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Antibacterial activity of phellodendron bark against *Streptococcus mutans*

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ABSTRACT

Streptococcus mutans is a major cause of tooth decay due to its promotion of biofilm formation and acid production. Several plant extracts have been reported to have multiple biological activities such as anti-inflammation and anti-bacterial effects. In this study, we investigated the antibacterial activity of three plant extracts, phellodendron bark (PB), yucca, and black ginger, and found that PB had a stronger effect than other extracts. Then, we investigated the minimum inhibitory concentration (MIC) of PB against 100 *S. mutans* strains. The MIC range of PB was 9.8–312.5 μg/mL. PB suppressed the growth kinetics of *S. mutans* in a dose-dependent manner, even at sub-MICs of PB. Then, we investigated the effect of PB on *S. mutans* virulence. PB suppressed biofilm formation at high concentrations, although PB did not affect the expression of glucosyltransferase genes. Additionally, PB suppressed the decrease in pH from adding excess of glucose. The expression of genes responsible for acid production was increased by the addition of excess glucose without PB, whereas their expression levels were not increased in the presence of 1× and 2× MIC of PB.

Although PB showed a bacteriostatic effect on planktonic *S. mutans* cells, we found that more than 2× MIC of PB showed a partial bactericidal effect on biofilm cells. In conclusion, PB not only showed antibacterial activity against *S. mutans* but also decreased the cariogenic activity in *S. mutans*.

INTRODUCTION

Recently, the emergence of multi-drug resistant bacteria has caused serious problems for chemotherapeutic therapy against bacterial infection (1). Therefore, the proper use of existing antibacterial agents against bacterial infections is required worldwide. In addition, the development of new antibacterial agents is expected. It is well known that several plant extracts including catechin, flavonol, and phenolic acids have biological activities such as anti-inflammation and antibacterial among others (2, 3, 4). Previously, we found that glycyrrhetinic acid (GRA) had antibacterial activity against *Staphylococcus aureus* including methicillin-resistant *S. aureus* (MRSA) and *Streptococcus mutans*, a cariogenic bacteria (5, 6). GRA is a major component of the extract from licorice, which is a Leguminosae perennial (7, 8, 9). Therefore, several plant extracts with

antibacterial activity may have potential use as chemotherapeutic agents in addition to the use as disinfectants and food additives.

Streptococcus mutans is a major cariogenic pathogen in humans (10, 11). Two major factors, glucan and acid production, are related to the formation of tooth decay (12, 13). *S. mutans* produces glucosyltransferases (GTFs), which mediate the reaction with the synthesis of water-insoluble sticky glucan using sucrose as a sole substrate. This glucan is responsible for the formation of dental plaque, which is a focus for tooth decay and periodontitis. In addition, *S. mutans* produces lactic acid by metabolizing various sugars, causing the demineralization of calcium phosphate in tooth enamel (12, 13). In addition, *S. mutans* is resistant to acidic environments by several acid tolerance factors. In *S. mutans*, H^+-ATPase is a proton pump responsible for de-acidification, and AgDS is part of the agmatine deiminase system operon for alkali production (14, 15, 16). These virulence factors are strongly related to tooth decay formation. Recently, *cnm*-positive *S. mutans* has been reported to induce cerebral hemorrhagic stroke as a result of bloodstream infection (17, 18, 19). Due to the collagen binding affinity of Cnm, Cnm-positive *S. mutans* adhere to vascular endothelial cells in

injured blood vessels and inhibit platelet aggregation for repairing blood vessels, resulting in continuous bleeding. Therefore, control of *S. mutans* in the oral cavity is important for the prevention of oral and systemic diseases.

In this study, we investigated three plant extracts, phellodendron bark (PB), yucca, and black ginger against *S. mutans* and found that PB had the strongest activity among extracts. Then, we investigated the effect of PB on *S. mutans* cariogenic activity including biofilm formation and acid production. Our results suggested the potential use of PB for the control *S. mutans* in the oral cavity.

MATERIALS AND METHODS

Bacterial strains and culture

S. mutans UA159 (20), RIMD3125001 (obtained by Riken BRC collection), MT8148 (21) and clinical isolates were used in this study. Additionally, *S. sobrinus* (except B13 was laboratory strain [22]), *S. sanguinis, S. salivarius*, and *S. gordonii* were obtained by Riken BRC collection or Japanese Society for Bacteriology. These strains were grown in trypticase soy broth (TSB) (Becton Dickinson Microbiology Systems, Cockeysville, MD) at 37°C with 5% CO_2.

Clinically isolated *S. mutans* strains were obtained from volunteers as described previously (6). Appropriate dilution of saliva were inoculated to Mitis-Salivarius agar medium (Becton Dickinson and company, Franklin Lakes, USA) containing 32 μg/ml of bacitracin. After 2days incubation at 37°C with 5% CO_2, single colony was pick up and inoculated in TSB. Finally, *S. mutans* was verified by PCR using *S. mutans* specific primer. *S. mutans* isolation was approved by the ethics committee of the Kagoshima University Graduate School of Medical and Dental Sciences (No.701).

Extracts from plants

Extracts of phellodendron bark (PB; *Phellodendron amurense*), yucca stem (*Yucca schidigera*) and black ginger root (*Kaempferia parviflora*) were obtained from Maruzen Pharmaceuticals Co., Ltd., Hiroshima, Japan. PB and black ginger were solubilized in 10% ethanol, and yucca was solubilized in distilled water. Stock solutions of each reagent were prepared at a concentration of 10 mg/ml and were diluted in medium to the appropriate concentrations indicated in each experiment.

MIC determination of plant extracts

The minimum inhibitory concentration (MIC) was measured using the micro-dilution method (23). Each extract was solubilized to 2500 μg/ml in TSB, and two fold serial dilutions were prepared in a 96-well microplate (Thermo Fischer Scientific, Roskilde, Denmark). Overnight cultures of *S. mutans* strains were adjusted to an OD_{660} of 1.0 (1×10^9 cells/mL) and diluted to 1:100 with TSB (1×10^7 cells/mL). Ten microliter of the dilution (1×10^5 cells/well) was added to each well (100 μL). The MICs were determined after 24 h incubation at 37°C.

Effect of phellodendron bark on the *S. mutans* growth curve

PB had strong antibacterial activity on *S. mutans* strains compared with the other extracts; therefore, we investigated the effect of PB on *S. mutans* in this study. Two methods were utilized to examine the effect of PB on the growth of *S. mutans*. First: 5 mL TSB containing various concentrations of PB (1× to 1/16× MIC) in a glass tube was prepared. Then, 0.5×10^6 *S. mutans* UA159 cells were added to each glass tube. The optical density (OD) at 660 nm was monitored at 1 h interval for 10 hours. Second: a small number (10^8 cells) of *S. mutans* UA159

cells were inoculated into 5 mL TSB, and incubated at 37°C with 5% CO_2. Various concentrations of PB were added to the medium when the OD at 660 nm reached 0.2. Then, the OD at 660 nm was monitored for 6 h. The data were analyzed for statistically significant differences compared with the control at each time point using one-way ANOVA followed by Dunnett's post hoc test.

Effects of phellodendron bark on cell viability in non-growing conditions

As shown above, we observed the antibacterial effect of PB on growing *S. mutans* cells, we then investigated the antibacterial effect on *S. mutans* cells in non-growing conditions. Overnight cultures of *S. mutans* UA159 cells were washed with 10 mM sodium phosphate buffer (pH 6.8), and then diluted to 10^7 cells/mL in the same buffer. Then, 10 μL of the diluted samples was added to 500 μL of the same buffer containing various concentrations of PB. After 2 h, appropriate dilutions were plated onto TSA. After 2 days' incubation at 37°C with 5% CO_2, colony forming units were counted and the survival ratios were calculated compared with untreated sample. Three independent experiments were performed. The data were analyzed for statistically significant differences

compared to untreated control using one-way ANOVA followed by Dunnett's post hoc test.

Effect of phellodendron bark on biofilm formation

Biofilm assays were performed using a method described previously (6). The 96-well microtiter plates (Thermo Fischer Scientific, Roskilde, Denmark) were used for this assay. Briefly, 10^5 cells from an overnight culture was applied to 100 μL of 2% sucrose containing TSB with serial dilutions of PB (10 mg/mL to 0.01 mg/mL). After a 16 h incubation, the culture was removed and each well was washed with distilled water three times. Then, 100 μL of 0.1% safranin solubilized in distilled water was added to the wells. After 10 min, the solution was removed and each well was washed with distilled water three times. The absorbance at 490 nm was measured at each well. Three independent experiments were performed. The data were analyzed for statistically significant differences compared with the control by a one-way ANOVA followed by Dunnett's post hoc test.

Then, we investigated the expression of *gtfB* and *gtfC* of *S. mutnas.* When bacterial cells were reached at OD_{660}=0.4, 1/2, 1, 2× MIC of PB were added for 1 hours. Then, bacterial cells were collected. Total RNA was extracted from the cells using a FastRNA Pro Blue kit (MP Biomedicals, Solon, OH, USA), according to the manufacturer's protocol. Then, cDNAs were constructed from total RNA (1 μg) using a first-strand cDNA synthesis kit (Roche, Tokyo, Japan). Quantitative PCR was performed using a LightCycler system (Roche, Tokyo, Japan). Primers (*gyrA*, *gtfB* and *gtfC*) to amplify genes were constructed, and *gyrA* was used as an internal control. All primers used in this study are shown in Supplemental Table 1. For quantitative PCR, three independent experiments were performed, and the mean ± SD was calculated.

ATP efflux from *S. mutans* biofilm cells

To investigate the viability of *S. mutans* biofilm cells, ATP efflux was measured by using BacTiter-Glo reagent (Promega, Madison, WI) in accordance with the manufacturer's protocol. Biofilm cells without PB were prepared as described above, and then, culture medium was removed and each well was washed with 10 mM phosphate buffer (pH 6.8) twice. Then, various concentrations of PB (1×

MIC and 2× MIC) were added to the wells. After 1 h incubation at 37°C with 5% CO_2, phosphate buffer was removed and centrifuged at 10,000×g for 5 min. The supernatant was used for the measurement of ATP concentrations. Equal volumes of the supernatant and reagent were mixed. After 5 min incubation at room temperature, the bioluminescence response in relative light units was detected using a TriStar^2 LB942 multimode plate reader (BERTHOLD Technologies, Bad Wildbad Im Schwarzwald, Germany). The ATP concentration of each sample was determined using standard ATP solutions (0.001–10 μM). Three independent experiments were performed. The data were analyzed for statistically significant differences compared to untreated control by a one-way ANOVA followed by Dunnett's post hoc test.

pH drop assay

The pH drop assay was performed as described previously (6). *S. mutans* UA159 cells grown at OD_{660} of 0.8 were collected and washed with 0.5 mM potassium phosphate buffer containing 37.5 mM KCl and 1.25 mM $MgCl_2$ (pH 6.5). Then, bacterial cells were suspended with the same buffer to reach OD_{660} of 0.8. PB at 1× and 2× MIC was added to the suspension. Glucose solution (20% wt/vol) was

added to the bacterial solution at a final concentration of 1% glucose. The bacterial suspension was incubated at 37°C with 5% CO_2. pH was monitored at appropriate intervals. Three independent experiments were performed. The data at 1 h were analyzed for statistically significant differences compared with glucose only by a one-way ANOVA followed by Dunnett's post hoc test.

Then, we investigated the genes related to lactic acid production (*ldh, pykF, enolase*) and acid tolerance (*atpD* and *aguD*) with the method described previously (6). Bacterial preparation was same as for the pH drop assay. At 1 h after glucose addition, bacterial cells in 5 mL suspension were collected. RNA extraction, cDNA synthesis and quantitative PCR were performed with the methods described above. The primers used in this assay are shown in Supplemental Table 1.

Analysis of biofilms by confocal laser scanning microscopy (CLSM)

A small portion (10^8 cells) of an overnight culture of *S. mutans* UA159 was inoculated into 5 mL fresh TSB containing 2% sucrose in a glass-bottomed dish. After 24 h at 37°C with 5% CO_2, the bacterial culture was removed and biofilm

cells were washed with 10 mM sodium phosphate buffered (Na-Pi) (pH 6.8) three times. Then, 3 mL Na-Pi containing a final concentration of 2×, and 4× MIC of PB was added to the dish for 1 h. Then, bacterial cells were stained using a BD Cell Viability kit (BD Biosciences, San Jose, CA, USA), which enables visualization of dead (red) or all cells (green). CLSM was performed using a Carl Zeiss LSM700 microscope (Carl Zeiss MicroImaging Co. Ltd., Tokyo, Japan). The microscope was equipped with detectors for monitoring red fluorescence (excitation wavelength 555 nm, emission wavelength 585 nm) and green fluorescence (excitation wavelength 488 nm, emission wavelength 590 nm). Confocal images were obtained using a 63 × /1.4 oil lens for an optical section thickness of approximately 0.7 μm. The experiment was performed three times independently.We qualified the effect of PB on biofilm cells. After obtaining images of dead or live cells in each horizontal section, image processing was performed using ZEN 2009 (Carl Zeiss). All layers were stacked to construct an image of the three-dimensional architecture of the biofilm and an image of the vertical section. Additionally, image analysis was performed using ImageJ 1.44i (National Institutes of Health, Bethesda, MD, USA). The ratio of the area

occupied by bacteria to the total area scanned in each layer and the ratios of live bacteria to total bacteria including live and dead cells were calculated. Finally, the average rate of surviving cells to the total number of cells in all sections was calculated to compare the effect of PB among strains.

Lactate dehydrogenase assay

OM-1 cells, human oral carcinoma epithelial cell line were seeded in a 96-well plate in Dulbecco's modified Eagle's medium (Sigma Chemical Co, St. Louis, MO, USA) containing 10% fetal calf serum and the cells reached approximately 80% confluence, then were subsequently cultured with various concentrations of PB (37.5 to 600 μg/mL) for 24hours. To determine maximum lactate dehydrogenase (LDH) release with 100% cell death, 2% Triton X-100 was added to the cell culture. Cell culture supernatants were collected, and LDH release determined using the cytotoxicity detection kit (SIGMA-Aldrich, St. Louis, MO, USA), according to the manufacturer’s instructions. LDH activity was determined by spectrophotometric absorbance using a standard plate reader with a wavelength of 490 nm. LDH release (%) for each time point was calculated relative to the 100% cell death value and the 0% value at non-blank cell. Three

independent experiments were performed. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test.

RESULTS

Antibacterial activity of three plant extracts against *S. mutans*

We evaluated the MIC of three plant extracts against oral streptococcal strains. All extracts showed antibacterial activity against these strains (Table 1), but PB showed a stronger effect than other extracts. The MIC of PB against cariogenic bacteria, *S. mutans* and *S. sobrinus*, was 156 or 312.5 μg/mL, whereas the MICs of the other extracts were more than 625.0 μg/mL. Then, we evaluated the MIC of PB against 100 *S. mutans* strains (Table 2). The MIC of PB varied from 9.8 to 312.5 μg/mL. Most strains showed MICs of 156.3 (42%) and 312.5 μg/mL (44%).

PB inhibited *S. mutans* growth and showed bactericidal effect on *S. mutans* cells

We evaluated the effect of PB on *S. mutans* growth using two methods. Fig. 1 shows *S. mutans* growth with various concentration of PB, in which PB suppressed *S. mutans* growth in a dose-dependent manner. Sub-MIC of PB, even

at 1/16× MIC of PB, significantly suppressed *S. mutans* growth. After 10 h incubation, the growth ratio compared with the growth without PB at 1/8×, 1/4×, and 1/2× MIC was 87.5%, 75.6%, and 11.9%, respectively. Then, we investigated the effect of PB on the growth of bacterial cells (Fig. 2). When PB was added to OD_{660} of 0.2, 1× MIC of PB suppressed *S. mutans* growth slightly, and 2× MIC of PB inhibited *S. mutans* growth strongly.

Furthermore, we investigated the antibacterial activity of PB under non-growing condition (Fig. 3). Below 1×MIC of PB had no effect on cell viability. Compared to no addition of PB, 2×MIC and 4×MIC of PB showed 43.6 % and 22.4% viability, respectively.

PB suppressed the biofilm formation

Fig. 4 shows the effect of PB on *S. mutans* biofilm formation. Biofilm formation was inhibited by the addition of PB in a dose-dependent manner. However, sub-MIC of PB did not inhibit biofilm formation.

Glucosyltransferases of *S. mutans* are mainly involved in biofilm formation. We investigated the expression levels of *gtfs* in the presence of 1/2×, 1× and 2×

MIC of PB. As a result, *gtfB* and *gtfC* expression levels were not affected by the presence of PB (Supplemental Fig. 1).

We also evaluated bactericidal activity by measuring ATP efflux in *S. mutans* biofilm cells (Fig. 5). ATP efflux was observed with the addition of more than 1× MIC of PB.

PB suppressed pH drop by addition of glucose

By addition of glucose (1% wt/vol), the pH in the *S. mutans* suspended solution dropped strongly to pH 5.9 (after 30 min) and pH 5.1 (after 1 h) (Fig. 6). The pH drop was suppressed in the presence of 1× MIC and 2× MIC of PB, being maintained at pH 5.6 and pH 5.8 (1 h), respectively.

Then, we investigated the expression levels of genes responsible for acid production and acid tolerance (Fig. 7). The expression of all genes (*ldh, pykF, eno, aguD*, and *atpD*) was strongly induced by the addition of glucose. However, these expression levels were not induced by the addition of glucose in the presence of 1× and 2× MIC of PB.

High concentration of PB showed bactericidal effect on biofilm cells

By addition of 2× MIC of PB, the proportion of dead cells did not increase compared with biofilm cells without PB (Fig. 8a). However, the addition of 4× MIC of PB resulted in a small proportion of dead cells compared with the control (without PB treatment). Then, we quantified the proportion of dead and live cells and found that dead cells with the addition of PB were mainly observed in the surface layer of the biofilm (Fig. 8b and 8c).

PB showed no cytotoxic activity on OM-1 cells.

To examine whether PB had cytotoxic effects to oral epithelial cells, we performed LDH cytotoxic assay. The results showed that PB (1/8 MIC to 2MIC) caused no significant increase in lactate dehydrogenase release from OM-1 cells (supplemental Fig. 2).

DISCUSSION

In this study, we evaluated the antibacterial activity of three plant extracts against *S. mutans*. Among these, PB showed stronger activity than the other extracts. PB (also known as Phellodendri Cortex) is an extract from

Phellodendron amurense Rupr or Phellodendron chinense Schneid (24). Traditionally, PB has been used for therapeutic effects against cirrhosis, dysentery, hepatic diseases, cerebral diseases and other conditions (25, 26, 27). Recently, PB has been reported to have comprehensive effects such as anti-inflammatory, antimicrobial, anticancer, antioxidant, anti-ulcer, and others (27). A few previous reports regarding antibacterial activity were found, although there was no detailed analysis (27, 28, 29). In this study, PB had antibacterial activity against oral streptococci especially *S. mutans* (Table 1). We found that PB showed antibacterial activity against 100 *S. mutans* strains, although susceptibility varied among strains (Table 2). Growth kinetics analyses showed that PB had a growth inhibitory effect on *S. mutans* cells (Fig. 1 and 2) indicating that PB has a bacteriostatic effect on *S. mutans*. However, we found more than 2× MIC of PB showed bactericidal activity under non-growing condition (Fig. 3). We also investigated the antibacterial activity of PB against *S. mutans* biofilm cells and found that a high concentration (4× MIC) of PB showed bactericidal effect using confocal analysis. Additionally, we found that ATP was gradually released from 1× MIC of PB (Fig. 5). Although we found that different doses were

required for the bactericidal effect between CLSM analysis and the ATP release assay, this was due to the higher sensitivity of the ATP release assay compared with CLSM analysis. PB contains alkaloids, limonoids, phenolic compounds, quinic acid, lignans, and flavonoid, and so on (27). Since these compounds were reported to have antibacterial activity (30-35), the antibacterial effect of PB is considered to be related to these components. Therefore, high concentrations of PB may cause a combination of the effect of several antibacterial components in PB, resulting in a partial bactericidal effect on biofilm cells.

In the biofilm assay, PB suppressed biofilm formation at 1× MIC of PB (Fig. 4). Although the growth of *S. mutans* in planktonic culture was suppressed by sub-MIC of PB, the growth of biofilm cells was not inhibited by sub-MIC of PB. We speculated that this difference was due to the different susceptibility to PB between planktonic and biofilm cells. Since *gtfB* and *gtfC* were strongly associated with biofilm formation (12), we investigated their expression levels, but we found no difference with the addition of PB (Supplemental Fig. 1). In the pH drop assay, we observed suppression of the pH drop with 1× and 2× MIC of PB (Fig. 6). This effect coincided with the observation of decreased expression of

genes for lactic acid production and also acid tolerance in the presence of PB (Fig. 7). Lactate dehydrogenase mediates the production of lactic acid from pyruvic acid, and pyruvate kinase mediates the production of pyruvic acid from phosphoenolpyruvate (PEP) (36, 37). With excess sugar in the medium, *S. mutans* produce many metabolic intermediates such as glucose 6-phosphate and fructose 1,6-bisphosphate, causing an increase in the expression of pyruvate kinase and lactate dehydrogenase, respectively. Enolase is responsible for the production of PEP. PEP is also responsible for the PEP: carbohydrate phosphotransferase system (38). As for factors involved in acid tolerance, *atpD* is a part of the F_1F_o-ATPase operon responsible for proton pumping (14, 15) and *aguD* is part of the agmatine deiminase system operon for alkali production (16). Previously, we found that disodium succinoyl glycyrrhetinate, a derivative of glycyrrhetinic acid extracted from licorice suppressed the expressions of these genes by addition of glucose due to the suppression of carbohydrate metabolism (5). Since PB also suppressed the expression of these genes by addition of glucose, it is considered that PB suppresses sugar metabolism although we still do not know the precise mechanism of PB.

Recently, the control of oral bacteria has been subject to more attention because there are many reports regarding the relationship of oral bacteria with systemic disease such as aspiration pneumonia, endocarditis, diabetes, atherosclerosis, and rheumatoid arthritis (39-41). Therefore, the importance of oral care is widely accepted in society. By performing oral care treatment, dental plaque, which is a bacterial aggregate and a cause for oral diseases, is effectively removed. To support oral care, mouthwashes are sometimes used. Chlorhexidine gluconate, cetylpyridinium chloride, and thymol are major components of mouthwashes (42-44). In addition, many natural products such as plant extracts have been demonstrated to have different activity including anti-bacterial activity and anti-biofilm activity on *S. mutans* cells (45-49). Magnolol and honokiol derived from magnolia bark showed bactericidal and anti-biofilm activity (47), while some extracts showed mainly anti-biofilm activity due to the inhibition of CSP or electricity (48, 49). Since PB showed bacteriostatic effect on *S. mutans* cells causing to suppression of biofilm formation, many natural products showed variable effects on *S. mutans* cells. PB was reported to have anti-inflammatory effects (27); therefore, administration of PB in the oral cavity may have not only

antibacterial effects but also anti-inflammatory effects. Furthermore, we found that PB (1/8 MIC to 2 MIC) had no effect on oral epithelial cells (Supplemental Fig. 1), assuming that PB could be applicable to oral cavity.

In conclusion, we demonstrated the antibacterial activity of PB against *S. mutans* strains. In addition, PB suppressed *S. mutans* virulence. Taken together with previous reports in the inflammatory effects of PB, it may have potential for clinical use against *S. mutans* to prevent the formation of dental plaque and tooth decay.

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Figure Legends

Figure 1. Effect of PB on the growth of *S. mutans* UA159

Aliquots of bacterial cells were added to 5 mL of TSB containing various concentrations of PB. The OD at 660 nm was measured. Three independent experiments were performed, and the mean ± SD was calculated.

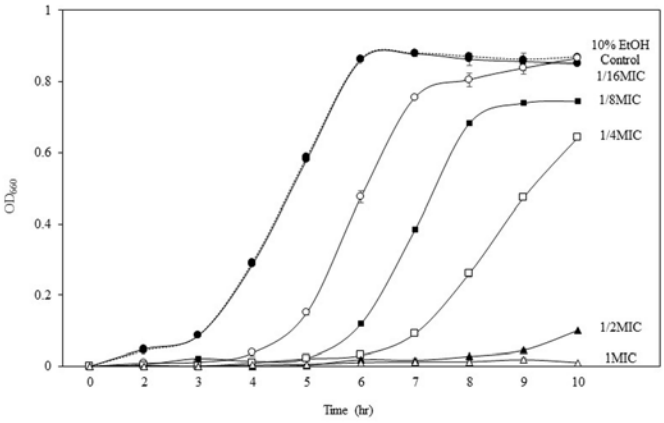


Figure 2. Effect of PB on the growth of *S. mutans* UA159 cells

Aliquots of bacterial cells were added to 5 mL of TSB. When the OD at 660 nm reached 0.2, various concentrations of PB were added to individual bacterial cultures. The OD was measured over time. The data were analyzed for statistically significant differences compared with the control by a one-way ANOVA followed by Dunnett's post hoc test. *P<0.05, **P<0.01, ***P<0.005,****P<0.001.

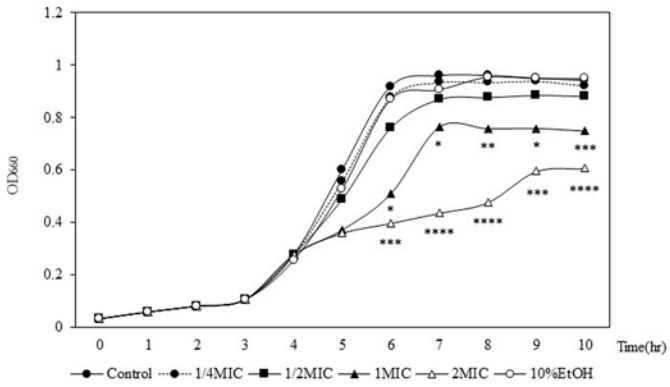


Figure 3. Effect of PB on non-growing *S. mutans* UA159 cells

S. mutans UA159 cells were reacted with various concentration of PB in 10 mM sodium phosphate buffer (pH 6.8) for 2 h. CFUs were counted and the survival ratio compared with no treatment were calculated. Three independent experiments were performed, and the mean ± SD was calculated. The data were analyzed for statistically significant differences compared to untreated control by a one-way ANOVA followed by Dunnet's post hoc test. *P<0.01,**P<0.005.

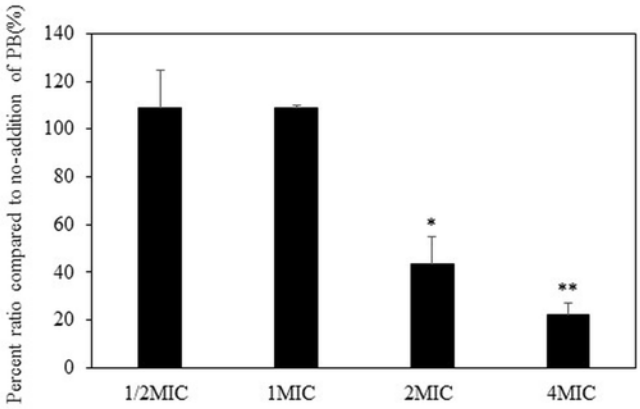


Figure 4. Effect of PB on biofilm formation

PB was serially diluted twofold with TSB containing 2% sucrose. The bacterial culture (1×10^5 cells) was inoculated to each well. After incubation for 24 h at 37°C with 5% CO_2, the wells were washed three times with distilled water, and biofilm cells were stained with 0.1% safranin for 10 min. Biofilm quantification was performed by evaluating the absorbance of each well at 490 nm. Three independent experiments were performed, and the mean ± SD was calculated. The data were analyzed for statistically significant differences compared with the control by a one-way ANOVA followed by Dunnett's post hoc test. *P<0.005, **P<0.001.

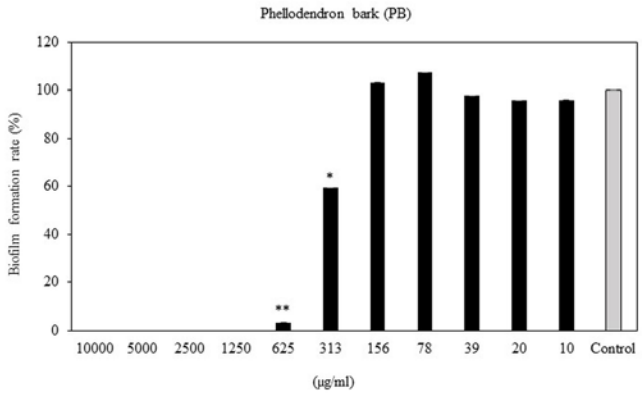


Figure 5. Effect of PB on the viability of biofilm cells

ATP efflux due to the addition of various concentrations of PB was measured using the method described in the Materials and Methods. Three independent experiments were performed, and the mean ± SD was calculated. The data were analyzed for statistically significant differences compared with the control by a one-way ANOVA followed by Dunnett's post hoc test. *P<0.001.

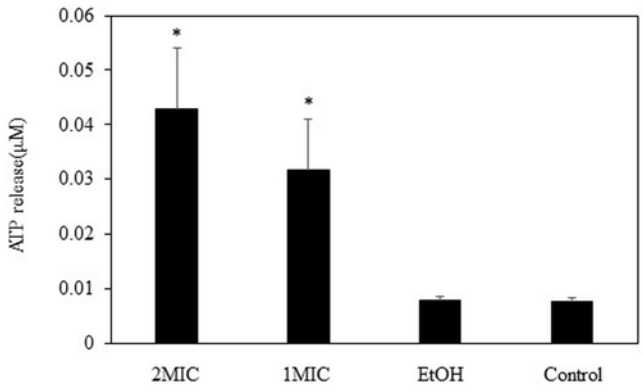


Figure 6. Effect of PB on pH change in *S. mutans* UA159

The pH value of *S. mutans* suspensions in the presence of 1× and 2× MIC of PB was monitored for 1h at 37°C. Three independent experiments were performed, and the mean ± SD was calculated. The data at 1 h were analyzed for statistically significant differences compared with the addition of glucose only by a one-way ANOVA followed by Dunnett's post hoc tests. *P<0.001.

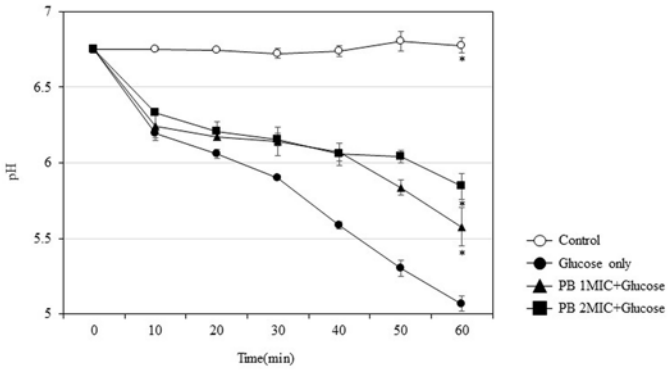


Figure 7. Effect of PB on the expression of acid production and acid tolerance genes

S. mutans cells in the presence of various concentrations of PB were incubated for 30 min. Then, bacterial cells were collected and then, RNA extraction, cDNA synthesis and quantitative PCR were performed using the methods described in the Materials and Methods. Three independent experiments were performed, and the mean ± SD was calculated. The data were analyzed for statistically significant increases compared with the control by a one-way ANOVA followed by Dunnett's post hoc tests. *P<0.05 **P<0.005,***P<0.001.

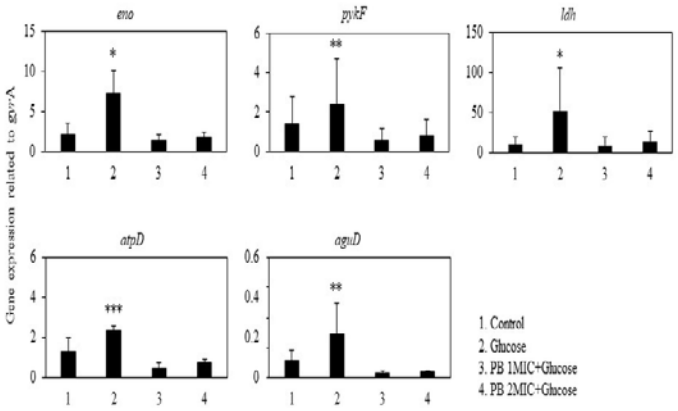
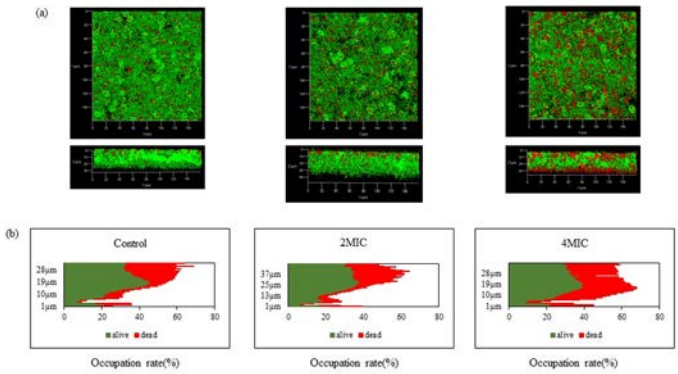
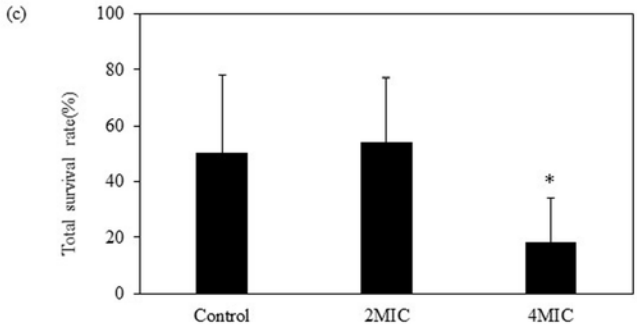


Figure 8. Effect of PB on biofilm cells by confocal laser scanning microscopy

(a) *S. mutans* UA159 biofilm cells were reacted with PB (2× and 4× MIC of PB) for 1 h. Confocal laser scanning microscopy was analyzed by the method described Materials and Methods. Finally, bacterial cells were stained using a BD Cell Viability kit, which enables the visualization of dead (red) or all cells (green). (b) The ratio of the area occupied by bacteria to the total area scanned in each layer and the ratio of live bacteria to the total bacteria including live and dead cells were calculated. (c) The average of the rate of surviving cells to the total number of cells in all sections was calculated to compare the effect of PB among strains. Three independent experiments were performed, and the mean ± SD was calculated. The quantification of the effect of PB was calculated by the method described Materials and Methods. The data were analyzed for statistically significant increases compared to control by a one-way ANOVA followed by Dunnett's post hoc tests.*$P<0.001$.





List of abbreviations

PB, phellodendron bark

MIC, minimum inhibitory concentration

CFU, colony forming unit

TSA, tryptic soy agar

TSB, tryptic soy broth

GTF, glucosyltransferase

LDH, lactate dehydrogenase

PEP, phosphoenolpyruvate

PK, pyruvate kinase

PTS, phosphotransferase system

Table 1. MIC of various plant-derived extract. 10%,50% EtOH ver.

Bacteria	Phellodendron Bark [1]	Yucca [2]	Black ginger [1]
S. mutans UA159	312.5 [3]	1250.0	625.0
S. mutans RIMD3125001	156.3	5000.0	5000.0
S. mutans MT8148	156.3	2500.0	625.0
S. sobrinus JCM5176	156.3	10000.0	1250.0
S. sobrinus B13	156.3	10000.0	2500.0
S. sanguinis GTC217	156.3	312.5	625.0
S. salivarius GTC215	625.0	5000.0	2500.0
S. gordonii JCM12995	78.1	312.5	625.0

1, resolve in 10% EtOH

2, resolve in distilled water

3, μg/mL

*, affected by EtOH in solution

Table 2. Susceptibility against phellodendron bark in 100 *S. mutans* isolated strains.

	Number of strains
MIC (μg/ ml)	phellodendron bark
312.5	44
156.3	42
78.1	13
9.8	1