

## Activity of panduratin A isolated from *Kaempferia pandurata* Roxb. against multi-species oral biofilms *in vitro*

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**Abstract:** The formation of dental biofilm caused by oral bacteria on tooth surfaces is the primary step leading to oral diseases. This study was performed to investigate the preventive and reducing effects of panduratin A, isolated from *Kaempferia pandurata* Roxb., against multi-species oral biofilms consisting of *Streptococcus mutans*, *Streptococcus sanguis* and *Actinomyces viscosus*. Minimum inhibitory concentration (MIC) of panduratin A was determined by the Clinical and Laboratory Standards Institute (CLSI) broth microdilution assay. Prevention of biofilm formation was performed on 96-well microtiter plates by coating panduratin A in mucin at 0.5-40 µg/ml, followed by biofilm formation at 37°C for 24 h. The reducing effect on the preformed biofilm was tested by forming the biofilm at 37°C for 24 h, followed by treatment with panduratin A at 0.2-10 µg/ml for up to 60 min. Panduratin A showed a MIC of 1 µg/ml for multi-species strains. Panduratin A at 2 × MIC for 8 h exhibited bactericidal activity against multi-species planktonic cells for 8 h. At 8 × MIC, panduratin A was able to prevent biofilm formation by > 50%. Biofilm mass was reduced by > 50% after exposure to panduratin A at 10 µg/ml for 15 min. Panduratin A showed a dose-dependent effect in preventing and reducing the biofilm. These results suggest that panduratin A is applicable as a natural anti-biofilm

agent to eliminate oral bacterial colonization during early dental plaque formation. (J Oral Sci 51, 87-95, 2009)

Keywords: anti-biofilm; *Kaempferia pandurata* Roxb.; panduratin A; multi-species oral biofilm.

### Introduction

Dental plaque is a multi-species biofilm consisting of more than 700 species of oral bacteria on tooth surfaces, and mutans streptococci are the major group of early primary colonizers (1). An effective approach for control of dental plaque is to prevent formation of the biofilm or to remove existing biofilm from the system by using anti-biofilm agents. In biofilm experimental models, the efficacy of an anti-biofilm agent is dependent on its ability to desorb either single- or multi-species biofilms from the test surface. The use of chlorhexidine gluconate, a commercial antimicrobial agent, for clinical application is limited because it has a bitter taste and can cause teeth to stain on frequent use (2,3). Thus, it is important to develop alternative antiplaque agents from natural sources that exhibit few or no side-effects and have a similar acceptance and efficacy for the treatment of plaque-related diseases such as dental caries and inflammatory periodontitis (4).

Several *in vitro* studies of oral biofilm models have been conducted with the aim of preventing and reducing oral biofilms with natural anti-biofilm agents. Some natural pre-coating agents such as delmophinol, xanthorhrizol, catechin and cranberry constituent have been reported to prevent the formation of single oral streptococci biofilms (5-8). Macelignan isolated from *Myristica fragrans* Houtt.,

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xanthorrhizol isolated from *Curcuma xanthorrhiza* Roxb., salvipisone diterpenoids isolated from *Salvia sclarea* L., and *Nidus vespae* extract have also demonstrated anti-biofilm activities against existing single oral biofilms (9-13). However, only a few studies have employed natural anti-biofilm agents against multi-species oral biofilms.

Panduratin A, isolated from *Kaempferia pandurata* Roxb., is a natural chalcone compound with a molecular weight of 407 (14). Panduratin A possesses some biological properties, such as anti-cariogenic, anti-inflammatory, anticancer, antiproliferative, proapoptotic, and anti-oxidant effects (14-18). The bactericidal activity of panduratin A against planktonic *Porphyromonas gingivalis* cells has been reported (17). However, its efficacy for treatment of oral biofilm has not yet been investigated. The present study was conducted to evaluate the preventive and reducing effects of panduratin A against multi-species oral biofilms *in vitro*.

## Materials and Methods

### Plant material and chemicals

The dried rhizomes of *Kaempferia pandurata* Roxb. were collected in Jakarta, Indonesia, and identified by Dr. N. I. Baek, Department of Oriental Medicinal Materials and Processing, Kyunghee University (Yongin, Korea). A voucher specimen (H082) is deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). All chemical reagents were purchased from Sigma Co. (St. Louis, MO, USA).

### Extraction and isolation

The ground *Kaempferia pandurata* Roxb. (100 g) was extracted with 95% ethanol (400 ml), and the extract

(11.95 g) was further fractionated with ethyl acetate (2 × 200 ml). The ethyl acetate fraction was applied to a silica gel column (5 × 45 cm, 650 g of silica gel; 70-230 mesh; Merck KGaA, Darmstadt, Germany) and eluted with *n*-hexane-chloroform-ethyl acetate 15:5:1.5 (2 l, v/v/v) to give seven fractions (fractions 1 to 7). Fraction 3 (0.9 l, 1.51 g) was further separated with *n*-hexane-ethyl acetate-methanol 18:2:1 (1.5 l, v/v/v), yielding fraction 3-B (1.1 g). Fraction 3-B was eluted with 100% methanol (0.8-0.9 ml) using recycling preparative HPLC [(JAIGEL W-252 column, 20.0 mm i.d. × 500 mm L; Japan Analytical Industry Co., Ltd., Tokyo, Japan); detection by UV absorption at 365 nm; mobile phase used was 100% MeOH; flow rate 3 ml/min] and compound 3-B (300 - 400 ml, 0.9 g) was finally obtained as a single compound. Careful comparison of several spectral data for compound 3-B, including <sup>13</sup>C-NMR, <sup>1</sup>H-NMR, <sup>13</sup>C-DEPT, <sup>1</sup>H-<sup>1</sup>H COSY, <sup>1</sup>H-<sup>13</sup>C HSQC, <sup>1</sup>H-<sup>13</sup>C HMBC and FAB-MS, with those in the literature (17,19) suggested the chemical structure to be panduratin A (98%, Fig. 1) or (4-methoxy-2,6-dihydroxyphenyl)-[3'-methyl-2'-(3''-methylbut-2''-enyl)-6'-phenylcyclohex-3'-enyl] methanone. Optical rotation was measured with a Perkin-Elmer 241 polarimeter (Perkin-Elmer Life and Analytical Sciences, Inc., MA, USA) as  $[\alpha]_D^{25} + 0.0066$  (*c* 0.1, CHCl<sub>3</sub>).

### Instrumentation

NMR spectra were recorded on a Bruker Avance-600 spectrometer (Bruker Optik GmbH, Ettlingen, Germany) at 600 MHz for <sup>1</sup>H and <sup>13</sup>C in CDCl<sub>3</sub> with TMS as an internal standard. Complete proton and carbon assignments were based on 1D (<sup>1</sup>H, <sup>13</sup>C, <sup>13</sup>C-DEPT) and 2D (<sup>1</sup>H-<sup>1</sup>H COSY, <sup>1</sup>H-<sup>13</sup>C HSQC, <sup>1</sup>H-<sup>13</sup>C HMBC) NMR experiments. Mass spectra (FAB-MS) were measured using JMS-700 (JEOL Ltd., Tokyo, Japan). All instrumental data are available upon request.

### Preparation of agents and artificial saliva

Panduratin A was dissolved in 100% dimethyl sulfoxide (DMSO), then diluted to concentrations of 0.5-40 μg/ml (for the preventive effect) or 1-50 μg/ml (for the reducing effect) using 1% sterile mucin. A commercial antimicrobial agent, chlorhexidine gluconate (1,1'-hexamethylenebis[5-*p*-chlorophenylbiguanide]) (Sigma-Aldrich, St. Louis, MO, USA), was dissolved in sterile distilled water, then diluted to concentrations of 0.5-40 μg/ml (for the preventive effect) or 1-50 μg/ml (for the reducing effect) using sterile distilled water. The artificial saliva, 1% type III mucin from porcine stomach (Sigma-Aldrich), was diluted with adherence buffer and autoclaved at 121°C for 15 min.

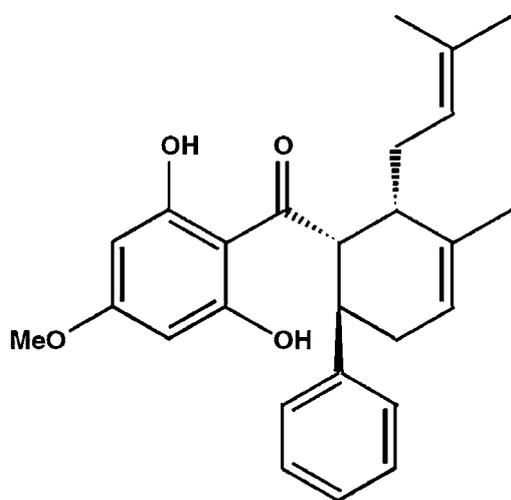


Fig. 1 Structure of panduratin A.

### Bacterial strains and inoculum cell preparation

The bacteria used in this study were *Streptococcus mutans* ATCC 25175, *S. sanguis* ATCC 10556 (American Type Culture Collection, Rockville, MD, USA) and *Actinomyces viscosus* KCCM 12074 (Korean Culture Center of Microorganisms, Seoul, Korea). All bacteria were grown aerobically at 37°C for 24 h in brain heart infusion (BHI) broth (DIFCO Laboratories, Detroit, MI, USA).

To prepare a bacterial cell suspension for multi-species biofilm formation, each overnight culture was harvested by centrifugation (4,500 rpm, 5 min), washed twice with 50 mM phosphate-buffered saline (PBS, pH 7.2), resuspended in adherence buffer (10 mM KPO<sub>4</sub>, 50 mM KCl, 1 mM CaCl<sub>2</sub>, 0.1 mM MgCl<sub>2</sub>, pH 7.0) and adjusted to a concentration of 1 × 10<sup>6</sup> CFU/ml. Multi-species inocula contained a mixture of *S. mutans*, *S. sanguis* and *A. viscosus* cells with a similar ratio and approximate cell density of 1 × 10<sup>6</sup> CFU/ml. A standard curve of turbidity against colony-forming units was used to obtain the number of cells.

### Determination of minimum inhibitory concentration (MIC)

The MIC of panduratin A against multi-species strains was determined using the Clinical and Laboratory Standards Institute (CLSI) recommended broth microdilution assay (2003). A stock solution of panduratin A at a concentration of 1,000 µg/ml was prepared in 10% DMSO. Briefly, 2-fold serial dilutions of panduratin A were prepared with BHI medium at a total volume of 200 µl per well in 96-well polystyrene microtiter plates (SPL Plastic Labware, Pocheon, Korea). The final concentrations of panduratin A ranged from 0.25 to 125 µg/ml. The microtiter plate wells were inoculated with 20 µl per well of multi-species bacterial cell suspension at a final concentration of 1 × 10<sup>6</sup> CFU/ml. After incubation at 37°C for 24 h, the absorbance was measured at 596 nm using a microplate reader (Versa Max, Sunnyvale, CA, USA) to assess the cell growth. Chlorhexidine gluconate was used as a positive control. The negative (untreated) control consisted of BHI medium and cell suspension without the agent, and the blank control contained only the medium. The MIC endpoint was defined as the lowest concentration of the test agent that completely inhibited growth or produced at least 90% reduction of absorbance in comparison with the untreated control. All experiments were performed in triplicate and the average values were reported as MICs.

### Preventive effects of panduratin A on planktonic cells

Aliquots of 5 ml of BHI medium and an initial multi-species inoculum of 1 × 10<sup>6</sup> CFU/ml were prepared for preventing the growth of multi-species planktonic cells. Final concentrations of panduratin A at 1/2 × MIC, 1 × MIC, 2 × MIC, 4 × MIC, 8 × MIC and 16 × MIC were used, and bacterial growth was measured at 0, 4, 8, 12 and 24 h after incubation at 37°C. A 100-µl sample was collected from each culture tube, diluted serially with BHI medium, and a 50-µl aliquot was spread on a BHI agar plate for multi-species strains. After incubation at 37°C for 24 h, the number of viable cells (CFU/ml) was counted. All experiments were conducted in triplicate.

### Preventive effects of panduratin A against biofilm formation

In this study, a 96-well polystyrene microtiter plate was employed for prevention of biofilm formation by the modified method of Rukayadi and Hwang (8). A 50-µl aliquot of panduratin A in sterile mucin at 0.5-40 µg/ml was used to coat each well, then incubated and shaken gently at room temperature for 3 h, followed by air-drying overnight. After the coating treatment, multi-species inocula (1 × 10<sup>6</sup> CFU/ml) were grown using BHIS media (BHI broth supplemented with 3% sucrose). For treatment, 20 µl of a cell inoculum was added to the wells containing BHIS in a total volume of 200 µl per well (final concentration of cells 1 × 10<sup>5</sup> CFU/ml). Chlorhexidine gluconate was used as a positive control treatment. The negative control (untreated) was BHIS medium and the cell suspension without the agent, and the blank control contained only the medium. After incubation at 37°C for 24 h, absorbance at 596 nm was recorded to assess culture growth. The culture supernatants from each well were then decanted, and planktonic cells were removed by washing twice with 200 µl of 50 mM PBS (pH 7.2) and air-dried for 1 h.

Biofilm cells were stained with 110 ml of 0.4% crystal violet solution for 30 min, then rinsed thoroughly with 300 µl of sterile distilled water until the control wells appeared colorless. Biofilm formation was quantified by addition of 200 µl of 95% ethanol as a destaining solution to each crystal violet-stained well, and 100 µl of destaining solution was immediately transferred to the new well. The amount of remaining crystal violet stain in the destaining solution was determined using a tunable microplate reader at 596 nm. The percentage of inhibition was calculated using the equation  $(1 - OD_{596} \text{ of the test agent} / OD_{596} \text{ of the untreated control}) \times 100$ . All experiments were performed in triplicate with two repeats.

## Reducing effects of panduratin A on the established biofilm

Artificial multi-species oral biofilms were prepared in commercially available presterilized polystyrene flat-bottom 96-well tissue culture test microtiter plates by the method of Stepanovic et al. (20) with slight modification. Briefly, each plate was conditioned with 200  $\mu$ l of mucin as artificial saliva, incubated, and shaken gently at room temperature for 3 h. Then, excess mucin was removed and the plate was air-dried overnight. Multi-species oral biofilms were initially grown using BHI broth supplemented with 3% sucrose (BHIS). For testing the positive control (chlorhexidine gluconate) and treatment (panduratin A) preparations, 20  $\mu$ l of multi-species inoculum at an approximate cell density of  $1 \times 10^6$  CFU/ml was added to each well with 180  $\mu$ l BHIS medium to give a 10-fold dilution. Thus, the final cell density of each culture was calculated to be about  $1 \times 10^5$  CFU/ml. For the negative control (untreated), the same steps were taken except that BHIS broth (200  $\mu$ l) was used without agent treatment or addition of the cell inoculum. The plate was incubated at 37°C for 24 h. Biofilm cells were treated with 50  $\mu$ l panduratin A or chlorhexidine gluconate (1, 5, 10, 25 and 50  $\mu$ g/ml as prepared above), giving final concentrations of 0.2, 1, 2, 5 and 10  $\mu$ g/ml, respectively. Agent exposure times were 1, 5, 10, 15, 30 and 60 min. Biofilm cells were stained and quantified as described previously. Panduratin A activity was defined as the percentage absorbance of the biofilm remaining after agent treatment in comparison with the untreated control. All experiments were performed in triplicate with three repeats.

## Statistical analysis

Data were expressed as mean ( $n = 3$ ) and standard deviation (S.D.) by computational analysis from triplicate independent experiments. Statistical analysis of untreated, panduratin A-treated and chlorhexidine gluconate-treated multi-species oral biofilms was performed by analysis of variance (SPSS 11.0 for Windows). The differences between the individual groups were compared using paired Student's *t*-test. The level of significance was taken as  $P < 0.05$ .

## Results

Growth of multi-species planktonic strains was shown in Fig. 2A. The initial number of  $1 \times 10^6$  CFU/ml planktonic cells grew well up to  $\sim 1 \times 10^{11}$  CFU/ml during incubation for 0-24 h. The MIC of panduratin A against multi-species oral strains was measured by susceptibility test along with the activity of chlorhexidine gluconate for comparison. Panduratin A and chlorhexidine gluconate showed the

same MIC value of 1  $\mu$ g/ml for multi-species strains (mixture of *S. mutans*, *S. sanguis* and *A. viscosus*). The preventive effects of panduratin A against multi-species planktonic cells are presented in Fig. 2B. In the absence of panduratin A, planktonic cells reached  $8 \times 10^{10}$  CFU/ml in 24 h. At sub-MIC, panduratin A did not inhibit the growth of multi-species planktonic cells. At its MIC point, panduratin A demonstrated a bacteriostatic effect during the period of growth of planktonic cells. Treatment with  $2 \times$  MIC panduratin A for 8 h exerted bactericidal activity against planktonic cells. Overall, at its super-MIC, panduratin A showed a significant bactericidal effect in terms of both dose and growth with time.

We also employed these MICs when investigating the preventive effects against biofilm formed by multi-species strains. As seen in Fig. 3A, in the absence of panduratin A, biofilm formation by multi-species strains on 96-well plates increased in a time-dependent manner during incubation for 0-24 h. The preventive effect of panduratin A at various concentrations against multi-species biofilm formation is shown in Fig. 3B. Panduratin A at MIC (1  $\mu$ g/ml) showed  $< 25\%$  inhibition of biofilm formation, and prevented biofilm growth by  $> 50\%$  at  $8 \times$  MIC or 8  $\mu$ g/ml. Its activity was almost comparable with that of chlorhexidine gluconate.

Panduratin A and chlorhexidine gluconate at various concentrations and exposure times showed similar properties in terms of removal of multi-species oral biofilms at 24 h (Table 1). Exposure to 10  $\mu$ g/ml panduratin A for 15 min reduced the biofilm mass by  $> 50\%$  ( $P < 0.05$ ). A longer period of exposure (up to 60 min) to panduratin A at 10  $\mu$ g/ml reduced the biofilm mass to  $> 70\%$ . It is noteworthy that the reducing effect of panduratin A showed a trend similar to that of chlorhexidine gluconate.

## Discussion

The use of an *in vitro* oral biofilm model provides an alternative approach for examining the preventive effects of anti-biofilm agents against oral biofilm formation and reduction of established biofilm cells. This method allows direct comparison of biofilm development between treated and negative control (untreated) groups, and also experimental reproducibility. Human oral biofilms consist of major oral bacterial genera that are common to saliva and plaque, such as *Streptococcus*, *Actinomyces*, *Fusobacterium*, *Prevotella* and *Veillonella* (21). The general principles of microbial succession that are involved in plaque development include primary colonization by pioneer Gram-positive bacteria, including the well known *S. mutans*, *S. sanguis* and *A. viscosus*, which attach to tooth surfaces (22). *In vitro* biofilm-based models have been

evaluated using multi-species rather than single-species culture to judge the suitability of anti-biofilm agents for treatment of plaque-related diseases. Therefore, we employed the *in vitro* biofilm formed by three oral primary strains, *S. mutans*, *S. sanguis* and *A. viscosus*, that are

responsible for the initial step of early dental plaque formation (22,23).

In a previous study, panduratin A, a chalcone compound isolated from *K. pandurata* Roxb., was shown to possess bactericidal activity against *Porphyromonas gingivalis*

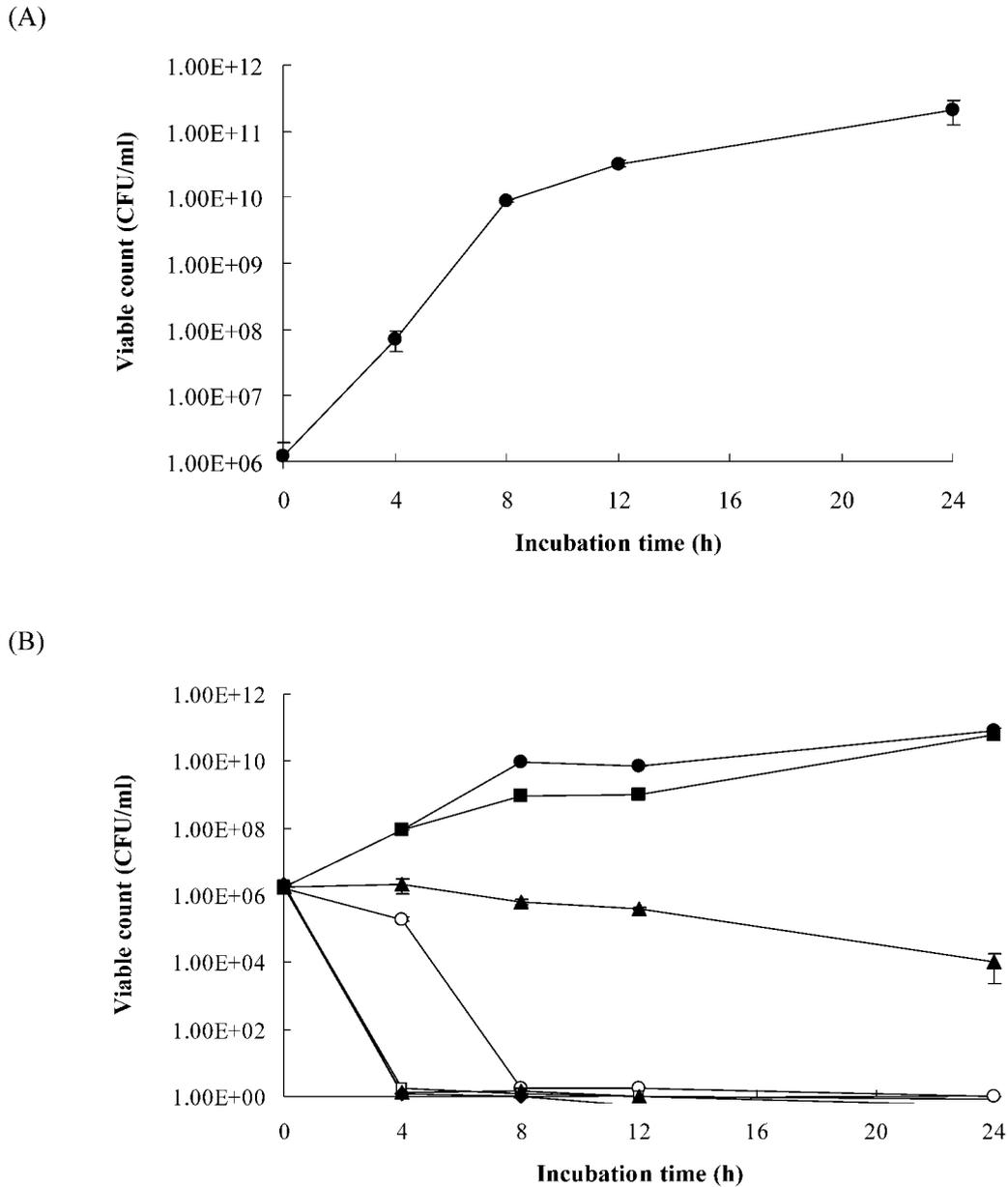


Fig. 2 (A) Growth of multi-species planktonic cells during 0-24 h. Data represent mean values (n = 3) of viable counts (CFU/ml) of planktonic cells. Error bars represent standard deviations; n = 3 from triplicate independent experiments. (B) Preventive effects of panduratin A on multi-species planktonic cells during 0-24 h. ●, control (0.05% DMSO); ■, 0.5 × MIC (0.5 µg/ml), ▲, 1 × MIC (1 µg/ml); ○, 2 × MIC (2 µg/ml); □, 4 × MIC (4 µg/ml); △, 8 × MIC (8 µg/ml); ◆, 16 × MIC (16 µg/ml). Data represent mean values (n = 3) of the viable count (CFU/ml) of planktonic cells in treated groups compared with those in untreated groups. Error bars represent standard deviations; n = 3 from triplicate independent experiments.

planktonic cells (17). Our results demonstrated that panduratin A at  $2 \times \text{MIC}$  also completely eradicated multi-species planktonic cells (Fig. 2B). However, its mode of action as an anti-bactericidal agent against planktonic cells has not been investigated. It has been reported that plant phenolic compounds and plant essential oils inhibit planktonic growth by disrupting bacterial cell walls or

membranes and inhibiting glucose uptake (24,25).

In this study, panduratin A was found to be active in preventing multi-species biofilm growth in a dose-dependent manner (Fig. 3B). At  $8 \mu\text{g/ml}$ , it effectively prevented biofilm formation by up to 50%. Confocal laser scanning microscopy (CLSM) analysis also revealed that the growth of biofilm was inhibited after coating treatment

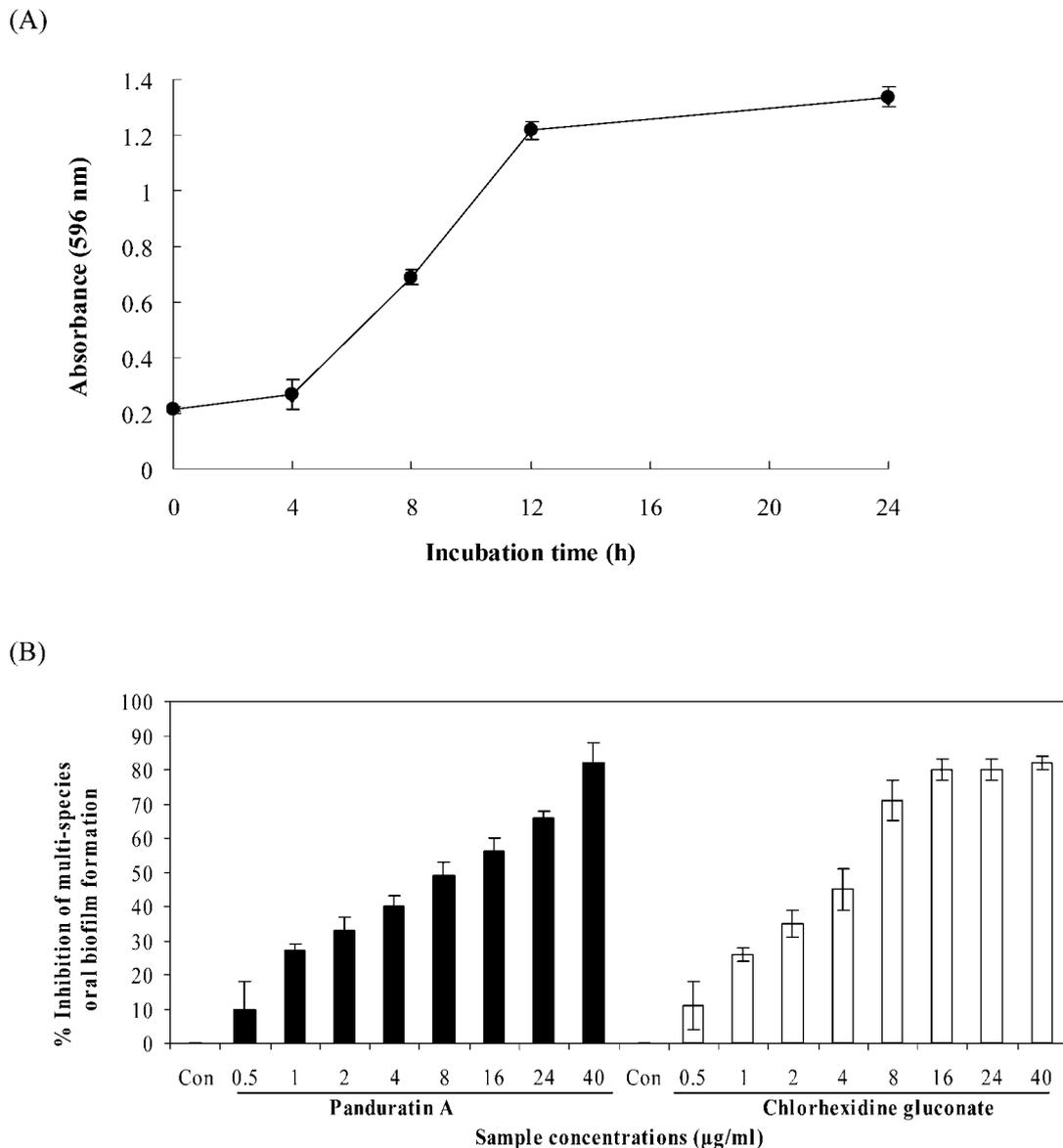


Fig. 3 (A) Growth of multi-species biofilm formation during 0-24 h. Data represent mean values ( $n = 3$ ) of absorbance (596 nm) of biofilms. Error bars represent standard deviations;  $n = 3$  from triplicate independent experiments. (B) Preventive effect of panduratin A at 0.5-40  $\mu\text{g/ml}$  on multi-species biofilm formation. Chlorhexidine gluconate was used for comparison. Values are expressed as the mean percentage absorbance (596 nm) of biofilms in treated wells compared with that in untreated wells (considered to be 100%). Error bars represent standard deviations;  $n = 3$  from triplicate independent experiments. \*Not significantly different from the control treatment assayed by analysis of variance and paired Student's  $t$ -test. All entries in the figure are significantly different ( $P < 0.05$ ) from the controls unless otherwise indicated.

Table 1 Effect of panduratin A at different concentrations and exposure times on the 24 h multi-species oral biofilms

Sample concentrations ( $\mu\text{g/ml}$ )	24 h multi-species biofilm					
	Time exposure of sample (min)					
	1	5	10	15	30	60
<b>Panduratin A</b>						
0.2	97 $\pm$ 3*	92 $\pm$ 2*	90 $\pm$ 4*	82 $\pm$ 2	74 $\pm$ 6	59 $\pm$ 2
1	90 $\pm$ 3*	88 $\pm$ 3	77 $\pm$ 6	74 $\pm$ 3	68 $\pm$ 2	47 $\pm$ 3
2	85 $\pm$ 6	80 $\pm$ 1	72 $\pm$ 1	63 $\pm$ 5	57 $\pm$ 3	42 $\pm$ 2
5	77 $\pm$ 4	76 $\pm$ 5	64 $\pm$ 2	54 $\pm$ 5	50 $\pm$ 2	38 $\pm$ 5
10	72 $\pm$ 2	68 $\pm$ 2	55 $\pm$ 4	44 $\pm$ 3	41 $\pm$ 7	29 $\pm$ 5
<b>Chlorhexidine gluconate</b>						
0.2	97 $\pm$ 2*	92 $\pm$ 3*	81 $\pm$ 4	79 $\pm$ 6	74 $\pm$ 2	72 $\pm$ 6
1	92 $\pm$ 4*	84 $\pm$ 3	76 $\pm$ 6	68 $\pm$ 2	66 $\pm$ 4	59 $\pm$ 2
2	90 $\pm$ 2*	79 $\pm$ 2	71 $\pm$ 2	63 $\pm$ 5	60 $\pm$ 2	48 $\pm$ 3
5	85 $\pm$ 5	73 $\pm$ 5	63 $\pm$ 3	59 $\pm$ 2	55 $\pm$ 5	43 $\pm$ 2
10	82 $\pm$ 5	68 $\pm$ 2	55 $\pm$ 5	41 $\pm$ 2	39 $\pm$ 7	31 $\pm$ 7

Values are expressed as the mean percentage of absorbance (596 nm) of biofilm in treated wells compared with that in untreated wells (considered to be 100%). Error bar is standard deviation of the mean percentage of absorbance (596 nm) of biofilms derived from three replicates per experiment. The experiments were conducted three times.

\*Not significantly different from the control treatment assayed by analysis of variance and a paired Student's *t*-test. All entries in the figure are significantly different ( $P < 0.05$ ) from the controls unless otherwise indicated.

with panduratin A at various concentrations (data not shown). Our findings demonstrated that panduratin A possessed less preventive effects on the growth of biofilm cells than that of planktonic cells. This indicates that bacteria in the biofilm are strongly protected and less susceptible to antimicrobial agents than in planktonic form (26). In plaque-related diseases, the target microorganisms are in the form of biofilms. The cells in a biofilm exhibit properties distinct from those of planktonic cells, such as increased resistance to biocides and antimicrobial agents (27). Experiments comparing biofilms of oral bacterial strains with broth cultures using chlorhexidine gluconate have demonstrated that much higher concentrations of chlorhexidine gluconate are required to significantly inhibit existing biofilm cells (28,29).

Furthermore, our study demonstrated that panduratin A was able to remove 24-h multi-species oral biofilms in the dose- and time-dependent manner (Table 1). Exposure to panduratin A at 10  $\mu\text{g/ml}$  for 15 min removed  $> 50\%$  of the established biofilm cells. CLSM images also showed that panduratin A at 10  $\mu\text{g/ml}$  reduced biofilm viability within 30 min, but did not kill the cells completely (data not shown). The underlying mechanism by which panduratin A prevents oral biofilm formation and reduces

the existing biofilms still remains unclear. Bacterial biofilms are comprise microcolonies surrounded by a rigid inter-microbial matrix for barrier protection (30). Panduratin A might disrupt the biofilm cells at the outer edge of the biofilm that are in direct contact with the agent, thus relasing dead cells from the periphery. In general, multi-species biofilms are more resistant to antibacterial agents and difficult to reduce in comparison to single-species biofilms. Kara et al. (31) reported that dual biofilms of *S. mutans* and *Veillonella parvula* were more resistant to chlorhexidine gluconate at 0.1 and 0.4 mg/ml than single-species biofilms of either strain. However, simple comparisons are difficult because of differences in biofilm composition, the antimicrobial agents used, and their concentrations.

Taken together, our results strongly indicate that panduratin A acts as a potent anti-biofilm agent that has dual actions by preventing biofilm formation and disrupting the existing biofilm. However, these studies were conducted on artificial multi-species biofilms *in vitro*, and the actual effects of panduratin A on dental plaque need to be evaluated in further clinical tests.

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