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ORIGINAL ARTICLE

Bioactivity-guided separation of anti-acidogenic substances against *Streptococcus mutans* UA 159 from *Polygonum cuspidatum*

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OBJECTIVES: The aim of this study was to separate the anti-acidogenic substances against *Streptococcus mutans* UA 159 from *Polygonum cuspidatum*.

MATERIAL AND METHODS: The anti-acidogenic substances were separated by a series of liquid–liquid fractionations followed by normal-phase silica gel liquid chromatography, based on high-performance liquid chromatography and glycolytic pH-drop assay. The effectiveness of the separated substances on the acidogenicity of *Streptococcus mutans* UA 159 was examined using sodium fluoride as a positive control. The chemical composition and quantities of the components of the substances was also assessed by qualitative–quantitative chromatographic analysis.

RESULTS: Among the substances separated from *P. cuspidatum*, F3 showed the strongest inhibitory effect on the acidogenicity of *S. mutans* UA 159 in a dose-dependent manner without displaying any bactericidal activity. F3 decreased the acidogenicity of *S. mutans* even at 12.5 μg ml^{-1} ($P < 0.05$). F3 consisted mainly of resveratrol and emodin ($C_{14}H_{12}O_3$ and $C_{14}H_4O_2(OH)_3CH_3$, respectively), which made up approximately 60% of the weight of F3.

CONCLUSION: F3 can be considered as a promising agent for controlling the acidogenicity of *S. mutans* and subsequent dental caries formation.

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Keywords: *Streptococcus mutans*; *Polygonum cuspidatum*; acidogenicity; anti-acidogenic substances; separation

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Introduction

Streptococcus mutans is an important bacterium in the initiation of dental caries (Hamada and Slade, 1980; Loesche, 1986). The leading role of *S. mutans* is determined mainly by its acidogenicity and aciduricity (Bender *et al*, 1985). The acidogenicity of *S. mutans* results in low pH values in the dental plaque matrix, which contributes to the selection of aciduric microorganisms and demineralization of the tooth enamel (Duarte *et al*, 2006). Therefore, approaches aimed at disrupting the ability of *S. mutans* to utilize dietary carbohydrates to form acids on the tooth surface could be precise and selective for the prevention of dental caries.

In recent years, there has been increasing interest in natural products as agents for preventing dental caries. Natural products have been used in traditional medicine for thousands of years and have shown promise as a source of components for the development of new antibacterial, antifungal, antiviral, anticancer, and antihypertensive drugs (Newman *et al*, 2003). They have recently undergone a more thorough investigation of their potential in preventing oral diseases, particularly dental plaque-related diseases, such as dental caries. However, the development of natural substances in dentistry is in the initial stages although there have been some research in analyzing the chemical properties and workings of these components (Hayacibara *et al*, 2005).

Polygonum cuspidatum (Polygonaceae) is a perennial species with spreading rhizomes and reddish-brown stems and has been used traditionally in East Asia to treat inflammatory diseases (Zhou *et al*, 2003; Park *et al*, 2004). In addition, it has been often used to control dental diseases in Korea. More than 30 chemical constituents have been isolated from the root of *P. cuspidatum*. Of these, resveratrol, polydatin, anthraglycoside B, emodin, and physcion have the predominant bio-activities contributing to the traditional efficacy of the plant (Yi *et al*, 2007).

We reported previously that *P. cuspidatum* inhibits *in vitro* growth and activity of several virulence properties

of mutans streptococci, including bacterial adhesion, water-insoluble glucan formation, acidogenicity, and aciduricity (Song *et al*, 2006, 2007). Nevertheless, there is little information on the biological properties of the specific components from *P. cuspidatum*, which may be useful for preventing dental caries. As part of an ongoing study on the prevention of dental caries by natural products, the aim of this study was to separate anti-acidogenic substances from *P. cuspidatum*, based on high-performance liquid chromatography (HPLC) and glycolytic pH-drop assay, and then examine the inhibitory effect of the separated substances on the acidogenicity of *S. mutans*.

Materials and methods

Experimental scheme, plant material, and fractionation

Figure 1 shows the experimental scheme for the separation of anti-acidogenic substances from *P. cuspidatum* root. Initially, *P. cuspidatum* root was purchased from an herbal drug market (Jeonju, Korea). A voucher specimen (PD0501) has been deposited at the Department of Preventive Dentistry, Chonbuk National University. The dried and finely ground *P. cuspidatum* root was extracted with methanol at room temperature for 24 h and concentrated under vacuum (methanol extract). A part of the methanol extract (ME) obtained was suspended in 70% aqueous methanol and fractioned serially with *n*-hexane and ethyl acetate. The yield of the *n*-hexane fraction (HF), ethyl acetate fraction (EF), and aqueous methanol fraction (AF) was 4.9% (w/w), 39.5% (w/w), and 49.8% (w/w), respectively. The fractions were stored at −20°C until tested. They were then dissolved in dimethyl sulfoxide (DMSO; 99.9%) and methanol immediately before glycolytic pH-drop assay and HPLC analysis, respectively.

Separation of anti-acidogenic substances

For the separation of the anti-acidogenic substances, EF was fractionated successively by column chromatography over silica gel (Kieselgel, 70–230 mesh, Merck, Darmstadt, Germany) using *n*-hexane: dichloromethane:ethyl acetate [(1:1:1), (1:1:2)]; *n*-hexane:ethyl acetate:methanol [(1:1:1), (1:1:2)]; *n*-hexane:methanol (1:4); and methanol, successively, as mobile phase. The fractions were collected and monitored by thin layer chromatography (TLC, silica gel 60, F254, Merck, 0.2-mm layer thickness). The fractions were classified into six major substances (F1–F6) according to their TLC profiles and stored at −20°C until tested. The fractions were dissolved in DMSO and methanol immediately before glycolytic pH-drop assay and HPLC analysis, respectively.

Bacterial strain, media, and glycolytic pH-drop assay

Streptococcus mutans UA159 (serotype c), a proven virulent cariogenic dental pathogen and the strain selected for genomic sequencing, was used (Ajdic *et al*, 2002). The bacterium was grown in ultrafiltered (Prep/Scale; Millipore, Billerica, MA, USA) tryptone-yeast extract broth (2.5% tryptone and 1.5% yeast extract, pH 7.0) with 1% glucose at 37°C under 5% CO_2.

The effects of ME, its fractions, and the substances separated on the acidogenicity of *S. mutans* UA159 were measured using glycolytic pH-drop assay, described previously by Belli *et al* (1995), with some modifications. For this assay, the cells of *S. mutans* UA159 from the suspension cultures were harvested, washed once with salt solution (50 mM KCl + 1 mM $MgCl_2$), and resuspended in a salt solution containing the test agents (25–1000 μg ml^{-1}) or vehicle control (2% DMSO or 4% DMSO + 6% ethanol, v/v). The pH was adjusted to 6.8–7.0 with 0.01 M KOH solution. Sufficient glucose was then added to obtain a concentration of 1% (w/v), and the decrease in pH was assessed over a period of 45 min using a glass electrode (Futura Micro Combination pH electrode, 5 mm diameter, Beckman Coulter Inc., Fullerton, CA, USA).

HPLC conditions

A JASCO LC-2000 Plus Series system (JASCO Corporation, Tokyo, Japan) equipped with online vacuum degasser, pump, thermostated column compartment, a UV/VIS detector, and ChromPass software was used. HPLC separation was performed on the KYA TECH HPLC column HIQ sil C18HS (4.3 μm, 4.6 × 150 mm; KYA TECH corporation, Tokyo, Japan) and a C18, 4.6 × 12.5 mm guard column (Agilent tech, Santa Clara,

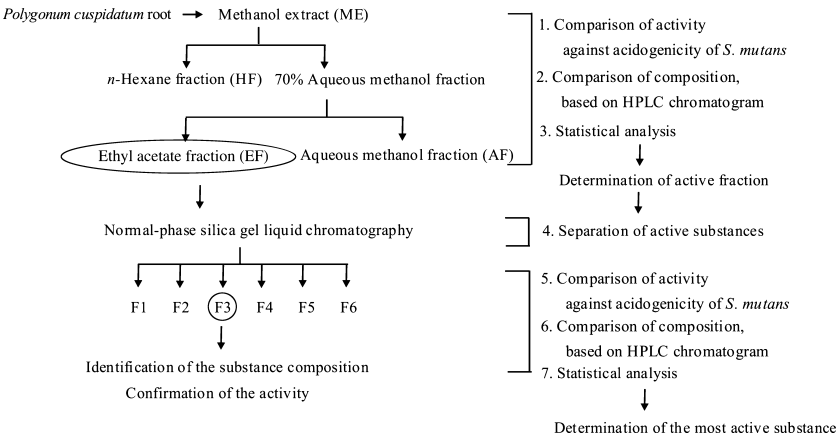

Figure 1 Experimental scheme for the separation of the anti-acidogenic substances from *Polygonum cuspidatum* root

CA, USA). A portion of ME, EF, separated substances, and standard compounds were redissolved in HPLC grade methanol (100–200 μg ml^{-1}), then filtered through a syringe filter (0.45 μm; Satorius Sedium Biotech, Aubagne, France). The redissolved sample (20 μl) was injected. The gradient mobile phase consisted of 0.4% formic acid in deionized water (solvent A) and acetonitrile (solvent B). The gradient program was as follows: 15–20% B over 0–20 min, 20–40% B over 20–40 min, 40–100% B over 40–60 min followed by 100% B for 60–65 min (Qian *et al*, 2006). The flow rate was 1 ml min^{-1} and the column temperature was maintained at 30°C. To determine the appropriate wavelength for HPLC analysis, the full UV/VIS spectra (200–800 nm) were obtained using a spectrophotometer (Jasco V-630). Based on the UV/VIS spectra (data not shown), the UV/VIS detector for the chromatograms was set to 290 and 306 nm.

Qualitative–quantitative chromatographic analysis

The standard reference compounds, emodin, resveratrol, physcion, and polydatin, were purchased from Sigma-Aldrich (St. Louis, MO, USA), Chroma Dex (Santa Ana, CA, USA), or ALEXIS Corporation (Switzerland). Stock solutions of emodin (100 μg ml^{-1}), resveratrol (100 μg ml^{-1}), and polydatin (100 μg ml^{-1}) were prepared in HPLC grade methanol. Physcion was dissolved in DMSO (100 μg ml^{-1}). For qualitative analysis, the retention times and UV spectra of the samples were compared with those of the standard compound solutions. Quantitative analysis was performed by measuring the amount of standard compounds in the samples, which was calculated from the corresponding calibration curve. The calibration curve was obtained by plotting the peak area of the standard compounds as a function of the concentration. The concentration used to calibrate the standard compounds was 0–100 μg ml^{-1}. Each calibration solution was injected twice and the curves were constructed with the averages. All the calibration curves showed an R^2 of 0.99.

Statistical analysis

All experiments, with the exception of HPLC analysis, were carried out in duplicate, and each experiment was repeated at least four times. The data are presented as mean ± s.d. The intergroup differences were estimated by one-way analysis of variance (ANOVA), followed by a *post hoc* multiple comparisons, Tukey test, for a comparison of multiple means. Values were considered statistically significant when P value was <0.05. The statistical analyses were performed using SPSS 12 software (SPSS Inc., Chicago, IL, USA).

Results

Selection of anti-acidogenic fraction

The effects of ME, HF, EF, and AF on the acidogenicity of *S. mutans* UA 159 were investigated to determine if they contain any anti-acidogenic components. The results of the inhibitory effects of ME, HF, EF, and AF on the acidogenicity of *S. mutans* are shown in Figure 2. Generally, ME, HF, EF, and AF had strong anti-acidogenic effects at 1 mg ml^{-1}. All the values (5–45 min incubation) were significantly different from the vehicle control ($P < 0.05$). Among the tested, ME and EF had the strongest anti-acidogenic effects. ME and EF produced significant decreases in pH after 45 min incubation at 1 mg ml^{-1} when compared with HF and AF ($P < 0.05$).

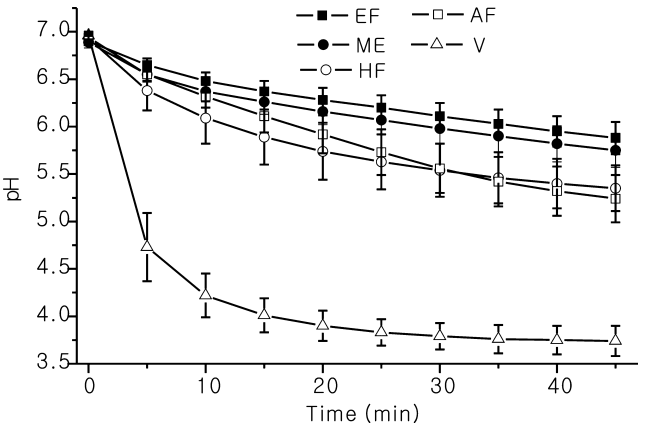

Figure 2 Influence of the methanol extract of *Polygonum cuspidatum* root and its fractions on glycolytic pH-drop of *Streptococcus mutans* UA159 in suspensions at 1 mg ml^{-1}. The vehicle control was 2% DMSO (v/v, final concentration) in the absence of the methanol extract or its fractions. ME, EF, HF, AF, and V mean methanol extract, ethyl acetate fraction, *n*-hexane fraction, aqueous methanol fraction, and vehicle control, respectively

The composition of ME and EF was also determined by HPLC qualitative analysis. Five major components, resveratrol, polydatin, anthraglycoside B, emodin, and physcion, were identified in ME and EF, based on the HPLC chromatogram (Figure 3). Generally, the composition of EF was similar to that of ME, but all the

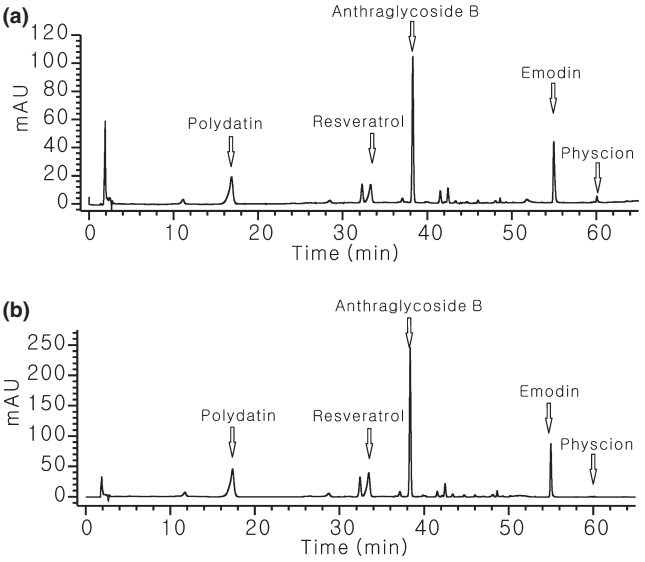

Figure 3 Representative HPLC chromatograms of the methanol extract from *Polygonum cuspidatum* root (a) and its ethyl acetate fraction (b) at 290 nm

components identified in EF were more concentrated than in ME, with the exception of physcion.

Comparison of anti-acidogenic effects of the separated substances

Six substances were separated from EF by normal phase column chromatography. Figure 4 shows the influence of the separated substances on the acidogenicity of *S. mutans* UA159. Among the substances tested, F3 had the strongest anti-acidogenic effect on *S. mutans* at 500 μg ml^{-1} ($P < 0.05$) without displaying any bactericidal activity (MICs of F3 against *S. mutans* were ≥2 mg ml^{-1}, J. G. Jeon, T. C. Yang, S. K. Kim & S. H. Ban, unpublished data). The other substances (F2, F4, and F5) from EF also disrupted the acidogenicity of *S. mutans* significantly at 500 μg ml^{-1} ($P < 0.05$).

High-performance liquid chromatography qualitative and quantitative analysis was performed to determine which components in F2, F3, and F4 have inhibitory effects on the acidogenicity of *S. mutans* UA 159, along with their concentrations. Figure 5 and Table 1 show the chemical structures and amounts of the components in F2, F3, and F4, respectively. Based on HPLC analysis, the strongest anti-acidogenic substance, F3, consisted mainly of resveratrol and emodin ($C_{14}H_{12}O_3$ and $C_{14}H_4O_2(OH)_3CH_3$, respectively). Resveratrol and emodin comprised approximately 60% of the F3 weight. F2 consisted mainly of emodin and F4 contained mainly polydatin, $C_{20}H_{22}O_8$. Emodin and polydatin comprised approximately 48% and 47% of the weight of F2 and F3, respectively.

Inhibitory effects of F3 on acidogenicity of Streptococcus mutans

As F3 had the strongest inhibitory effect on the acidogenicity of *S. mutans* UA 159, the inhibitory effect of F3 was re-experimented using sodium fluoride (NaF)

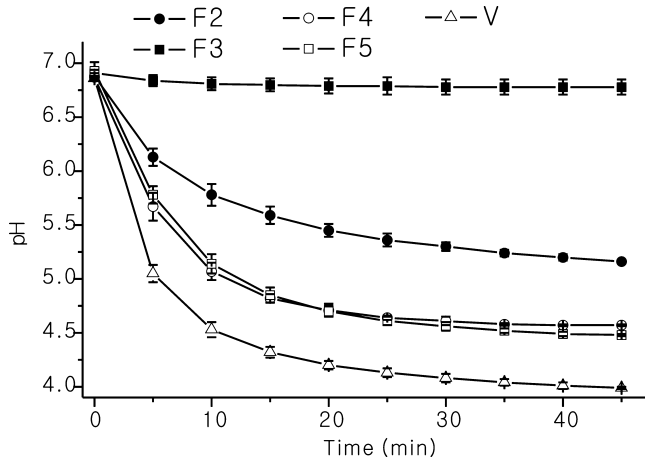

Figure 4 Influence of the fractions (F2–F6) from EF on glycolytic pH-drop of *Streptococcus mutans* UA159 in suspensions at 500 μg ml^{-1}. Vehicle control (V) was 4% DMSO + 6% ethanol (v/v, final concentration) in the absence of the test agents. EF means the ethyl acetate fraction from *Polygonum cuspidatum* root. All the agents tested, except for F4 and F5, were significantly different from each other ($P < 0.05$)

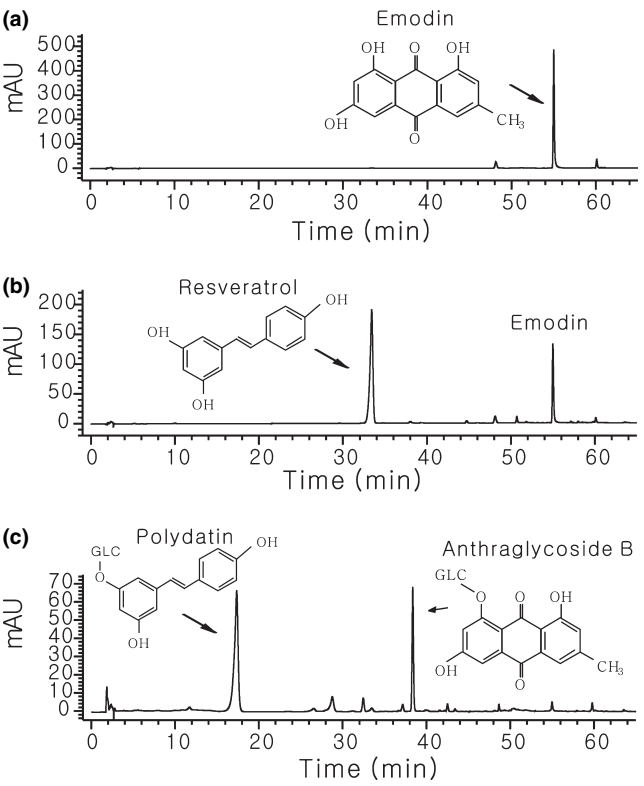

Figure 5 Representative HPLC chromatograms of F2 (a), F3 (b), and F4 fraction (c) from EF at 290 nm and the chemical structures of their main components. EF means the ethyl acetate fraction from *Polygonum cuspidatum* root

Table 1 Contents of the four constituents in the F2, F3, and F4 fractions from ethyl acetate fraction (EF)

Component	*Amount in F2* (μg mg^{-1})	*Amount in F3* (μg mg^{-1})	*Amount in F4* (μg mg^{-1})
Polydatin	0 (0)	0 (0)	468 (46.8)
Resveratrol	0 (0)	448 (44.8)	0 (0)
Emodin	476 (47.6)	146 (14.6)	12.2 (1.22)
Physcion	6.3 (0.63)	0 (0)	1 (0.1)

Values in parenthesis are expressed in percentage.
EF means the ethyl acetate fraction from *Polygonum cuspidatum* root. The figures in parentheses represent the percentage of each component in the fractions.

as a positive control to confirm the inhibitory effects. As shown in Figure 6, F3 decreased the acidogenicity of *S. mutans* UA 159 in a dose-dependent manner, even at 12.5 μg ml^{-1} ($P < 0.05$). Furthermore, the anti-acidogenic effect of F3 was similar to that of sodium fluoride at 25 μg ml^{-1} after 45 min incubation ($P > 0.05$).

Discussion

Despite the complexity of human oral flora, one species of mutans streptococci, *S. mutans*, has been implicated as a primary etiologic agent of dental caries. One of the most important virulence properties of the species is the ability to produce glucosyltransferases (GTFs) and synthesize

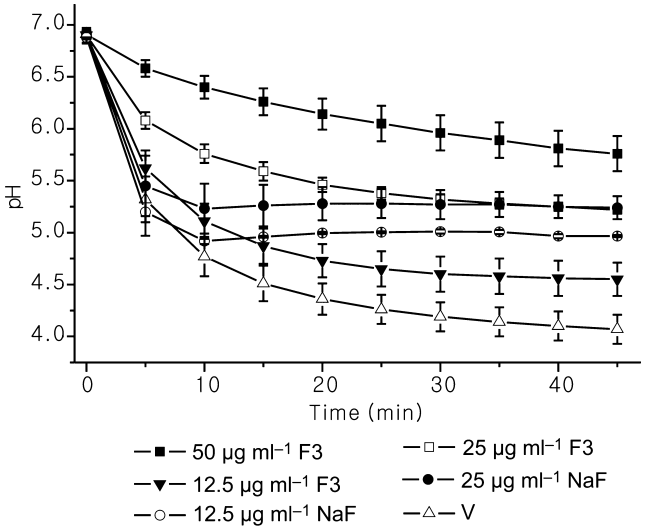

Figure 6 Influence F3 and NaF on glycolytic pH-drop of *Streptococcus mutans* UA159 in suspensions. The vehicle control was 4% DMSO + 6% ethanol (v/v, final concentration) in the absence of test agents. All the agents tested were significantly different from the vehicle control ($P < 0.05$)

water-insoluble glucan from sucrose (Hamada and Torii, 1978; Schilling and Bowen, 1992). The acidogenicity and aciduricity of the species also serve as virulence properties because the formation of acid end products during the metabolism of dietary carbohydrates by bacteria is an essential property in the development of dental caries (Harper and Loesche, 1984; Kuramitsu, 1993). In this context, this study focused on the inhibitory effects of natural products on the acidogenicity of *S. mutans* in the control of dental caries.

There has been increasing interest in the use of natural products as chemotherapeutic agents. Recently, in the dental field, many studies have attempted to determine the potential use of natural products in dentistry by inhibiting the virulence properties of caries-related bacteria or eliminating the bacteria (Hu *et al*, 2000; Nascimento *et al*, 2008). However, most of them provided limited information due to the lack of chemical analysis of the natural products related to the activities for the control of dental caries. Therefore, in this study, an attempt was made to separate the anti-ancidogenic substances from the medicinal plants using a HPLC chromatogram and glycolytic pH-drop assay to clarify the relationship between the chemical characterization and bioactivity. Furthermore, to the best of our knowledge, this is the first study using glycolytic pH-drop assay as a bioactivity guide to separate the bioactive components from natural products.

The effects of the agents tested in this study on the acidogenicity of *S. mutans* were examined using glycolytic pH-drop assay. Acid sensitization can be observed rapidly in glycolytic pH-drop experiments in which the cells are given excess glucose. *S. mutans* rapidly degrades glucose and decreases the pH of the suspension until only they can maintain a cytoplasmatic pH because at least one of the glycolytic enzymes of *S.mutans* is extremely sensitive to pH (Bunick and Kashket, 1981; Dashper and Reynolds, 1992). In the glycolytic pH-drop assay, the rate of the decrease in pH reflects the acidogenic capacity of *S. mutans*, while the final pH of the suspensions can reflect the aciduricity and total amount of hydrogen ions produced by the bacterium (Gregoire *et al*, 2007). Therefore, the results of the glycolytic pH-drop experiments suggest the potential of these agents for inhibiting the aciduricity and acidogenicity of *S. mutans*. When an agent with bactericidal activity is examined using glycolytic pH-drop assay, the results of acidogenicity and aciduricity can be masked by the bactericidal activity To eliminate the masking effects by the bactericidal activity, all the substances separated were examined below the minimum inhibitory concentrations (MICs). Generally, the MICs of the separated substances against *S. mutans* were ≥500 μg ml^{-1} (J. G. Jeon, T. C. Yang, S. K. Kim & S. H. Ban, unpublished data).

In this study, there were no significant differences in pH between ME and EF at 1 mg ml^{-1} after 45 min incubation (Figure 2). Furthermore, as shown in Figure 3, the composition of ME and EF was similar. However, because the concentration of the components is also another important factor for separation, this study finally selected EF, which contained a higher concentration of all components identified than ME, for the further separation of anti-acidogenic substances.

To separate the anti-acidogenic substances from EF, normal phase chromatography, the stationary phase of which is more polar than the mobile phase, was used because normal or reverse phase chromatography is the mode of separation used most commonly to isolate natural products (Cannell, 1998). As expected, F2, F3, and F4, which showed strong or moderate bioactivity, were comprised mainly of non-polar components (emodin etc.), non-polar – polar components (resveratrol and emodin etc.), and polar components (polydatin and anthrquinone B etc.), respectively (Figure 5). It is noteworthy that none of the compounds identified in this study was investigated as potential sources for chemotherapeutic agents in the prevention of dental caries.

Fluoride is effective in reducing the acidogenicity of *S. mutans* through a variety of mechanisms, including inhibitory effects on F-ATPase and enolase (Marquis *et al*, 2003). In this study, the anti-acidogenic activity of F3 was compared with NaF to determine if it has sufficient activity to be incorporated into mouth rinses and toothpastes in the future. As shown in Figure 6, the activity of F3 was similar to that of sodium fluoride at 25 μg ml^{-1} after 45 min incubation ($P > 0.05$). However, it is noteworthy that the inhibitory mechanisms of F3 and NaF are slightly different. The activity of F3 appeared to depend more on the direct inhibition of glycolysis than NaF, which is related to the strong F-ATPase inhibitory activity.

Emodin and resveratrol, the main components of F3, have not been studied in the dental field. The inhibitory effects of F3 might be expected to relate to the amount of emodin or resveratrol, because almost 60% of the weight of F3 consists of these two components (Table 1). However, it could be not concluded that the

inhibitory effects of F3 are due to the two main components, because some studies showed that an extract has greater bioactivity than a mixture of its major components, and F3 also contained at least six minor components (Figure 5). As a result, the activity of F3 may depend on the activity of the major or minor components, or the synergic effects between these components.

Overall, the data in this study showed that F3, which was separated from *P. cuspidatum* and consisted mainly of resveratrol and emodin, is a promising naturally occurring agent displaying inhibitory effects on the acidogenicity of *S. mutans*. The anti-acidogenic activity of F3 was similar to that of sodium fluoride. Based on these results, F3 can be considered a promising agent for controlling the acidogenicity of *S. mutans* and subsequent dental caries formation.

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Author contributions

J-G Jeon contributed to the research design. Y-R Kwon, K-J Son, S Pandit and J-E Kim performed laboratory analyses. K-W Chang performed data analyses. J-G Jeon drafted the manuscript and all authors participated in the editing and final preparation of the manuscript.

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