caries Res., 34: 418-426.
Lee SD (1986). Dongeui Bogam (Ⅱ). Yeokang Publications, Seoul, pp. 773-845.
Marlise IK, Lena D, Senyo A (2010). Dynamics of *Streptococcus mutans* Transcriptome in Response to Starch and Sucrose during Biofilm Development. PLoS ONE, 5(10): e13478.
Marsh PD (1993). Antimicrobial strategies in the prevention of dental caries. Caries Res., 27(1): 72-76.
Marsh PD (1992). Microbiological aspects of the chemical control of plaque and gingivitis. J. Dent. Res., 71: 1431-1438.
Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S, Ooshima T (1999). Inhibitory effects of oolong tea extract on caries-inducing properties of mutans streptococci. Caries Res., 33: 441-445.
Matsumoto-Nakano M, Nagayama K, Kitagri H (2011). Inhibitory Effects of Oenothera biennis (Evening Primrose) Seed Extract on *Streptococcus mutans* and *S. mutans*-Induced Dental Caries in Rats. Caries Res., 45: 56-63.
Namba T, Tsunezuka M, Hattori M (1982). Dental caries prevention by traditional Chinese medicines. Part II. Potent antibacterial action of Magnoliae cortex extracts against *Streptococcus mutans*. Planta Med., 44: 100-106.
Ooshima T, Osaka Y, Sasaki H, Osawa K, Yasuda H, Matsumura M, Sobue S, Matsumoto M (2000). Caries inhibitory activity of cacao bean husk extract in *in-vitro* and animal experiments. Arch. Oral Biol., 45: 639-645.
Otten MPT, Busscher HJ, Vander MHC, Van Hoogmoed CG, Abbas F (2011). Acute and substantive action of antimicrobial toothpastes and mouthrinsed on oral biofilm *in vitro*. Eur. J. Oral Sci., 119: 151-155.
Pan PH, Finnegan MB, Sturdivant L, Barnett ML (1999). Comparative antimicrobial activity of an essential oil and an amine fluoride/stannous fluoride mouthrinse *in vitro*. J. Clin. Periodontol., 26: 474-476.
Shouji N, Takada K, Fukushima K, Hirasawa M (2000). Anticaries effect of a component from shiitake (an edible mushroom). Caries Res., 34: 94-98.
The Pharmacopoeia Commission of PRC (1997). Pharmacopoeia of the people's republic of China (Ⅰ). Chemical Industry Press, Beijing, China, p. 79.
Wennstrom J, Lindhe J (1985). Some effects of a Sanguinarine-containing mouthrinse on developing plaque and gingivitis. J. Clin. Periodontol., 12: 867-872.
Westergren G, Olsson J (1983). Hydrophobicity and adherence of oral streptococci after repeated subculture *in vitro*. Infect. Immun., 40: 432-435.
Wiater A, Choma A, Szczodrak J (1999). Insoluble glucans synthesized by cariogenic streptococci: a structural study. J. Basic Microbiol., 39: 265-273.
Woo WS (2001). Experimental methods for phytochemistry. Seoul National University Press, Seoul, pp. 1-62.