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Original Article

대황 추출물의 streptococci 증식 및 바이오필름 형성 억제 효과

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The inhibitory effects of *Rheum palmatum* extract on the growth of oral streptococci and biofilm formation

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Objectives: Oral streptococci play a significant role in the development of dental caries. Among them, *Streptococcus mutans* and *Streptococcus sobrinus* are the principal causative agents of dental caries. *Rheum palmatum* is a flowering plant of the family Polygonaceae with several known medicinal properties. However, its effects on oral streptococci have yet to be established. Therefore, we investigated the effects of *Rheum palmatum* for its potential use as an anticaries agent in inhibiting the growth of streptococci and preventing biofilm formation.
Methods: *Rheum palmatum* extract was diluted with sterile distilled water to obtain various extract concentrations. Several strains of oral bacteria, including *S. mutans* and *S. sobrinus*, were treated with the varying concentrations. The effects of the extract on bacterial growth was examined using the viable cell count method. Glucan synthesis was measured using a spectrophotometer at 650 nm optical density. Crystal violet staining was also carried out to observe the effect of the extract on biofilm formation.
Results: The growth of *S. mutans* and *S. sobrinus* was significantly inhibited by the *Rheum palmatum* solution at concentrations of 0.3% or more compared to the control group. The viable cell count results indicated that the number of bacterial colonies decreased 1.2-fold and 1.7-fold at concentrations of 1.25% and 2.5%, respectively, compared to the control group. Biofilm formation by *S. mutans* and *S. sobrinus* was suppressed more than 20-fold compared to the control group at extract concentrations of 1.25% or more.
Conclusions: The extract inhibited the growth of caries-causing bacteria, namely *S. mutans* and *S. sobrinus*. Furthermore, the extract inhibited the synthesis of glucan and biofilm formation by *S. mutans* and *S. sobrinus*. Therefore, this study suggests that the extract is a potential candidate as a therapeutic agent for controlling dental caries.

Key Words: Biofilm, Growth, *Rheum palmatum*, *Streptococcus mutans*, *Streptococcus sobrinus*

Introduction

There has been a significant decline in the prevalence of dental caries in some developed countries due to the increasing awareness of common dental hygiene techniques among the general population. However, no significant reduction has been seen in other parts of the world, including South Korea, and dental caries continues to be an important public health issue here[1]. There are more than 700 species of bacteria that exist in the mouth, occupying various oral surfaces. Some of them

adhere to teeth, damaging tooth structures, and resulting in the development of caries. Among these, is a group of streptococcal species called mutans streptococci (MS) and two important caries-causing bacteria in the group are *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*). These bacteria have excellent tooth adhesion properties, thereby increasing cariogenicity with acidogenicity, and are thus, an important cause of dental caries[2].

MS synthesize extracellular polysaccharides such as glucans and fructans from sucrose through the action of glucosyltransferase (GTFase) and fructosyltransferase (FTFase), respectively[3]. They serve as efficient energy sources for MS within the oral cavity[4].

Glucans are sticky glucose polymers that trap oral bacteria, leading to the formation of a biofilm. This serves as a first step in the pathogenesis of dental caries[2,3]. Insoluble glucans help *S. mutans* and *S. sobrinus* to adhere to the tooth surface and maintain the dental biofilm. *S. mutans* and *S. sobrinus* generate organic acids from glucan in addition to dietary sugars. Of these, lactic acid dissolves hydroxyapatite, a chemical component of tooth enamel[5]. Besides, glucans make the biofilm more condense and insoluble, and thus, act as a barrier that prevents penetration of saliva into the biofilm leading to a reduced salivary buffering capacity. This then causes the acid to stagnate within the biofilm, finally resulting in dental caries[6]. Therefore, suppressing the growth of *S. mutans* and *S. sobrinus* is important to lower the occurrence of dental caries.

Many medicinal plants targeting streptococci for the prevention of dental caries have been studied to date. *Camellia sinensis*[7] or *Magnolia officinalis*[8] have been reported to have antibacterial effects. However, toxicities and many side effects such as digestive disorders and hypersensitivity reactions have also been reported with the use of these plant extracts[9]. Thus, there was a need to identify an effective agent to control dental caries with fewer side effects.

Rheum palmatum is a species of flowering plant of the knotweed family Polygonaceae. It is commonly called Chinese rhubarb[10], Turkey rhubarb[11], or East Indian rhubarb[11]. *Rheum palmatum* is a herbaceous perennial related to the edible rhubarb and is primarily used in traditional medicine. In Chinese medicine, it has been used as a laxative and an anti-inflammatory drug in the treatment of various disorders. It has also been reported to possess antibacterial and antiproliferative properties[12]. Also, recent in vivo studies have shown that *Rheum palmatum* could play a beneficial role in the treatment of liver injuries and hepatitis[13]. However, the effects of *Rheum palmatum* on oral streptococci including *S. mutans* and *S. sobrinus* have not yet been investigated.

Previously, in the course of the development of preventive agents for dental caries, we had tested the antibacterial activities of several plant extracts which have been reported to be effective in the treatment of oral diseases in oriental medicine. Among them, the *Rheum palmatum* extract showed the best antibacterial effects. Thus, this study aimed to study the anticariogenic activity of *Rheum palmatum* including its inhibitory effects on the growth, glucan synthesis, and biofilm formation of the cariogenic bacteria, *S. mutans*, and *S. sobrinus*.

Materials and Methods

1. Bacterial culture

S. mutans and *S. sobrinus* were successively cultured twice in a brain heart infusion (BHI) broth (BD, MO, USA) in a 5% CO_2 incubator at 37℃ before use.

2. Preparation of *Rheum palmatum* extract and other natural extracts

Rheum palmatum extract and other natural extracts used in the study were taken from the Okcheondang located in Yeongcheon. The extraction was done with hydrothermal water using a reflux cooling extractor with 10 times (w/v) distilled water per 100 g. The extracts were filtered using a filter paper (Whatman No. 2) and then concentrated with a rotary vacuum evaporator (Buchi rotavapor R-100, Germany) before freeze drying (TFD5505, ilShin BioBase Co. Ltd., Korea). The concentration of the extracts was adjusted from 0.1% (1 mg/ml) to 10% (100 mg/ml) by diluting them in a phosphate-buffered saline solution (PBS).

3. Screening experiments of herbal extracts against *S. mutans* and *S. sobrinus*

5 ml of *Mentha piperascens*, *Ulmus macrocarpa Hance*, *Syzygium aromaticum*, *Polygonum tinctorium*, *Coptis chinesis*, and *Rheum palmatum* extracts suspended in PBS at concentrations of 0 to 5% were added into a 96-well plate and the bacteria were inoculated at 1×10^5 colony forming units (CFU)/well. After incubation at 37℃ for 24 h, the absorbance was measured at 650 nm using a spectrophotometer (Tecan, Männedorf, Switzerland). No herbal extracts were added to the control group.

4. Measurement of the growth of *S. mutans* and *S. sobrinus*

Equal quantities of BHI broth and *Rheum palmatum* extract were added to the 96-well plate and each bacterial species

was inoculated at 1×10^5 CFU/well. After incubating at 37℃ for 24 h, absorbance was measured at 650 nm using a spectrophotometer. *Rheum palmatum* extract was not added to the control group.

5. Estimation of viable cell count post-treatment

5 ml of *Rheum palmatum* extract was added into each tube at concentrations varying from 0 to 2.5%. *S. mutans* and *S. sobrinus* (1×10^4 CFU/ml) grown and diluted in the BHI broth were inoculated into the tube containing the *Rheum palmatum* extract. After 18 h of incubation in a CO_2 incubator, the mixture was diluted with PBS and inoculated with BHI agar. The number of viable cells was then measured after 48 h of incubation.

6. Measurement of biofilm formation

The BHI broth with 5% sucrose in a 24-well plate was inoculated with *S. mutans* and *S. sobrinus*, respectively, and mixed with the *Rheum palmatum* extract in concentrations ranging from 0 to 10%. After incubation at 37℃ for 72 h to induce biofilm production, the biofilm obtained was washed three times with PBS and then stained with 0.1% crystal violet solution for 10 min. After washing three times with PBS and drying in a hood for 15 min, the biofilm was dissolved by adding 100% ethanol and the absorbance was measured at 570 nm using a spectrophotometer.

7. Glucan synthesis test

S. mutans and *S. sobrinus* were inoculated to 1×10^5 CFU/ml into glass test tubes containing BHI broth with or without 5% sucrose and then the *Rheum palmatum* extract was added in concentrations varying from 0 to 2.5%. After incubation at 37℃ for 24 h, 1 ml of the culture supernatant was centrifuged and 200 μl of supernatant was transferred into the 96-well plate, and then the absorbance was measured at 650 nm by a spectrophotometer. No *Rheum palmatum* extract was added to the control group.

Results

1. Effect of *Rheum palmatum* extract on the growth of oral streptococci

In our previous study, we screened various natural extracts for their antibacterial properties. Based on this screening, *Rheum palmatum* was selected as the most effective. To examine its antibacterial activity, *S. mutans*, *S. sobrinus*, *S. oralis*, *S. mitis*, and *S. salivarius* were treated with the *Rheum palmatum* extract at 37℃ for 24 h and the optical density was then measured. All concentrations of the *Rheum palmatum* extract inhibited the growth of all the Streptococcal species used in the experiment, and the growth of all the test strains was inhibited at concentrations above 0.6% (Fig. 1).

Among the streptococci, *S. mutans* and *S. sobrinus* are the major causative agents of dental caries. A viable cell count assay

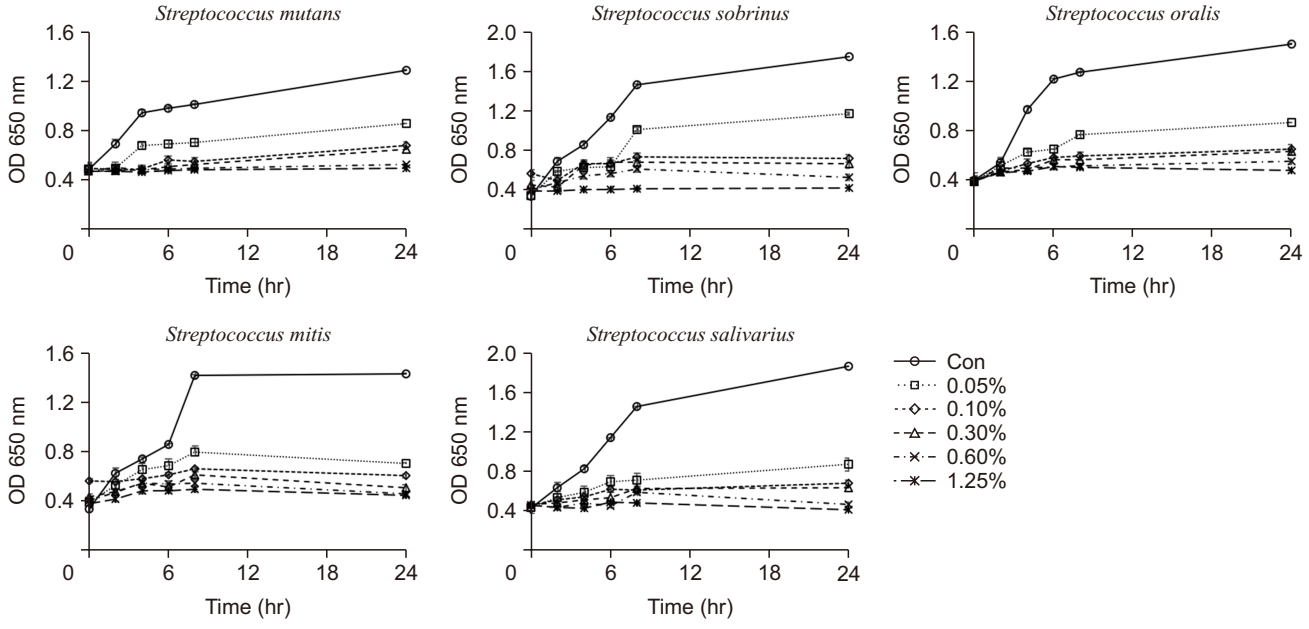

Fig. 1. Effect of *Rheum palmatum* extract on the growth of oral streptococci. Oral streptococcal species were treated with *Rheum palmatum* extract solution of 1.25% to 2.5% and optical densities were measured at 650 nm after incubation of 37℃ for 24 h.

was carried out to examine in detail, the effect of the *Rheum palmatum* extract on the growth of *S. mutans* and *S. sobrinus*. *Rheum palmatum* extract at concentrations of 1.25% and 2.5% significantly decreased the number of viable cells of *S. mutans* and *S. sobrinus*, compared to the control group. The *Rheum palmatum* extract thus inhibited viable cells in a concentration-dependent manner (Fig. 2).

2. Effect of *Rheum palmatum* extract on biofilm formation

We examined whether *Rheum palmatum* extract inhibited biofilm formation which is an early step in the development of dental caries. As shown in Fig. 3, the biofilm formation by *S. mutans* and *S. sobrinus* was decreased 20-fold or more at concentrations of 1.25 and 2.5% of *Rheum palmatum*, compared to the control.

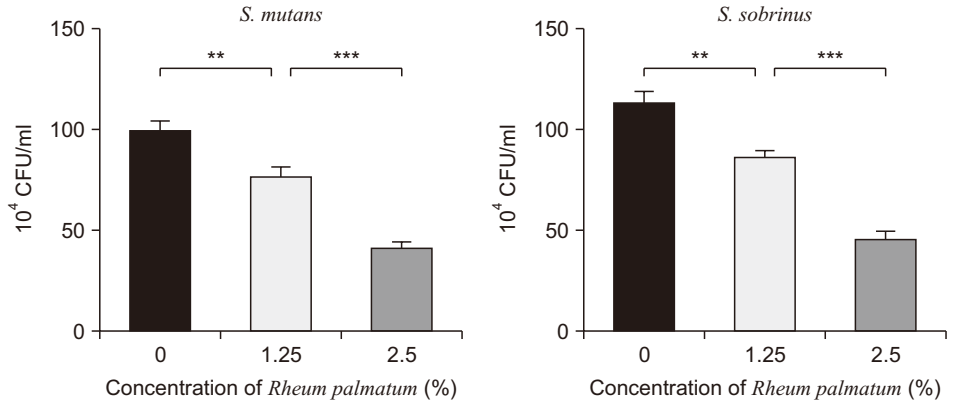

Fig. 2. Effect of *Rheum palmatum* extract on the growth of *S. mutans* and *S. sobrinus* by viable cell count. *S. mutans* and *S. sobrinus* were treated with or without *Rheum palmatum* extract at 37℃ overnight and viable cells were counted on BHI agar plates. **$P<0.01$, ***$P<0.001$.

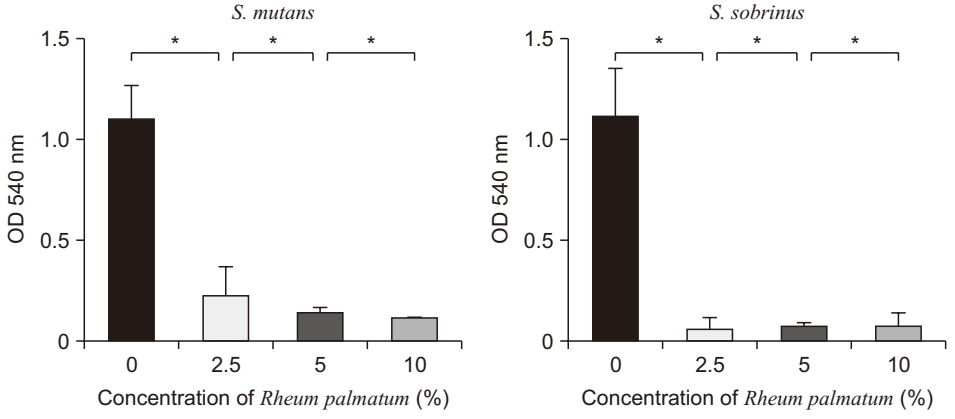

Fig. 3. Effect of *Rheum palmatum* extract on the biofilm formation. The biofilm was produced by *S. mutans* and *S. sobrinus* at 37℃ for 72 h, washed with PBS, stained with crystal violet and measured by detecting optical densities at 540 nm using spectrophotometer. *$P<0.05$.

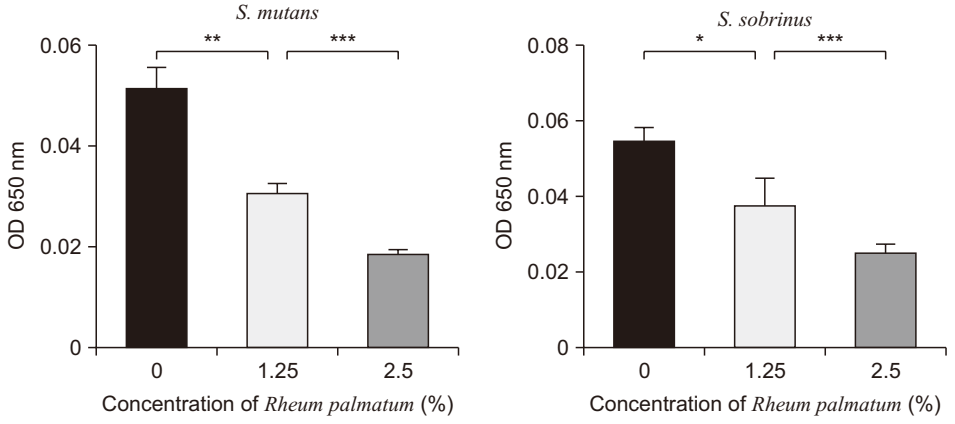

Fig. 4. Effect of *Rheum palmatum* extract on the glucan synthesis. *S. mutans* and *S. sobrinus* were grown in BHIS broth with or without *Rheum palmatum* extract at 37℃ overnight. The culture solution was centrifuged and the optical densities of the culture supernatants were measured at 650 nm to determine glucan synthesis. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

3. Effect of *Rheum palmatum* extract on glucan synthesis

We examined the effect of *Rheum palmatum* on glucan synthesis which is essential for bacterial adherence and biofilm formation. The *Rheum palmatum* extract was observed to inhibit glucan synthesis (Fig. 4). Specifically, the 1.25% and 2.5% concentration levels of the *Rheum palmatum* extract significantly inhibited glucan synthesis by both *S. mutans* and *S. sobrinus*, compared to the control.

Discussion

We screened the various candidate oriental medicines including *Rheum palmatum* for their antibacterial activity, inhibition of glucan synthesis and formation of biofilms. In accordance with our previous study, we found that *Rheum palmatum* extract most effectively inhibited the growth of *S. mutans* and *S. sobrinus*, synthesis of glucan, and formation of biofilms.

According to traditional Chinese medicine, *Rheum palmatum* is one of the most ancient and medically useful herbs[14]. *Rheum palmatum* has thick roots, hollow and erect stems, and small white-green or purple-red flowers clustered on its branches and belongs to the *Rheum L.* genus from the Polygonaceae family[14]. Anthraquinones are a major group of the polyphenol constituents of *Rheum palmatum*, and these include aloe-emodin, rhein, emodin, chrysophanol, and their glycosides[15]. Recent studies have reported anticancer[16], antiviral[17], antioxidant[18], and antidiabetic activities of anthraquinones[15]. Also, *Rheum palmatum* has long been used as an anti-inflammatory, antifibrotic and anticancer medicine in China[19]. In the ancient Chinese book "*Shen Nong Ben Cao Jing*", the rhizome of *Rheum palmatum* was classified as an important medicinal plant[20]. Furthermore, there is clinical evidence of the efficacy of *Rheum palmatum* in treating chronic hepatic diseases[20]. Although studies have been conducted to evaluate the role of *Rheum palmatum* in various diseases, its effect on dental caries has not been examined to date.

In this study, the *Rheum palmatum* extract showed a strong inhibitory effect on the growth of *S. mutans* and *S. sobrinus* in a dose-dependent manner. Specifically, the number of viable bacteria was significantly reduced when *S. mutans* and *S. sobrinus* were exposed to *Rheum palmatum* extract at a concentration of 2.5% for 18 h. To date, *Rheum palmatum* has been found to have antibacterial effects only on *Staphylococcus aureus* and *Pseudomonas aerginosa*[21]. The antibacterial effect of *Rheum palmatum* extract on *S. mutans* and *S. sobrinus* can make it a useful agent for use in the prevention of dental caries.

Next, we investigated the effects of *Rheum palmatum* extract on the formation of biofilms by *S. mutans* and *S. sobrinus*. The microbial population, including streptococci, forms a dental biofilm in the oral cavity that is composed of bacteria and bacterial products including polysaccharides enclosed in a matrix of extracellular material derived both from the cells themselves and the environment[2]. Compared to floating bacteria, the biofilm can withstand a variety of environmental stresses such as nutrient depletion, dehydration, pH shifts, and osmotic shock. Biofilms adversely affect oral health and are resistant to commonly used antibacterial agents, making treatment of dental caries a significant challenge[22]. Therefore, it is important to inhibit the biofilm formation by streptococci to prevent dental caries. In this study, the *Rheum palmatum* extract significantly reduced the biofilm formation by *S. mutans* and *S. sobrinus*.

Finally, we examined the effects of *Rheum palmatum* extract on glucan synthesis by streptococci. Glucans are a major factor contributing to the ability of streptococci to adhere to the tooth surface and for bacterial cells to aggregate within a biofilm. The presence of glucan, allows the bacteria to remain adhered to the teeth, despite the action of physical forces such as mastication[23]. For the prevention of dental caries, it is not only necessary to inhibit the biofilm formation, but also essential to inhibit the synthesis of glucans. *S. mutans* synthesizes glucans from sucrose using GTFase enzyme. Both soluble and insoluble glucans have strong adhesive properties[23]. Specifically, the insoluble glucans synthesized by the oral bacteria play an important role in the formation of biofilm by promoting their adhesion and accumulation[24]. Various Chinese herbal medicines[25], ribocitrin[26], and polyphenols of cocoa bean husk[27] have been studied to assess their GTFase inhibitory activity[28]. Of these, extracts of two Chinese herbal medicines, the *Coptis chinensis* extract[29] and *Radix pulsatillae* extract[30] have been shown to simultaneously inhibit the formation of the biofilm and the synthesis of glucans. The present results with *Rheum palmatum* extract are similar, as the extract inhibited not only the formation of biofilm but also glucan synthesis.

Conclusions

This study showed that the *Rheum palmatum* extract inhibited the growth, biofilm formation, and glucan synthesis of *S. mutans* and *S. sobrinus*. Therefore, *Rheum palmatum* extract could be suggested as a potential candidate for the control of dental caries by virtue of its antibacterial effects.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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