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Antibacterial Characteristics of *Curcuma xanthorrhiza* Extract on *Streptococcus mutans* Biofilm

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This study evaluated the antibacterial effects of a natural *Curcuma xanthorrhiza* extract (Xan) on a *Streptococcus mutans* biofilm by examining the bactericidal activity, inhibition of acidogenesis and morphological alteration. Xan was obtained from the roots of a medicinal plant in Indonesia, which has shown selective antibacterial effects on planktonic *S. mutans*. *S. mutans* biofilms were formed on slide glass over a 72 h period and treated with the following compounds for 5, 30, and 60 min: saline, 1% DMSO, 2 mg/ml chlorhexidine (CHX), and 0.1 mg/ml Xan. The Xan group exposed for 5 and 30 min showed significantly fewer colony forming units (CFU, 57.6 and 97.3%, respectively) than those exposed to 1% DMSO, the negative control group ($P<0.05$). These CFU were similar in number to those slides exposed to CHX, the positive control group. Xan showed similar bactericidal effect to that of CHX but the dose of Xan was one twentieth that of CHX. In addition, the biofilms treated with Xan and CHX maintained a neutral pH for 4 h, which indicates that Xan and CHX inhibit acid production. Scanning electron microscopy showed morphological changes in the cell wall and membrane of the Xan-treated biofilms; an uneven surface and a deformation in contour. Overall, natural Xan has strong bactericidal activity, inhibitory effects on acidogenesis, and alters the microstructure of *S. mutans* biofilm. In conclusion, Xan has potential in anti-*S. mutans* therapy for the prevention of dental caries.

***Keywords*:** antibacterial effects, biofilms, *Curcuma xanthorrhiza*, *Streptococcus mutans*

Streptococcus mutans is an important microorganism for early colonization on a tooth surface during the formative process of dental plaque (Loesche, 1986). The acidogenic properties of *S. mutans* with its ability to synthesize extracellular glucans are the major factors for the development and establishment of cariogenic biofilms (Gamboa *et al.*, 2004). Various chemical plaque control approaches have been suggested to reduce cariogenic biofilms. Among them chlorhexidine (CHX) and triclosan, which are synthetic chemical antimicrobial agents, are representative materials in oral care products. Although these compounds have broad and strong antibacterial characteristics (Ciancio, 2007), they also have long term side effects, such as intra oral staining and a disturbance of the oral bacterial ecology (Phan and Marquis, 2006). Therefore, there has been increasing attention focused on the discovery of new compounds without side effects during long term use. Many studies have focused on applying natural extracts clinically, such as green tea and oolong tea (Matsumoto *et al*., 1999; Maeyama *et al*., 2005).

This study examined a *Curcuma xanthorrhiza* extract (Xan) isolated from the ethyl-acetate fraction of the methanol extract of Javanese turmeric (*Curcuma xanthorrhiza* Roxb.), which is a medicinal agent in Indonesia. Xanthorrhizol is the main active component of Xan, and has a variety of pharmacological activities, such as anti-metastasis (Choi *et al*., 2005) and inhibitory effects on nephrotoxicity (Kim *et al*., 2005), as well as anti-cancer and anti-inflammatory effects (Itokawa *et al*., 1985; Claeson *et al*., 1993; Claeson *et al*., 1996). The bactericidal activity of Xan against several oral pathogens has also been reported using planktonic and simple biofilm models (Rukayadi and Hwang, 2006a, 2006b). Dental plaque is a representative biofilm. Therefore, a biofilm model that is similar to oral ecology will be needed to evaluate the antibacterial characteristics of new materials. A previous study reported that the CHX concentration needed to inhibit biofilms was 100 times higher than that of planktonic cells (Shani *et al*., 2000). Therefore, Xan will be also needed to evaluate antibacterial characteristics using more precise biofilm model which would be similar to oral ecological environment.

Thus, the antibacterial effect of natural Xan on a *S. mutans* biofilm was evaluated *in vitro* by examining the bactericidal activity, acidogenesis and morphology.

Materials and Methods

Test compounds

The *Curcuma xanthorrhiza* extract (Xan) was obtained from

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Table 1. Antibacterial effects of the Curcuma Xanthorrhiza extract against *S. mutans* biofilms

Test compounds	Exposure time					
	5 min		30 min		60 min	
	N	CFU ($\times 10^6$)	N	CFU ($\times 10^6$)	N	CFU ($\times 10^6$)
Saline	10	29.42 ± 10.50^a	16	17.98 ± 6.00^a	17	11.10 ± 4.14^a
1% DMSO	16	28.52 ± 16.02^a	19	20.85 ± 7.04^a	17	10.77 ± 4.60^a
2 mg/ml CHX	17	9.53 ± 5.17^b	19	3.63 ± 6.34^b	17	0.47 ± 0.28^b
0.1 mg/ml Xan	17	12.08 ± 12.14^b	19	0.56 ± 0.46^b	17	0.15 ± 0.10^b

CHX is chlorhexidine and Xan means the *Curcuma Xanthorrhiza* extracts. N indicates the number of samples. The data shown are the Mean±SD. The different superscripts in the same column indicate statistically significant difference from each group ($P<0.05$).

Bioproducts Research Center of Yonsei University and isolated from the ethyl acetate fraction of the methanol extract of *Curcuma xanthorrhiza* Roxb. (Indonesia) using the method reported by Hwang *et al*. (2000a, 2000b). Based on previous experiments (data not shown), 0.1 mg/ml Xan in 1% dimethyl sulfoxide (DMSO) was used as the Xan concentration in this study. Two mg/ml CHX (Sigma, USA) was used as the positive control. Saline and 1% DMSO were used as negative controls.

Bacterial strain, media, and growth conditions

Streptococcus mutans ATCC 25175 was used in this study. *S. mutans* was grown in a tryptone yeast extract broth containing 1% (w/v) sucrose at 37°C and 5% CO_2. Biofilms of *S. mutans* were formed on standard glass microscope slides in a 50 ml tube. Each slide was transferred daily to fresh medium over a 3 day period (Koo *et al.*, 2003). The *S. mutans* biofilms on each slide contained approximately 2×10^7 colony forming units per milliliter.

Measurement of bacterial viability

After exposing the biofilms to the solutions for 5, 30, and 60 min, they were placed in a 50 ml tube containing a sterile saline solution. The slide glass in the 50 ml tube was ultrasonicated at 50 W (Branson Sonic, USA) using 3×10 sec pulses with 2×5 sec intervals (Koo *et al.*, 2002; Koo *et al.*, 2003). The suspension was diluted serially from 10^{-1} to 10^{-6}, and plated on Brain Heart Infusion (BHI) agar. The plates were incubated in 5% CO_2 at 37°C for 48 h, and the CFU were determined by counting the number of colonies.

Measurement of acid production

The level of acid production from the *S. mutans* biofilms treated with the compounds was determined by measuring the pH (Koo *et al.*, 2006). After a 30 min treatment with the test compounds, the *S. mutans* biofilms on the slide glass were transferred to BHI media and incubated in 5% CO_2 at 37°C. The pH of the media was measured every hour for 4 h. These assays were repeated at least five times.

SEM and TEM analysis

Scanning electron microscopy (SEM) S-800 (Hitachi, Japan) was used to examine the changes in the *S. mutans* morphology. The *S. mutans* biofilm on the slide glass was treated with 1% DMSO, 2 mg/ml CHX or 0.1 mg/ml Xan for 30 min. The slide glasses were cut using a sharp diamond knife and fixed in 2% glutaraldehyde in 0.1 M PBS for 1 h at room temperature. The fixed samples were then dehydrated for 30 min in a graded series of ethanol, coated with gold and observed by SEM. Transmission electron microscopy (TEM) JEM 1011 (JEOL, Japan) was used to examine the intracellular changes in *S. mutans*. *S. mutans* harvested from the slide surface was fixed and dehydrated. The fixed cells were embedded, and small blocks of bacteria were cut using an ultramicrotome (Leica, Austria).

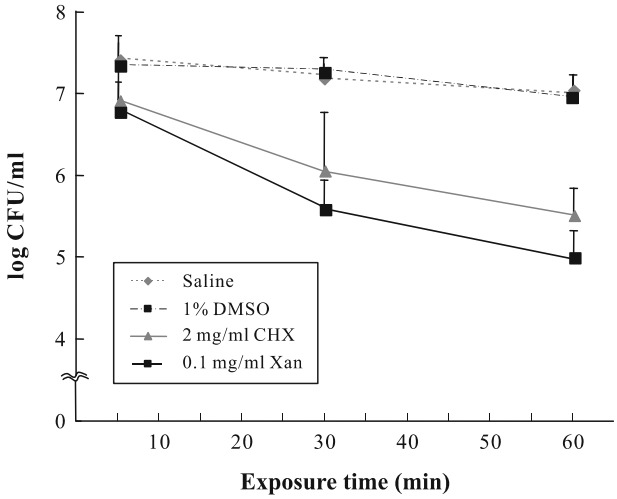

Fig. 1. The killing curve of *S. mutans* Biofilm by a *Curcuma xanthorrhiza* extract (Xan). After the biofilms had been exposed to the test solutions for 5, 30, and 60 min, The suspension was plated on BHI agar and incubated for 48 h. The number of colonies was counted to determine the CFU.

Statistical analysis

The difference between the groups was analyzed by one-way ANOVA and Scheffe's post hoc analysis using the SPSS 12.0 statistical package program (SPSS Inc., USA). A P value <0.05 was considered significant.

Result

Bactericidal activity of Xan

It was initially determined that Xan has bactericidal activity on *S. mutasn* biofilms. The groups treated with 0.1 mg/ml Xan and 2 mg/ml CHX showed significantly lower numbers

of surviving *S. mutans* colony forming units (CFU) than those of the negative control groups ($P<0.05$) (Table 1 and Fig. 1). 0.1 mg/ml Xan exhibited similar bactericidal activity to 2 mg/ml CHX. The Xan and CHX groups for 5 min showed 57.6% and 67.6% fewer CFU, respectively, than the 1% DMSO negative control group. The Xan group for 30 min showed greater bactericidal activity (97.3%) than that of the CHX group exposed for the same time (79.8%). The Xan group for 60 min showed 98.6% bactericidal activity, which is higher than the 95.6% observed for the CHX group for 60 min. These results show that Xan has similar bactericidal effects against *S. mutans* biofilms to that of CHX. However, the concentration of Xan used was one twentieth of the amounts of CHX used, which shows that Xan for 30 min has superior bactericidal activity to that of CHX for the same time (Fig. 1).

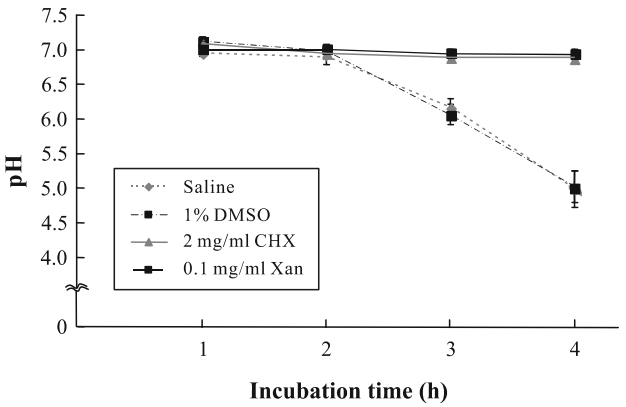

Fig. 2. Acidogenicity of *S. mutans* biofilms was determined by measuring the pH of media. After exposure to saline, the *S. mutans* biofilms were cultured for 4 h in 1% DMSO, 2 mg/ml Chlorhexidine (CHX) or 0.1 mg/ml *Curcuma xanthorrhiza* extract (Xan) for 30 min, and the pH of media was measured.

Inhibition of acid production

The pH of culture medium was recorded during 4 h after Xan treatment to determine the effect of Xan on acid production. The pH patterns of the Xan and CHX groups were significantly different from those of saline and 1% DMSO groups after 3 h ($P<0.05$). Both the Xan and CHX groups maintained a pH of approximately 7.0 for 4 h whereas the pH of the saline and 1% DMSO groups decreased rapidly from pH 7.0 to pH 6.2 and 5.0 after 3 h and 4 h, respectively. This suggests that 0.1 mg/ml Xan effectively inhibits acid production in the *S. mutans* biofilms, which is similar to that of 2 mg/ml CHX (Fig. 2).

Morphological changes in *S. mutans* biofilms by Xan

The mechanism responsible for the bactericidal activity of Xan was examined by observing the changes in the morphology of *S. mutans* by SEM and TEM after treating the biofilm with 0.1 mg/ml Xan and 2 mg/ml CHX for 30 min.

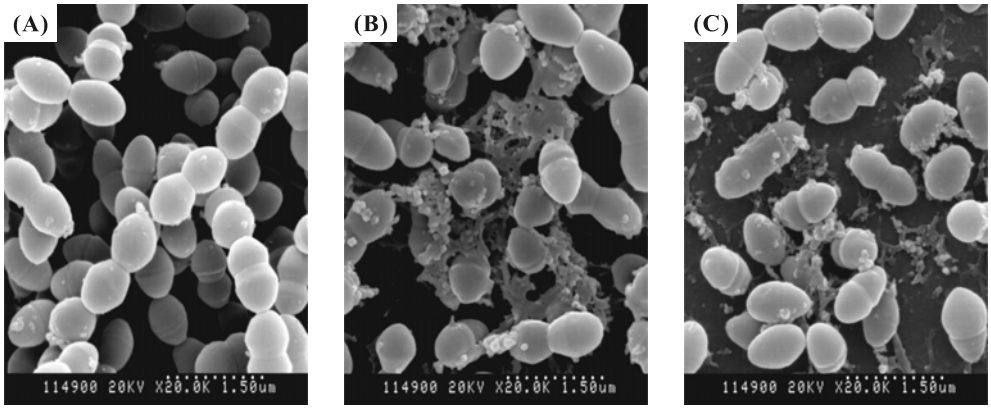

Fig. 3. SEM images of the *S. mutans* biofilm after 30 min treatment. (A) 1% DMSO, (B) 2 mg/ml Chlorhexidine (CHX), and (C) 0.1 mg/ml *Curcuma xanthorrhiza* extract (Xan).

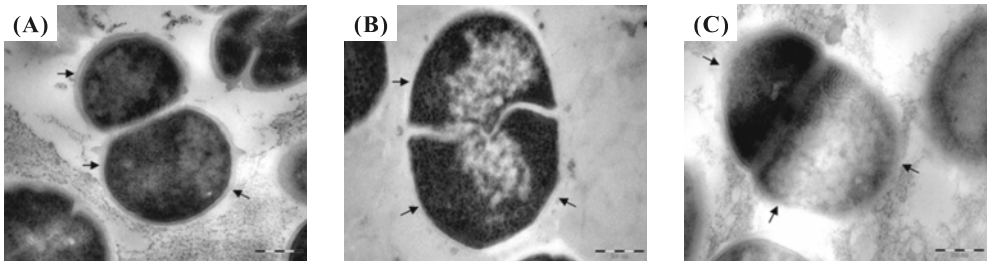

Fig. 4. TEM images of the *S. mutans* biofilm after 30 min treatment. The black arrows indicate the wall of *S. mutans*. The scale bar is 200 nm. (A) 1% DMSO, (B) 2 mg/ml Chlorhexidine (CHX), and (C) 0.1 mg/ml *Curcuma xanthorrhiza* extract (Xan).

Consistent with Table 1, SEM showed fewer intact bacteria in the Xan and CHX groups for 30 min than in the control group, and the *S. mutans* in the Xan and CHX groups for 30 min showed a more defective morphology and intracellular contents than the negative control group (Fig. 3). In addition, the Xan and CHX groups revealed an irregular cell wall structure, agglomerated material of debris and fibers, and fewer cells in chains in contrast to the smooth cell wall and typical long chains observed in the negative control groups.

TEM was used to examine the level of cell wall damage and intracellular modification. After the *S. mutans* biofilms had been exposed to 1% DMSO for 30 min as the negative control, TEM showed an obvious outline of the *S. mutans* cell wall and a peptidoglycan layer (Fig. 4A). However, the peptidoglycan layers of *S. mutans* in the CHX group had disappeared completely (Fig. 4B). *S. mutans* of the Xan group showed incomplete septa about the outline of the cell wall. In addition, there was some outflow of the intracellular contents from the bacteria (Fig. 4C). The antibacterial activity of Xan was similar to that of CHX but there was less injury to *S. mutans*.

Discussion

Streptococcus mutans (*S. mutans*) is a Gram-positive, facultative species of anaerobic bacteria that is commonly found in the human oral cavity and is a significant contributor to dental caries. *S. mutans* plays an important role in metabolizing sucrose to lactic acid, which leads to demineralization of the tooth enamel. In addition, the microbe plays the role as an early colonizer, which forms dental plaque that sticks to the teeth. Dental plaque is a representative example of a biofilm. A biofilm is multicellular aggregation of microorganisms attached to a surface that builds up into a thick layer. Because of these structural features, the biofilm shows different properties compared with planktonic cells. According to previous studies, a biofilm can better withstand the defensive acts of the host than planktonic cells and has a higher resistance to antibiotics (Gilbert *et al*., 1997; Bowden and Hamilton, 1998; Socransky and Haffajee, 2002). Therefore, a biofilm study is used to evaluate the antibacterial effect of antibiotics more accurately than a planktonic study. The 96-well plate method was the biofilm used in a former study to examine the effects of Xan on a *S. mutans* biofilm deposited on a well plate base. On the other hand, the biofilm model in this study was a biofilm of *S. mutans* attached to slide glass which was placed perpendicular to the ground. This is simulates the process of forming dental plaque without a gravity effect on the tooth surface. Therefore, the slide glass model in this study reflects the environment of the oral cavity better than the 96-well plate method.

According to preceding studies, various natural extracts were effective in preventive caries. Catechin is the representative natural component, which is the main ingredient of green tea. It was reported that a mouth rinse solution containing 2 mg/ml of epigallocatechin gallate can inhibit the acidogenesis of dental plaque (Maeyama *et al.*, 2005). Although an oolong tea extract was also shown to inhibit acid production and have antiglucosyltranferase activity, a minimum concentration of approximately 1 mg/ml of these natural extracts was required to produce an antibacterial effect on *S. mutans* biofilms (Maeyama *et al.*, 2005). On the other hand, Xan has excellent antibacterial efficacy against *S. mutans* biofilms at relatively lower concentrations (Rukayadi and Hwang, 2006a, 2006b). The antibacterial effect of Xan was also superior to those of the other natural extracts, such as catechin and oolong tea extracts (Matsumoto *et al.*, 1999; Sasaki *et al*. 2004; Hirasawa *et al.*, 2006).

There were significantly fewer CFUs of *S. mutans* in the group exposed to Xan than in the group exposed to 1% DMSO ($P<0.05$). In addition, the biofilm exposed to Xan maintained a constant pH of 7. This shows that Xan can stop the additional acid production of *S. mutans*. This result is comparable to the antibacterial effect of CHX, which is to be the most effective antiplaque agent. Therefore, Xan has potential as an antimicrobial agent in against *S. mutans* instead of CHX, which has various side effect in long term use.

The anticaries metabolism of natural antimicrobial agents can be classified into 2 mechanisms. One is to destroy the integrity of the cell wall and the other is to inhibit bacterial adhesion instead of killing the bacteria. According to the SEM images in this study, Xan might have destroyed the cell wall integrity. *S. mutans* exposed to Xan showed peptidoglycan layer damage and an increase in the level of dead cell debris compared with the control group. TEM indicated that exposure to Xan resulted in partial destruction of the peptidoglycan layer and leakage of the intracellular contents. This indicates that the superior antibacterial effect of Xan might be the result of bacterial peptidoglycan layer damage. This is similar to previous studies showing that a grapefruit seed extract is a natural antibacterial material that can weaken the function of the physiological active enzyme in microorganism cells and destroy the cell wall function (Miele, 1988).

Although the precise biological mechanism of Xan is unclear, these results show that it might also disturb or destroy the peptidoglycan layer. The main active component of Xan is xanthorrhizol, which consists of phenol and a hydrocarbon chain. Among these chemical structures, the hydroxyl group is responsible for the main active antibacterial portion. The antibacterial effects of xanthorrizol on planktonic *S. mutans* almost disappeared when the hydroxyl residue of xanthorrizol was acetylated during the extraction process (Shim, 2000). The antibacterial activity of xanthorrhizol is significantly higher than carvacrol (5-Isopropyl-2-methylphenol), which is a commercial germicide with a similar chemical structure to xanthorrhizol differing only in the length of the hydrocarbon chain (Chami *et al.*, 2005). This indicates that the –OH and hydrocarbon chain of xanthorrhizol might be essential for its activity.

Because this study only analyzed the antimicrobial effect of *S. mutans*, more study will be needed to examine the effects of Xan on other cariogenic bacteria besides *S. mutans*. In addition, because this study was an *in vitro* examination, more studies using clinical trials will be needed to determine its clinical relevance.

This study found that natural Xan has strong bactericidal activity, inhibits acidogenesis, and alters the microstructure

of a *S. mutans* biofilm. In conclusion, Xan might be a useful anti-*S. mutans* treatment for preventing dental caries.

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