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Inhibitory Effects of Resina Pini on the Growth and Glucosyltransferase activity of *Streptococcus mutans*

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Abstract – The purpose of this study is to evaluate the inhibitory effects of Resina Pini against *Streptococcus mutans* (*S. mutans*) that is one of the major causes of dental caries and oral diseases. Topically applied Resina Pini (RP) would be incorporated in saliva and thus the factor associated with water solubility should be considered. In this paper, therefore, effects of various treatment for RP and activities of water extracts from unprocessed and processed RP were compared. The crude RP (RP1) and the recrystallized RP (RP2) in ethanol solution showed strong antimicrobial activities (d.>15mm) against *S. mutans*. All RP samples exhibited considerable inhibitory effect against glucosyltransferase produced by *S. mutans* (IC_{50}= 91.2 to 276.2 μg/ml). The very considerable increase in cellular permeability of *S. mutans* was observed with RP1, RP2 and their water extracts. These results suggest that RP1 and RP2 may be a potential source for pharmaceutical products used for prevention and/or treatment of dental caries and periodontal disease.

Keywords – Resina Pini, glucosyltransferase, *Streptococcus mutans,* periodontal disease, Cellular permeability

Introduction

Streptococcus mutans (*S. mutans*) is considered one of the primary causative agents of dental caries and periodontal disease, which comprise the most common oral disease (Banas, 2004; Wu-Yuan, 1988). Microbial colonization of tooth and mucosal surfaces and the subsequent initiation of plaque formation are dependent on the adherence of bacteria to each other and to host cells (Nyvad and Kalin, 1987). Glucosyltransferase (GTase) produced by *S. mutans* catalyze glucosyl transfer from sucrose to a glucan chain and synthesis of insoluble glucan with 93% of α-(1 → 3) linkages and 7% of α-(1 → 6) linkages from sucrose (Tsumori and Kuramitsu, 1997). Glucans mediate the adherence of *S. mutans* and other oral bacteria flora on tooth and mucosal surfaces contribute to the formation of plaque. Hence, the inhibition of this process would result in the prevention of dental caries and periodontitis (Yanagida *et al.*, 2000). Failure of conventional periodontal therapy may be related to an incomplete elimination of periodontopathic bacteria (Mombelli, 2003; Schwach-Abdellaoui *et al.*, 2002). Therefore, the microbial etiology of gingivitis and periodontitis provides the rationale for use of adjunctive antimicrobial agents in the prevention and treatment of oral diseases (Trombelli and Tatakis, 2003). The active agents should prevent biofilm formation without affecting the biological equilibrium within the oral cavity (Goodson, 1994). The use of natural products has been a successful strategy for the discovery of new medicine and a number of natural extracts have been investigated recently (Harvey, 2000; Bacca *et al.*, 1997). Resina Pini is a resinous exudation obtained from *Pinus densiflora* and has been used traditionally for the purpose of precaution and treatment of periodontitis and other oral diseases (Yun, 1997). The main components of Resina Pini are rosine including levopimaric acid, neoabietic acid, dextropimaric acid, isodextropimaric acid. Besides the rosine, terpenoids including α-pinene, β-pinene, myrcene, β-phellandrene, linalool and linalyl acetate have been isolated from Resina pini (Song and Kim, 1994; Namba, 1980). Since Resina Pini may contain harmful components, processed Resina Pini has been often used in order to avoid the hazards caused by those toxicants and increase the therapeutic effects. Topically applied Resina Pini would be incorporated in saliva and thus the factor associated with water solubility should be considered.

The aim of this study was to evaluate and compare the inhibitory effects of various Resina Pini samples on *S. mutans*. Furthermore, the possibility of novel agent for prevention and/or treatment of dental caries and periodontal

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disease is discussed.

Experimental

Plant materials and extraction – Resina Pini and Ramus Mori Albae were purchased from an herb market in Daegu, Korea. The crude Resina Pini (unprocessed Resina Pini, RP1) was powdered and filtrated through gauze at 90°C. After coagulation in cold distilled water, the recrystallized Resina Pini (RP2) was obtained and air-dried. To prepare the Ramus Mori Albae-treated Resina Pini (RP3), the mixture of Resina Pini : Ramus Mori Albae 20:3 (w/w) was boiled with distilled water (500 ml) adequately. Then, the mixture was filtered and recrystallized after cooling in the same way described above. The powdered crude Resina Pini (unprocessed Resina Pini) and processed Resina Pinies were extracted with distilled water. Each 20 g of powdered plant materials was boiled with distilled water (500 ml) in rotary vacuum evaporator for 5 h at 90°C. The mixture was filtrated with Whatman 2 filter, and centrifuged at 4,000 rpm for 25 min. The supernatant was concentrated under vacuum below 60°C to furnish the dried extracts. The water extract of the crude Resina Pini (RP1-WE) yielded a brown crystalline residue (1.4% w/w). Similarly, the water extracts of the recrystallized Resina Pini (RP2-WE) and the Ramus Mori Albae-treated Resina Pini (RP3-WE) yielded a yellow brown crystalline solid (0.7% w/w) and a dark brown crystalline solid (2.2% w/w), respectively. The dried powder of plant material was dissolved in 10% DMSO (v/v) and filtered through a Millipore filter (0.22 m, Gelman lab) prior to use.

Bacteria – *S. mutans* KCTC 3065 was used in this study. *S. mutans* was cultivated in the Brain Heart Infusion (BHI, Difco, USA) broth at 37°C with shaking. The culture was stored at −70°C in BHI containing 30% glycerol (w/v).

Inhibitory effect on growth of *S. mutans* – The inhibitory effect of Resina Pini on the growth of *S. mutans* was determined by the disk diffusion tests according to the recommendations of the National Committee for Clinic Laboratory Standards (NCCLS, 1999). All samples were dissolved in distilled water and 50% ethanol (v/v) just prior to performance of the assays. *S. mutans* (0.5 McFarland turbidity) was streaked onto BHI agar plate. Disks (diameter, 8 mm) were impregnated into the water and ethanol solutions of tested materials and placed on the inoculated agar surface. For ethanol solutions of samples, disks were dried under sterile condition to remove the ethanol before placing. The plates were inverted and incubated for 18 hours in 5% CO_2 at 37°C before the results were determined. Antimicrobial activity was recorded by measuring the diameter (d.) of the clear inhibition zones around each disk. The negative control test consisted of the same plate with solvent alone (no tested material). The positive control test consisted of the same plate with 0.1% chlorhexidine gluconate (v/v) disk. All assays were performed in triplicate.

Inhibitory effect glucosyltransferase activity – All tested materials were dissolved in 10% DMSO (v/v) just prior to performance of the assays. *S. mutans* was grown for 24 h at 37°C in the BHI broth. Culture supernatant was obtained by centrifugation at 8,000×g for 30 min at 4°C. The supernatant was concentrated by 50% saturated ammonium sulfate precipitation. After centrifugation at 14,000×g for 30 min, the precipitates were used as the crude glucosyltransferase (GTase). 0.025 ml of GTase and 0.175 ml of Resina Pini samples were added to 0.8 ml of 0.0625 mol/l potassium phosphate buffer (pH 6.5) containing 12.5 μg/l sucrose and 0.25 μg/l sodium azide (Lee *et al.*, 2002). To measure GTase activity, the reaction mixture was incubated for 24 h at 37°C, in a total volume of 1 ml. Then, the water-insoluble glucan was sedimented and washed with 3.0 ml of distilled water. The sediment suspended in 3.0 ml of distilled water was ultrasonicated for 5 sec (Ultrasonic generator US-300, Nissei, Japan). For the analysis of glucan, the absorbance of the suspension was measured at 550 nm (UV/Visible Spectrophotometer Ultrospec 2000, Phamacia Biotec) against the corresponding blank (Kwon *et al.*, 1993).

Cellular permeability assay – Whole culture broth of *S. mutans* containing tested materials was incubated for 20 min at 37°C and centrifuged at 10,000×g for 20 min at 4°C. After the supernatant was removed, the harvested cells were resuspended in 0.1 M phosphate buffer (pH 7.0) and kept for 10 min at 37°C. The absorption was determined at 260 nm (Park and Shin, 1995).

Statistics – All values were expressed as a mean±S.D. Differences between treatments were analyzed statistically by unpaired Student's *t*-test. $p < 0.05$ or <0.01 was considered statistically significant.

Results

Antimicrobial activity on *S. mutans* – Table 1 shows the inhibition zone diameter values of the Resina Pini samples. Among the Resina Pini samples tested, RP1 and RP2 were showed inhibitory effects against *S. mutans*. In RP3, any significant change in the growth inhibition activity was not observed versus the negative control. RP1

Table 1. Antimicrobial activity of Resina Pini on *Streptococcus mutans*

Tested materials	Concentration (μg/ml)	Inhibition zone (mm)	
		Water solution	50% ethanol solution
Control		8.00±0.00	8.90±0.42
Raw Resina Pini	90	9.15±0.35	16.24±0.83*
	125	9.18±0.53	15.50±2.34*
	200	9.43±0.74	16.16±2.25*
	500	9.78±0.60	20.80±0.28*
Recrystallized Resina Pini	90	8.95±0.21	15.70±2.40*
	125	9.10±0.28	17.68±1.58*
	200	9.30±0.14	19.38±6.02*
	500	9.83±0.11	21.76±0.53*
Ramus Mori Albae-treated Resina Pini	90	< 8.00[a)]	< 8.00
	125	< 8.00	< 8.00
	200	< 8.00	< 8.00
	500	< 8.00	< 8.00

Results are mean±S.D. (n = 3). Control group were not exposed to Resina Pini samples. Chlorhexidine gluconate (100 μg/ml) used as positive control showed 23.25±3.18. *$p < 0.05$, significant to the control group. [a)] No effect: < 8.00 mm.

and RP2 in distilled water showed weak activity (d. < 10mm) against *S. mutans*. However, a significant growth inhibition of *S. mutans* was found in their ethanol solutions (final concentrations ranged from 90 to 500 μg/ml). They showed strong antimicrobial activities (d. > 15 mm) on *S. mutans*, similar to that of chlorhexidine gluconate (100 μg/ml) used as positive control. Chlorhexidine gluconate showed 23.25±3.18.

Inhibitory effect on glucosyltransferase activity – The inhibitory effects of Resina Pini samples on glucan synthesis by crude GTase obtained from *S. mutans* were examined as a function of their concentrations, and are illustrated in Fig. 1. The inhibitory effects of the samples were graphically expressed as the relative inhibition rate produced at a certain sample concentration as compared to the amount produced in the absence of any sample (the control test). All of the tested materials showed the inhibitory activity ranging from 12.02% to 88.96% at the tested concentration in a concentration-dependent manner. The highest Inhibitory value was 88.96% with RP2-WE at the concentration level of 500.0 μg/ml. As showing in Fig. 1A, RP1 and RP2 at a concentration of 500.0 μg/ml suppressed glucan synthesis at 73.60% and 80.33%, respectively. A significant inhibition of glucan synthesis by GTase was found at concentrations of more than 20.0 μg/ml for RP1 and RP2 ($p < 0.01$), when compared to the negative control by Student's *t*-test. Furthermore, both samples exhibited similar IC_{50} values (concentration needed for 50% inhibition of glucan synthesis), which were estimated at 116.5 μg/ml and 116.3 μg/ml, respectively. However, RP3 exhibited slightly lower effects than those obtained with RP1 and RP2. Maximum inhibitory value of RP3 was 58.14% at 500.0 μg/ml and

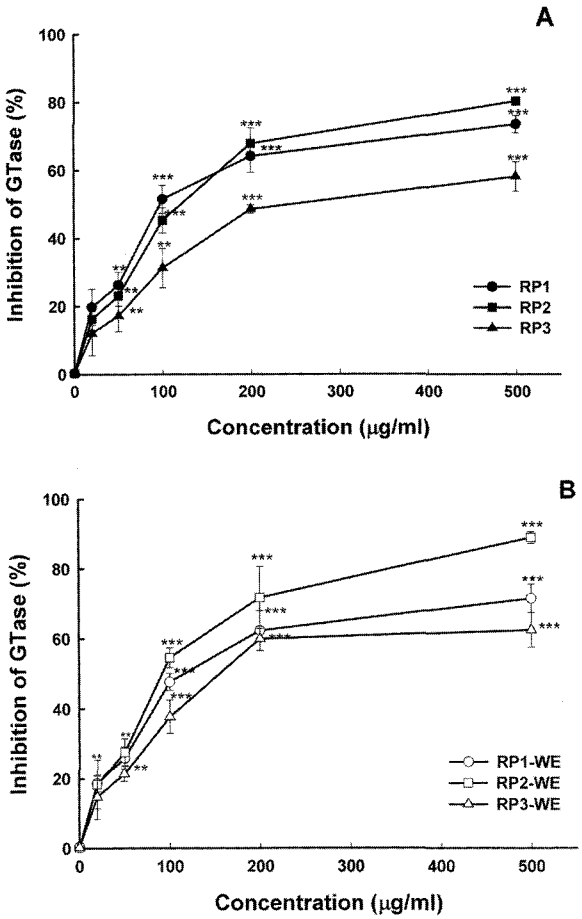

Fig. 1. Inhibitiory effects of (A) various Resina Pinies and (B) their water extracts on glucan synthesis by crude glucosyltransferase of *S. mutans*. The % inhibition of glucan synthesis means the relative amount (%) of glucan produced at a certain sample concentration as compared to the amount produced in the control test. Each value indicates mean±S.D. (n = 3). Statistically significant difference between control (no sample) and treatment with Resina Pini sample (**$p < 0.01$, ***$p < 0.001$).

IC_{50} was estimated at 276.2 μg/ml. Fig. 1B shows the inhibition of RP1-WE, RP2-WE and RP3-WE on GTase activity. RP1-WE, RP2-WE and RP3-WE reduced the glucan synthesis by *S. mutans* in a concentration-dependent manner and the highest values were 71.47%, 88.96% and 62.40%, respectively. A significant inhibition of glucan synthesis by GTase was found at concentrations of more than 20.0 μg/ml for RP1-WE, while RP2-WE and RP3-WE presented a significant inhibitory effect at concentrations of more than 50.0 μg/ml for ($p < 0.01$). The IC_{50} of RP1-WE, RP2-WE and RP3-WE were estimated at 129.3 μg/ml, 91.2 μg/ml and 180.6 μg/ml, respectively. Although the inhibitory values of glucan synthesis obtained with RP1-WE were slightly higher than those obtained with RP3-WE, treatments with both extracts were not significantly different from each other at $p < 0.05$.

Effect on cellular permeability of *S. mutans* – The cellular permeability of *S. mutans* exposed to the Resina Pini sample were established by the plot of absorbance (260 nm) of the culture *versus* concentration (50-500 μg/ml final concentration), as illustrated in Fig. 2. All Resina Pini samples increased the cellular permeability of *S. mutans* significantly ($p < 0.001$) and the effects were dependent on the concentrations in the reaction mixture. Moreover, The values of Resina Pini samples treatmented at 500.0 μg/ml concentration were comparable to that of control, about five times more. The absorbance of the control culture (not exposed to samples) was 0.386±0.013. As shown in Fig. 2A, treatments with RP1 and RP2 at the tested concentrations exhibited stronger effect than that obtained with RP3. Furthermore, RP1 and RP2 showed close absorbance values for cellular permeability of *S. mutans*, but treatments with both samples were found to be significantly different from each other at the tested concentration ($p < 0.01$). The highest absorbance values reached by unprocessed or processed Resina Pini-treated cultures were 2.310±0.011 for RP1, 2.166±0.007 for RP2 and 1.832±0.011 for RP3, respectively. Fig. 2B shows the effect of cellular permeability with increasing concentrations of RP1-WE, RP2-WE and RP3-WE. The treatment of three water extracts caused a significant increase in the cellular permeability of *S. mutans* at the tested concentration ($p < 0.001$), when compared to the negative control (not exposed to extracts), except RP3-WE at a concentration of 50 μg/ml. In addition, RP1-WE and RP2-WE exhibited higher absorbance values than those obtained with RP3-WE. Although the concentration-response relation of RP1-WE and RP2-WE was represented by curves with close slopes, both the extracts treatments

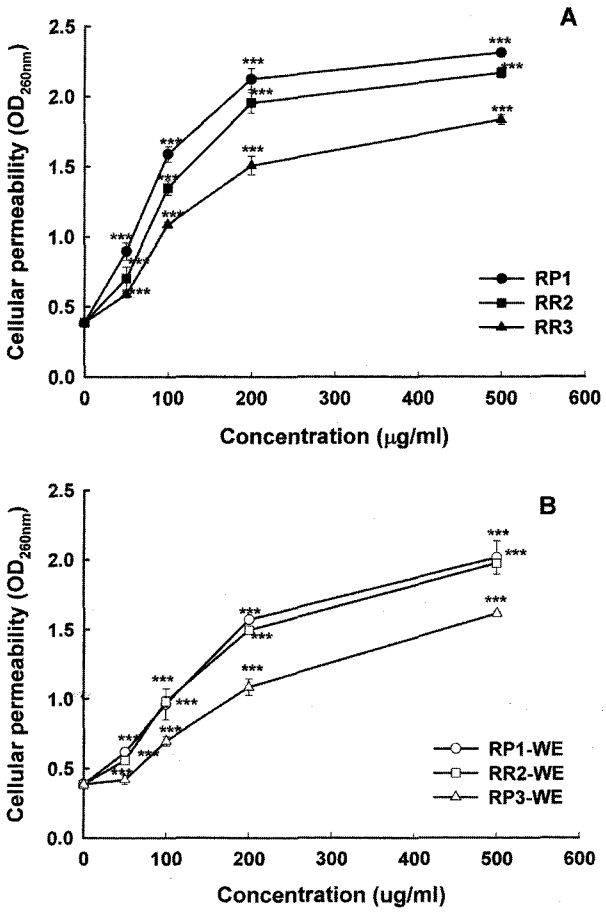

Fig. 2. Effects of (A) various Resina Pinies and (B) their water extracts on cellular permeability of *S. mutans*. The values represent OD_{260} mean±S.D. (n = 3). Statistically significant difference between control (no sample) and treatment with Resina Pini sample (***$p < 0.001$).

were found to be significantly different from each other at $p < 0.01$ when compared by Student's *t*-test. The highest absorbance values of water extracts were 2.014±0.020 for RP1-WE, 1.973±0.008 for RP2-WE and 1.614±0.016 for RP3-WE, respectively.

Discussion

Besides the purpose of prevention and treatment of dental caries and periodontitis, Resina Pini has been used externally as folk medicine in Korea to treat various diseases such as itch, burning sensation, acne, wounds, skin diseases, cough, ulcers, neuralgia, rheumatoid arthritis, *etc*. In addition, Resina Pini was administered orally for the treatment of pulmonary tuberculosis, pulmonary abscess and peptic ulcer before the development of modern medicine (Yun, 1997). The processed Resina Pini with or without Ramus Mori Albae for the detoxification has been commonly used in the traditional prescription.

Ramus Mori Albae is a dried twig of *Morus alba L.*, used as antineuralgic, antihypertensive and diuretic (Kim, 1995). Mulberrin that is one of the ingredients of Ramus Mori Albae is reported to possess potential anti-inflammatory activity (Park *et al.*, 1990). Hence, in the present study, six samples including the crude Resina Pini (unprocessed Resina Pini, RP1), two types of processed Resina Pini (RP2 and RP3) and their three water extracts were selected for the *in vitro* studies.

Oral streptococci, especially *S. mutans* is one of the major pathogens causing dental caries and oral diseases. These species utilizes sugars including sucrose to produce glucosyltransferase (GTase) and synthesize glucans from sucrose to generate the plaque and increase the enamel demineralization and tissue damage (Tanzer, 1989). Bacterial plaque has been implicated as the principal etiological factor in both dental caries and periodontal diseases. Therefore, the inhibition of GTase activity is one of the important factors in the inhibition of bacterial adherence that deserves attention during the investigation of new medicine to be employed in dental care and prevention of periodontal disease. Among the six samples tested, RP1, RP2 and their water extracts (RP1-WE and RP2-WE) exhibited strong inhibitory activity as evidenced by IC_{50} < 130.0 μg/ml (Fig. 1). But the IC_{50} values of RP3 and RP3-WE were found to be higher than those of them. Especially, RP1, RP2 and their water extracts exhibited similar inhibitory profiles for the synthesis of glucan, which suggests that the inhibitory effect on GTase activity could be due to bioactive components of Resina Pini.

Promotion of the cellular permeability is another antimicrobial factor of *S. mutans*. Most bacteria have a delicate cell membrane, which contains a cytoplasm enclosed within the membrane. The cell membrane is selectively permeable and capable pf transporting nutrients inward and wastes outward. Therefore, the effect of Resina Pini samples on the cellular permeability of *S. mutans* was evaluated and all the samples of Resina Pini at the tested concentration caused significant increase of the cellular permeability leading to the leakage of cytosolic components. Interestingly, RP1, RP2 and their water extracts showed stronger effect than those of RP3 and RP3-WE. Furthermore, this result is agreement with data of GTase inhibition.

Based on these findings, it would be supposed that the inhibitory effect on gluan synthesis might be due to the antimicrbial activity against *S. mutans*. Therefore, the antimicrobial activity of the crude Resina Pini and two processed Resina Pini against *S. mutans* was assessed using the disk diffusion test. RP1 and RP2 in 50% ethanol solution (v/v) exhibited very strong inhibitory activity (d.>15 mm) on the growth of *S. mutans* but RP3 did not showed any activity (d.<8 mm). It is apparent from this study that only Resina Pini has antimicrobial activity against *S. mutans*.

These biological activities such as antimicrobial, GTase inhibitory activities of Resina Pini observed in the present study support its traditional use. In conclusion, our results suggest that the crude Resina Pini and processed Resina Pini could be successfully incorporated into pharmaceutical products employed in prevention and/or treatment of dental caries and periodontal disease.

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