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In Vitro Anti-Cariogenic Activity of Dichloromethane Fraction from *Rheum undulatum* L. Root

Ju-Hee Song, Tae-Cheol Yang, Kee-Wan Chang, Seong-Kyu Han[1], Ho-Keun Yi[2], and Jae-Gyu Jeon

Department of Preventive Dentistry, Chonbuk National University, Jeonju 561-756, Korea, [1]Department of Oral Physiology, Chonbuk National University, Jeonju 561-756, Korea, and [2]Department of Oral Biochemistry, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju 561-756, Korea

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This study aimed to evaluate *in vitro* effects of *Rheum undulatum* L. root on the development of dental caries, especially its effects on viability, dental plaque formation, and glycolytic acid production of *Streptococcus mutans* and *Streptococcus sobrinus*. Methanol extract of *Rheum undulatum* L. root and its fractions were prepared and tested. Among the test extract and fractions, dichloromethane fraction (DF) showed the most active antibacterial activity (inhibition zone: 13-17 mm) against *S. mutans* and *S. sobrinus* in a disc diffusion method. Minimal inhibitory concentrations (MICs) of DF against these bacteria ranged from 0.25 to 0.5 mg/mL. Furthermore, DF significantly inhibited the caries-inducing factors of these bacteria. At sub-MIC levels, DF inhibited *in vitro* dental plaque formation by *S. mutans* and *S. sobrinus* (IC_{50}= 0.079 and 0.142 mg/mL, respectively), which was caused, in part, by the inhibitory effect on the activity of glucosyltransferases. A significant reduction of glycolytic acid production was found at the concentration as low as 0.032 mg/mL for *S. mutans* and 0.063 mg/mL for *S. sobrinus*. The possible bioactive compounds that are inducing *in vitro* anti-cariogenic activity of DF are unknown. Based on the preliminary phytochemical analysis, the activity of DF may be related to the presence of anthraquinones, cardiac glycosides, coumarines, sterols/terpenes, and phenolics. These results indicate that DF is probably useful for the control of dental plaque formation and subsequent dental caries development.

Key words: Acid production, Dental caries, Dental plaque, *Rhei undulatium*, *Streptococcus mutans*, *Streptococcus sobrinus*

INTRODUCTION

Dental caries is a transmissible infectious disease (Tanzer, 1995). The essential process of this disease involves bacterial adherence to tooth surfaces, dental plaque formation, and localized demineralization of tooth enamel by acids of bacterial origin produced from the fermentation of dietary carbohydrates (Hajishengallis and Michalek, 1999). Mutans streptococci, one of the major oral bacteria, are considered to be important for dental caries (Loesche, 1986). Among this group, *Streptococcus mutans* and *Streptococcus sobrinus* are the most frequently isolated from the human oral cavity (Hamada and Slade, 1980; Loesche, 1986), and are associated with the pathogenesis of dental caries. These bacteria produce glucosyltransferases (GTFs) and synthesize water-insoluble glucan from sucrose, which mediates the adherence and accumulation of the bacteria on tooth surfaces, and contributes to the formation of dental plaque (Madison *et al.*, 1991). Furthermore, the bacteria increase the magnitude of the pH drop following carbohydrate fermentation and increase the probability of enamel demineralization (Fejerskov *et al.*, 1992).

A widely adopted approach for prevention of dental caries is the topical application of chemoprophylactic agents (Sreenivasan and Gaffar, 2002). These agents, e.g. chlorhexidine and antibiotics, act by lowering the number of microorganisms or inhibiting dental plaque formation (Gaffar *et al.*, 1997). However, they have several undesirable side effects, including tooth staining and emergence of bacterial resistance (Eley, 1999). The side effects stimulate the search for alternative agents. Recently,

Correspondence to: Jae-Gyu Jeon, Department of Preventive Dentistry, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, 664-14 Duckjin-dong, Jeonju 561-756, Korea
Tel: 82-63-270-4036, Fax: 82-63-270-4035
E-mail: dentjjk@chonbuk.ac.kr

medicinal plants and natural products have been shown to be alternatives to the agents for dental caries prevention (Rasheed and Haider, 1998; Koo *et al.*, 2002).

Rhei Rhizoma is one of the important herbal drugs widely used as a purgative and anti-inflammatory agent in East Asia (Yang *et al.*, 2004). Moreover, it has been traditionally used as a controlling agent for dental diseases in Korea (Hur, 1994). It is a dried root and derived from the outer corky layer of the genus Rheum. *Rheum undulatum*, *R. palmatum*, *R. tanguticum*, *R. coreanum*, and their hybrids have been generally used in Korea, Japan, and China (Lee *et al.*, 2003). Many reports are devoted to the chemical composition and pharmacological activities of Rhei Rhizoma (Ko *et al.*, 1999; Ko, 2000). However, although Rhei Rhizoma has been used as a controlling agent for dental diseases, little is known about its biological effects on dental diseases, especially on the development of dental caries.

The purpose of this study was to evaluate *in vitro* effects of *R. undulatum* root on the development of dental caries, especially its effects against bacterial viability and caries-inducing factors of *S. mutans* and *S. sobrinus*.

MATERIALS AND METHODS

Plant material and fractionation

R. undulatum root was purchased from an herbal drug market in 2004 (Jeonju, Korea) and identified by Professor Young-Sung Ju. A voucher specimen (No. PD0502) was deposited at the Institute of Oral Bioscience, Chonbuk National University. The powdered plant material (200 g) was macerated with methanol for 24 h at room temperature. The extract was filtered, concentrated under reduced pressure at 40°C, and then lyophilized (yield: 23.5%, w/w). Part of the methanol extract (40 g) obtained was suspended in 70% aqueous methanol and serially fractioned with *n*-hexane, dichloromethane, and ethyl acetate. The yield of *n*-hexane fraction (HF), dichloromethane fraction (DF), ethyl acetate fraction (EF), and aqueous methanol fraction (AF) was 2% (w/w), 3.5% (w/w), 21.9% (w/w), and 65.5% (w/w), respectively. Methanol extract (ME) and its fractions obtained were kept at -20°C until tested, and dissolved in dimethyl sulfoxide (DMSO) just prior to performance of the assays.

Test bacterial strains and media

Sixteen bacterial strains, 12 Gram-positive and 4 Gram-negative, were used in this study (Table I). All the bacterial strains were used for the determination of antibacterial activity. Only *S. mutans* KCTC 3298 and *S. sobrinus* KCTC 3288 were used in the sub-minimal inhibitory concentration (MIC) assays. All gram-positive bacteria, with the exception of lactobacilli, were cultured in Brain Heart Infusion (BHI; Difco, Detroit, U.S.A.) medium under aerobic conditions at 37°C. Lactobacilli were cultured in De Man, Rogosa, Sharpe (MRS; Oxoid LTD., Basingstoke, Hampshire, England) medium under anaerobic conditions (Anaerobic System, Forma Scientific Co., U.S.A.) with N_2 85%, H_2 10%, and CO_2 5% at 37°C. Gram-negative bacteria were cultured in BHI medium supplemented with hemin (500 μg/mL), yeast extract (5 mg/mL), vitamin K_1 (2 μg/mL) (MBHI) under anaerobic conditions at 37°C.

Antibacterial activity and MIC determination

The screening of antibacterial activity of ME and its fractions was conducted using a disc diffusion method (Sahin *et al.*, 2003). The ME and its fractions were dissolved in DMSO to a final concentration of 100 mg/mL. 100 μL of prepared culture containing 10^8 CFU/mL of bacteria was spread on BHI, MRS or MBHI agar. The discs (6 mm in diameter) placed on the inoculated agar were impregnated with 10 μL (1 mg/disc) of ME or its fractions. Ten μL of DMSO was used as vehicle control. The inoculated plates were incubated for 24 h for Gram-positive bacteria, and 48-72 h for Gram-negative bacteria. Antibacterial activity was evaluated by measuring the zone of inhibition against the test bacteria.

MIC values were determined for the fraction with the highest antibacterial activity in the disc diffusion assay, using a micro-well dilution method (Sahin *et al.*, 2003). Inoculum suspensions were prepared from 18-48 h broth cultures. Diluted 100 μL suspensions of the bacterial strains were added to 100 μL of each concentration of the fraction diluted with the liquid media to get 1.5×10^6 CFU/mL. The concentrations of the fraction ranged from 0.032 to 2 mg/mL. DMSO (2%, v/v) was used as vehicle control. MIC was defined as the lowest concentration of the fraction that restricted growth to a lower than 0.05 at 550 nm.

Effect of DF on bacterial growth at sub-MIC levels

S. mutans KCTC 3298 or *S. sobrinus* KCTC 3288 was grown at 37°C in BHI broth containing 1% glucose and the highest antibacterial fraction (or vehicle control). The bacterial suspension was adjusted to 0.5 MacFarland standard turbidity prior to inoculation. The fraction was two-fold diluted at sub-MIC levels. The end-point of bacterial growth after 24 h incubation was determined spectrophotometrically by measuring the optical density at 550 nm (Matsumoto *et al.*, 1999).

Effect of DF on dental plaque formation

To assess the effect of the highest antibacterial fraction on plaque formation by *S. mutans* and *S. sobrinus*, bacteria were incubated at 37°C with an angle 30° for 18 h in glass tubes as detailed by Hamada and Torii (1978) and Koo *et al.* (2000). Individual 18-24 h colonies from

BHI agar plates were suspended in the same broth and the suspensions were adjusted to 0.5 MacFarland standard turbidity. *S. mutans* KCTC 3298 or *S. sobrinus* KCTC 3288 was grown in BHI broth plus 1% (w/v) sucrose containing the highest antibacterial fraction (or vehicle control). The fraction was two fold-diluted at sub-MIC levels. After incubation, the adherent portion was washed and re-suspended in an ultrasonic bath. The amount of the adherent portion was measured spectrophotometrically at 550 nm.

GTF preparation and effect of DF on water-insoluble glucan synthesis

The effect of the highest antibacterial fraction on water-insoluble glucan synthesis by GTFs was tested. Crude mixtures of GTFs were prepared according to the previously described method (Wiater *et al.*, 1999). *S. mutans* KCTC 3298 or *S. sobrinus* KCTC 3288 was grown in BHI broth at 37°C for 18 h. The cell-free GTF (CF-GTF) was precipitated from the culture supernatant by adding solid ammonium sulfate and recovered as detailed elsewhere (Hamada *et al.*, 1989). The cell-associated GTF (CA-GTF) was extracted from whole cells by treatment with urea as previously described (Hamada *et al.*, 1989; Wiater *et al.*, 1999).

To measure water-insoluble glucan synthesis, we used a reaction mixture containing 1 mL of 0.25 M sucrose, 100 μL of each enzyme, and 20 μL of the highest antibacterial fraction (or vehicle control) in a total volume of 2 mL 0.1 M potassium phosphate buffer (pH 6.0) containing 0.02% PMSF and 1 mM NaN_3. The fraction was two-fold diluted at sub-MIC levels. The mixture was incubated at 37°C for 18 h. The water-insoluble glucans produced by the enzymes were measured spectrophotometrically at 550 nm.

Effect on glycolytic acid production

We tested the effect of the highest antibacterial fraction on glycolytic acid production of *S. mutans* or *S. sobrinus* at sub-MIC levels. For this assay, bacterial cells were grown in BHI broth and collected by centrifugation (10,000 g for 10 min at 4°C) at the logarithmic phase of growth. The cell pellet was suspended in 2 mM potassium phosphate buffer containing 150 mM KCl and 5 mM $MgCl_2$ (Kakuta *et al.*, 2003). The reaction mixture (2 mL) contained 0.5 mL of bacterial cells (*S. mutans* KCTC 3298; O.D = 1.22, *S. sobrinus* KCTC 3288; O.D = 1.68, at 550 nm), 0.5 mL of the fraction, and 1 mL of 2% glucose solution. The reaction was started by addition of the mixture containing the fraction and glucose. The glycolytic pH-drop was monitored using a pH meter over a 30 min time period.

Preliminary phytochemical analysis

The chemical constituents present in the highest antibacterial fraction were screened according to the method of Wagner and Bladt (2001). The chromatographic analyses were performed by thin layer chromatography (TLC; silica gel 60 F254, Merck, Germany) using ethyl acetate-methanol-water (100:13.5:10, v/v) as the mobile phase. Approximately 10 μL of the fraction was spotted onto the TLC plate. TLC spots were observed under UV_{254nm} and UV_{366nm}, before and after spraying with adequate TLC reagents (Gibbons and Gray, 1988; Wagner and Bladt, 2001); Dragendorff reagent for alkaloids, 10% ethanolic KOH for anthraquinones and coumarines, Kedde reagent for cardiac glycosides, Liebermann-Burchard reagent for sterols/terpenes, Anisaldehyde-sulphuric acid reagents for saponins, and 5% $FeCl_3$ for phenolics (Gibbons and Gray, 1988; Wagner and Bladt, 2001).

Statistical analysis

Data were analyzed with SPSS (Version 10.0 for Windows). Oneway analysis of variance was performed, followed by Tukey test, for comparison of mutiple means. The level of significance was $p<0.05$.

RESULTS AND DISCUSSION

Inhibitory effect on bacterial viability

The means of the zones of bacterial growth inhibition by ME and its fractions are shown in Table I. Among the test extracts, DF displayed the most effective antibacterial activity ($p<0.05$), inhibiting all the bacterial strains tested (inhibition zone ranging from 8 to 17 mm). DF also showed significantly higher inhibition against *S. mutans* and *S. sobrinus* (inhibition zone ranging from 13 to 17 mm, $p<0.05$) than those of ME and the other fractions. We thus chose DF for the other assays. As shown in Table I, DF expressed a broad-spectrum antibacterial activity. MIC values of DF varied from 0.125-0.5 mg/mL, depending on the strains. All the test strains of *S. mutans* and *S. sobrinus* were also sensitive to the DF (MICs; 0.25-0.5 mg/mL), but *S. mutans* appeared to be more susceptible to DF than *S. sobrinus*. Furthermore, at sub-MIC levels, DF significantly reduced the growth end-points of *S. mutans* and *S. sobrinus* after 24 h incubation at the concentration as low as 0.032 mg/mL ($p<0.05$) (Fig. 1A and 1B).

Although *R. undulatum* root has been extensively studied for its biological activities, this is the first report on antibacterial activity of the plant. Since *S. mutans* and *S. sobrinus* are associated with dental caries, we chose them as the main test bacteria. Lactobacill and four Gram-negative bacteria, such as *A. actinomycetemcomitans*, were also selected because of their association with dental caries and periodontal disease, respectively. In this study, DF showed considerable antibacterial activity against *S. mutans* and *S. sobrinus,* and Gram-negative periodon-

Table I. Antibacterial activity of the methanol extract of *R. undulatum* root and its fractions

Bacterial strain	Inhibition zone[a] (mm)					MIC[b] (mg/mL)
	ME	HF	DF	EF	AF	DF
Gram-positives						
Streptococcus mutans KCTC 3298	10	11	17	10	7	0.25
Streptococcus mutans KCTC 3306	7	-	13	9	8	0.25
Streptococcus mutans KCTC 3289	7	-	14	7	7	0.25
Streptococcus sobrinus KCTC 3307	7	-	13	10	7	0.5
Streptococcus sobrinus KCTC 3288	7	10	15	8	-	0.5
Streptococcus gordonii KCTC 3286	10	11	14	11	7	0.25
Streptococcus cricetus KCTC 3292	11	10	17	13	8	0.5
Streptococcus sanguis KCTC 3284	8	9	15	9	7	0.25
Streptococcus pyogens KCTC 3096	10	8	15	10	8	0.05
Actinomyces viscosus KCTC 9146	8	9	16	11	9	0.25
Lactobacillus acidophillus KCTC 3111	7	-	8	-	-	-
Lactobacillus oris KCTC 3502	8	7	8	-	-	-
Gram-negatives						
Actinobacillus actinomycetemcomitans KCTC 2581	7	9	15	10	-	0.25
Porphyromonas gingivalis ATCC 49417	10	-	16	13	7	0.25
Prevotella intermedia ATCC 25261	8	9	16	8	8	0.25
Fusobacterium nucleatum ATCC 51190	13	-	17	15	9	0.125

KCTC: the Korean Collection for Type Culture, ATCC: the American Type Culture Collection, -: activity absent, ME: methanol extract, HF: *n*-hexane fraction of ME, DF: dichloromethane fraction of ME, EF: ethyl acetate fraction of ME, AF: aqueous methanol fraction of ME.
[a]Includes the disc diameter (6 mm), 1 mg/disc, Vehicle controls (10 μL of DMSO) showed no activity.
[b]Vehicle controls (2% DMSO) showed no MIC.

topathogenic bacteria. This finding indicates that DF may be useful for prevention of periodontal diseases resulting from Gram-negative periodontopathogens, as well as dental caries resulting from Gram-positive mutans streptococci. The possible bioactive compounds that are inducing antibacterial activity of DF are unknown. As shown in Table II, preliminary phytochemical analysis of DF indicated the presence of common phytoconstituents, such as anthraquinones, cardiac glycosides, coumarines, sterols/terpenes, and phenolics. Based on the results, the antibacterial activity of DF may be related to the presence of phenolics, coumarines, and sterols/terpenes, since results from previous studies have shown that these secondary metabolites from plants possess antimicrobial properties (Ojala *et al.*, 2000; Cos *et al.*, 2004).

Inhibitory effect on dental plaque formation and acid production

We investigated whether DF inhibits *in vitro* dental plaque formation by growing *S. mutans* or *S. sobrinus*, since the adherence and accumulation of *S. mutans* and *S. sobrinus* to the tooth surface have been considered important for the development of dental caries. The plaque formation assay was performed using sub-MIC levels, since false positive results could be generated due to antimicrobial effects of the fraction. As shown in Fig. 2A and 2B, DF inhibited plaque formation by the growing *S. mutans* and *S. sobrinus* at the concentrations as low as 0.016 mg/mL and 0.032 mg/mL ($p<0.05$), respectively. The IC_{50} of DF was 0.079 mg/mL (*S. mutans*) or 0.142 mg/mL (*S. sobrinus*).

To study the inhibitory mechanisms of *in vitro* dental plaque formation, the effects of DF on water-insoluble glucan synthesis by GTFs of *S. mutans* and *S. sobrinus* were examined. DF dose-dependently decreased GTF activity from *S. mutans* at the concentration as low as 0.063 mg/mL ($p<0.05$) (Fig. 3A), and *S. sobrinus* at 0.125 mg/mL ($p<0.05$) (Fig. 3B). DF was more effective in inhibiting CA-GTF activity (70% inhibition for *S. mutans* and 65% inhibition for *S. sobrinus*) than CF-GTF activity (35% inhibition for *S. mutans* and 32% inhibition for *S.* sobrinus) at the highest sub-MIC concentrations ($p<0.05$).

The inhibitory effects of DF on plaque formation could result from reduction of the bacterial the growth at sub-MIC levels. As shown in Fig. 1A and 1B, growth end-points of *S. mutans* and *S. sobrinus* after 24 h incubation was reduced in the presence of DF, suggesting that the total number of *S. mutans* or *S. sobrinus*, which can

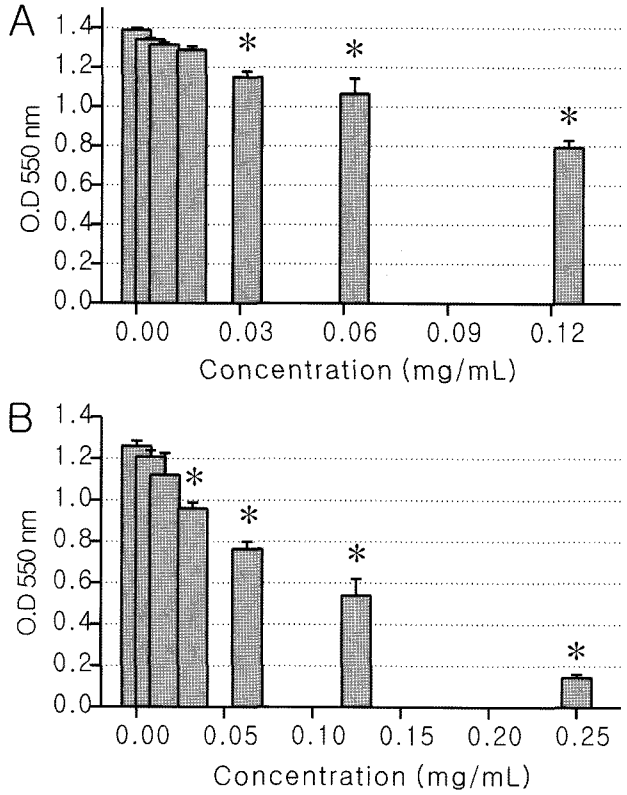

Fig. 1. Inhibitory effects of dichloromethane fraction (DF) of *R. undulatum* root on the growth of *Streptococcus mutans* KCTC 3298 (A) and *Streptococcus sobrinus* KCTC 3288 (B) after 24 h incubation at sub-MIC levels. Data represent mean ± standard error. *$p<0.05$: significantly different from vehicle controls (0.125% DMSO for *Streptococcus mutans* KCTC 3298, 0.25% DMSO for *Streptococcus sobrinus* KCTC 3288).

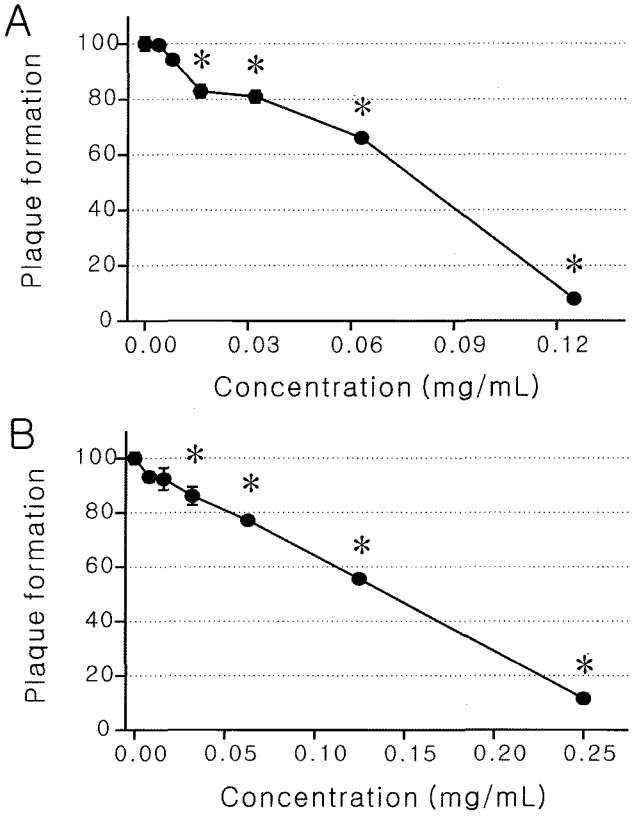

Fig. 2. Inhibitory effects of dichloromethane fraction (DF) of *R. undulatum* root on the plaque formation by *Streptococcus mutans* KCTC 3298 (A) and *Streptococcus sobrinus* KCTC 3288 (B) at sub-MIC levels. The value of plaque formation means the relative amount (%) of plaque at a certain DF concentration as compared to the amount detected in vehicle controls (0.125% DMSO for *Streptococcus mutans* KCTC 3298, 0.25% DMSO for *Streptococcus sobrinus* KCTC 3288). Data represent mean ± standard error. *$p<0.05$: significantly different from vehicle controls.

Table II. Phytochemical screening of dichloromethane fraction (DF) of *R. undulatum* root

Class of constituents	DF of *R. undulatum* root
Alkaloids	-
Anthraquinones	+
Cardiac glycosides	+
Coumarines	+
Phenolics	+
Saponines	-
Sterol/terpenes	+

+, detected: -, not detected.

adhere and produce GTFs, in the presence of DF was lower than that of the control after 18 h incubation. The inhibitory effects also related to GTF inhibitory activity of DF, whereby DF prevented the synthesis of water-insoluble glucan. It is well known that GTFs of *S. mutans* and *S. sobrinus* produce water-insoluble glucan from sucrose (Hamada and Slade, 1980; Loesche, 1986), which is important caries-inducing factors. Therefore, inhibition of water-insoluble glucan formation is one of the strategies to prevent dental caries (Hamada and Slade, 1980). Water-insoluble glucan is mainly produced by CA-GTF of *S. mutans* and CF-GTF of *S. sobrinus* (Hamada *et al.*, 1989; Hashimoto *et al.*, 2001). However, since other studies have shown that GTFs are present in cell-associated or cell-free forms and most water-insoluble glucans are synthesized by CF-GTF (Mukasa *et al.*, 1985; Wiater *et al.*, 1999), we examined CA-GTF and CF-GTF at the same time. Our data indicate that DF is effective in the prevention of water-insoluble glucan formation by both CF-GTF and CA-GTF (Fig. 3A and 3B). However, future studies need to confirm the effect of DF on the activity of purified GTFs. The possible biological active components of DF that modulate GTF inhibition are unknown. Because plant polyphenols have the ability to bind strongly to proteins (Mehansho *et al.*, 1987) and a non-competitive

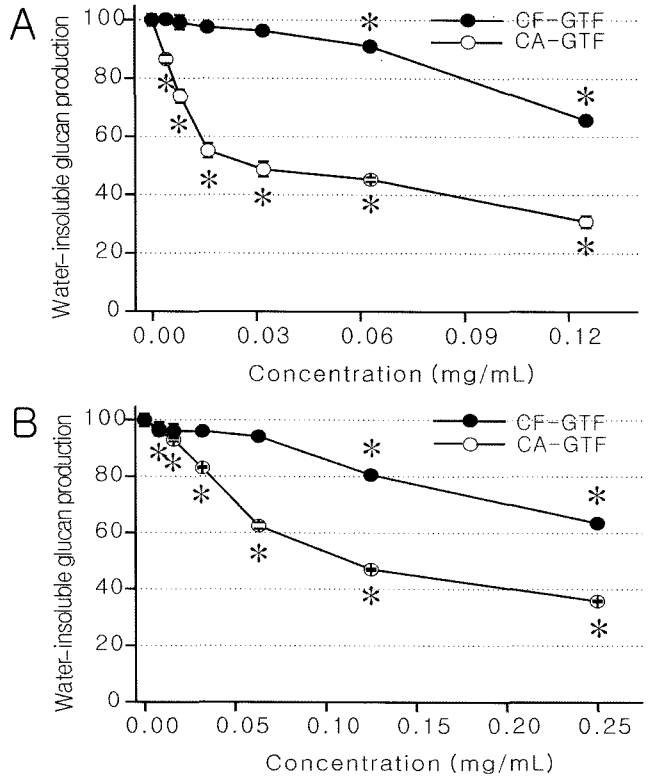

Fig. 3. Inhibitory effects of dichloromethane fraction (DF) of *R. undulatum* root on the activity of cell-free glucosyltransferase (CF-GTF) and cell-associated glucosyltransferase (CA-GTF) by *Streptococcus mutans* KCTC 3298 (A) and *Streptococcus sobrinus* KCTC 3288 (B) at sub-MIC levels. The value of water-insoluble glucan production means the relative amount (%) of water-insoluble glucan produced at a certain DF concentration as compared to the amount produced in vehicle controls (0.125% DMSO for *Streptococcus mutans* KCTC 3298, 0.25% DMSO for *Streptococcus sobrinus* KCTC 3288). Data represent mean ± standard error. *$p<0.05$: significantly different from vehicle controls.

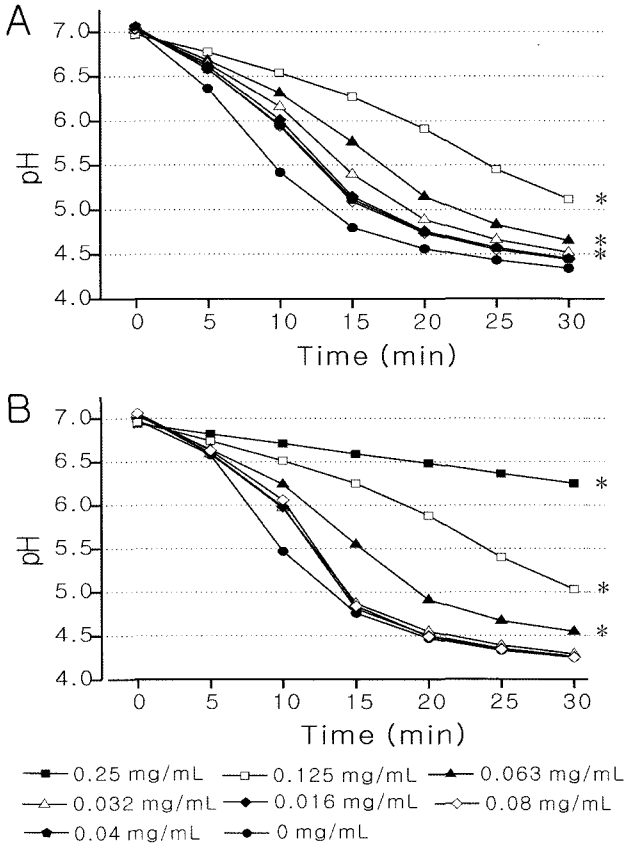

Fig. 4. Inhibitory effects of dichloromethane fraction (DF) of *R. undulatum* root on glycolytic acid production by *Streptococcus mutans* KCTC 3298 (A) and *Streptococcus sobrinus* KCTC 3288 (B) for 30 min incubation at sub-MIC levels. *$p<0.05$: significantly different from vehicle controls (0.125% DMSO for *Streptococcus mutans* KCTC 3298, 0.25% DMSO for *Streptococcus sobrinus* KCTC 3288).

inhibitory effect on GTF activity (Jagtap and Karkera, 2000), the GTF inhibitory activity of DF may result from the effect of the phenolics in DF.

The production of acids from fermentable carbohydrates is another important caries inducing factor of *S. mutans* and *S. sobrinus* that deserves attention during the examination of medicinal plants for prevention of dental caries. As shown in Fig. 4A and 4B, DF dose-dependently reduced the glycolytic pH-drop by cell suspension of *S. mutans* and *S. sobrinus*. A significant reduction of glycolytic acid production was found at the concentrations as low as 0.032 mg/mL for *S. mutans* and 0.063 mg/mL for *S. sobrinus* ($p<0.05$) after 30 min incubation. Since secondary metabolites are known to influence the permeability of natural and synthetic membranes and inhibits enzymes (Havsteen, 1983), it is possible that DF inhibit enzyme activity associated with the glycolyzing systems of *S. mutans* and *S. sobrinus*.

In summary, the results of the present study demonstrated that DF from *R. undulatum* root inhibited viability of oral pathogenic bacteria. Moreover, DF significantly inhibited *in vitro* dental plaque formation and acid production by *S. mutans* and *S. sobrinus* at the concentrations lower than MIC. These results indicate that DF might be useful for the control of dental plaque formation and subsequent dental caries development. However, this is the first time that this plant has been bioassayed in the dental field. Thus, more biochemical and phytochemical investigations are necessary in order to find the active compounds which showed antibacterial activity and the biological activities on caries inducing factors of *S. mutans* and *S. sobrinus*.

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