ORIGINAL ARTICLE

In vitro activity of xanthorrhizol against *Streptococcus mutans* biofilms

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Abstract

Aims: We determined the effect of xanthorrhizol (XTZ) purified from the rhizome of *Curcuma xanthorrhiza* Roxb. on the *Streptococcus mutans* biofilms *in vitro*.

Methods and Results: The biofilms of *S. mutans* at different phases of growth were exposed to XTZ at different concentrations (5, 10 and 50 μmol l^{-1}) and for different time exposures (1, 10, 30 and 60 min). The results demonstrated that the activity of XTZ in removing *S. mutans* biofilm was dependent on the concentration, exposure time and the phase growth of biofilm. A concentration of 5 μmol l^{-1} of XTZ completely inhibited biofilm formation by *S. mutans* at adherent phases of growth, whereas 50 μmol l^{-1} of XTZ removed 76% of biofilm at plateau accumulated phase when exposed to *S. mutans* biofilm for 60 min.

Conclusions: Xanthorrhizol isolated from an edible plant (*C. xanthorrhiza* Roxb.) shows promise as an antibacterial agent for inhibiting and removing *S. mutans* biofilms *in vitro*.

Significance and Impact of the Study: XTZ could be used as a potential antibacterial agent against biofilm formation by *S. mutans*.

Introduction

Streptococcus mutans is known to be a major causative organism for dental plaque, which is acknowledged to be associated with biofilms and can also be a source of infective endocarditis (Banas 2004). The widely adopted approach for reducing plaque development in the topical application of bactericides such as triclosan, chlorhexidine and cetylpyridinium chloride have resulted in a reduction in the plaque development and lowering the number of micro-organisms in saliva (Eley 1999). However, in general, they are nonselective in their efficacy and their frequent use can lead potentially to a change in oral microbiota and occurrence of resistant strains (McMurry *et al.* 1998).

Some plant extracts clearly demonstrate antibacterial properties, although the mechanisms of activity are still poorly understood (Dorman and Deans 2000). The spice extracts, cinnamon bark oil, papua-mace extract, and clove bud oil were all reported to inhibit the growth of many oral bacteria (Saeki *et al.* 1989). Sanguinarine, an alkaloid extract from the rhizome of the *Sanguinaria canadensis*, has been reported to possess a broad spectrum against a wide variety of oral bacteria (Joann and Sigmund 1985). In particular, green tea extract, which is customarily drunk after every meal in Japan, is known to contain several polyphenols that inhibit the growth of *S. mutans* (Sakanaka *et al.* 1989).

Xanthorrhizol (XTZ) (Fig. 1) isolated from java turmeric (*Curcuma xanthorrhiza* Roxb.) has been reported to possess antibacterial activity against several oral pathogens, and it showed very fast bactericidal activity against *S. mutans* (Hwang *et al.* 2000a,b). However, there is no report of XTZ against an oral bacterial biofilm or dental plaque. In this study, we used a polystyrene microtitre plate assay to determine the effect of XTZ extracted from the rhizome of *C. xanthorrhiza* Roxb. on the *S. mutans* biofilms *in vitro*.

Materials and methods

Bacterial strain, media, growth conditions, XTZ and chemicals

Experiments were conducted with *S. mutans* ATCC 25175. The *S. mutans* strain was maintained routinely

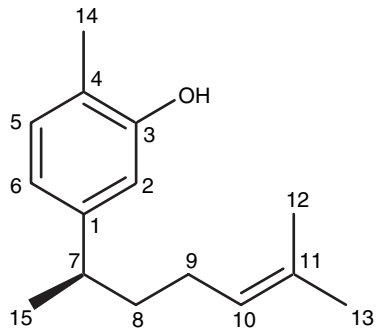

Figure 1 Structure of xanthorrhizol.

with weekly subculture on streptococci media, brain heart infusion (BHI) medium (Difco) at pH 7·0 ± 0·2 with shaking (200 rev min^{-1}) in a controlled shaker bath or on BHIA (BHI supplemented 15 g of agar per litre broth) at 37°C. XTZ was isolated from the ethyl acetate fraction of the methanol extract of *C. xanthorrhiza* Roxb. according to the method of Hwang *et al.* (2000b). XTZ was dissolved in 1% dimethyl sulfoxide (DMSO) to make a stock solution of 25, 50 and 250 μmol l^{-1}. One per cent of DMSO did not kill *S. mutans*. Chlorhexidine (1,1′-hexamethylenebis[5-*p*-chlorphenylbiguanide]) was purchased from Sigma (St Louise, MO, USA). The artificial saliva containing 1% (w/v) hog gastric mucin (type III; Sigma) in an adherence buffer (10 mmol l^{-1} KPO_4, 50 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 0·1 mmol l^{-1} $MgCl_2$, pH 7·0) was autoclaved at 121°C for 20 min.

Inoculum cell preparation

An inoculum cell suspension was prepared as follows: a single colony of *S. mutans* was propagated in BHI broth at 37°C overnight with 200 rev min^{-1} agitation and a quantity of 1 ml of overnight cultures in BHI was centrifuged (3000 ***g*** at 4°C for 1 min), followed by washing the pellets twice with 1 ml of 50 mmol l^{-1} pH 7·0 phosphate-buffered saline (PBS). The pellets were re-suspended in 1 ml of adherence buffer to a final concentration of approx. 2×10^5 cells ml^{-1}. A standard curve of turbidity against colony-forming unit was used to obtain the number of cells.

In vitro biofilm formation, treatment and quantification

The formation of *S. mutans* biofilms were studied in commercially available presterilized, polystyrene, flat-bottom 96-well microtitre plates by the method described previously (Loo *et al.* 2000; Li *et al.* 2002) with modification. The plates were first conditioned with 200 μl of artificial saliva solution. The plates were then incubated at room temperature for 2 h with gentle shaking and air-dried after removal of excess artificial saliva solution. The growth of biofilm formation was initiated by addition of 175 μl of BHI broth supplemented with 3% (w/v) of sucrose (BHIS) to each well of a 96-well microtitre plate. A 96-well microtitre plate containing 175 μl of BHIS broth per well was inoculated with 25 μl of a previously prepared inoculum cell suspension at a density of 2×10^5 cells ml^{-1}. The final density of inoculum in each well of a 96-well microtitre plate was $2{\cdot}5 \times 10^4$ cells ml^{-1}. The microtitre plates were then incubated at 37°C without agitation. Biofilm formation by *S. mutans* grown in BHI media usually showed three accumulation phases: (i) the adherent phase (0–4 h), (ii) the active accumulated phase (4–20 h), and (iii) the slow or plateau accumulated phase (after 20 h) (Li *et al.* 2001). In this study, we divided *S. mutans* biofilm formation into four accumulation phases: 4 h, adherent phase; 12 h, active accumulated phase; 20 h, beginning plateau accumulated phase and 24 h, plateau accumulated phase.

Streptococcus mutans biofilms at different phases of biofilm growth, i.e. adherent, active accumulated, beginning plateau accumulated, and plateau accumulated phase containing 200 μl biofilm were treated with 50 μl of XTZ (25, 50 and 250 μmol l^{-1} as prepared above), which made the final concentration of XTZ 5, 10 and 50 μmol l^{-1}, respectively. The exposed time to XTZ was 1, 10, 30 and 60 min.

Biofilm formation was quantified by a crystal violet assay recently described by others (Djordjevic *et al.* 2002; Jin *et al.* 2003). Briefly, the biofilm-coated wells of microtitre plates as described above for biofilm formation and treatment were vigorously shaken in order to remove all nonadherent bacteria. The remaining attached bacteria were washed twice with 200 μl of 50 mmol l^{-1} PBS (pH 7·0) and air-dried for 45 min. Then, each of the washed wells was stained with 110 μl of 0·4% aqueous crystal violet solution for 45 min. Afterwards, each well was washed twice with 350 μl of sterile distilled water and immediately de-stained with 200 μl of 95% ethanol. After 45 min of de-staining, 100 μl of de-staining solution was transferred to a new well and the amount of the crystal violet stain in the de-staining solution was measured with a tunable microplate reader ($VERSA_{MAX}$, Sunnyvale, CA, USA) at 595 nm. The activity of XTZ was expressed as the percentage of the absorbance of biofilm treated by XTZ compared with the control (untreated). The experiment was repeated with four replicates per experiment. Plates without XTZ served as a negative control and plates treated with chlorhexidine at 5·0, 10 and 50 μmol l^{-1} final concentration were included in each trial.

Statistical analysis

Data were expressed as mean ($n = 4 \times 4$) and standard deviation (SD). Mean and SD were computed. Statistical significance was determined by analysis of variance (SPSS 11·0 for Windows). Fisher's exact tests were applied to contingency tables (2×2) to compare effectiveness of *S. mutans* biofilm treatment with XTZ between 10 and 50 μmol l^{-1} XTZ. A *P*-value of less than 0·05 was taken as statistically significant.

Results

Xanthorrhizol (5, 10, and 50 μmol l^{-1}) was used to treat 4-, 12-, 20- and 24-h-old biofilms to see whether it could adversely affect *S. mutans* biofilms on each phase of biofilm growth: 4 h, adherent phase; 12 h, active accumulated phase; 20 h, beginning plateau accumulated phase and 24 h, plateau accumulated phase. The results of these experiments demonstrated that the activity of XTZ in removing *S. mutans* biofilm was dependent on the concentration, exposure time and the phase growth of biofilm (Table 1). A similar result was also obtained when *S. mutans* biofilms were exposed to chlorhexidine at 5·0, 10 and 50 μmol l^{-1} final concentrations (data not shown).

The biofilms of *S. mutans* at the adherent phase (4 h) were completely removed by all variations of the XTZ treatment. The growth of *S. mutans* biofilm at the active accumulated phase was also completely removed after exposure to 50 μmol l^{-1} XTZ for 30 min. Treatment with 50 μmol l^{-1} of XTZ for 60 min of exposure at the beginning plateau accumulated phase (20 h) resulted in 89% removal of *S. mutans* biofilm relative to the untreated control. Moreover, treatment with 50 μmol l^{-1} of XTZ resulted in 76% removal of *S. mutans* at plateau accumulated phase (24 h) relative to untreated control when exposed to *S. mutans* biofilm for 60 min.

Fisher's exact tests were used to compare the effectiveness of XTZ in removing *S. mutans* biofilm between treatments with 10 and 50 μmol l^{-1} (Table 2). At the active accumulated phase of *S. mutans* biofilm growth, treatment with 10 min of exposure to 50 μmol l^{-1} XTZ was more effective than exposure to 10 min of 10 μmol l^{-1} XTZ ($P < 0{\cdot}003$). At the beginning plateau accumulated phase, treatment with 50 μmol l^{-1} XTZ for 60 min exposure time was more effective than treatment with 60 min of 10 μmol l^{-1} XTZ ($P < 0{\cdot}013$). Finally, *S. mutans* biofilm at the plateau accumulated phase could be removed more effectively with 60 min of exposure to 50 μmol l^{-1} XTZ than exposure to 60 min of 10 μmol l^{-1} XTZ ($P < 0{\cdot}036$).

Table 1 The effect of xanthorrhizol at different concentrations and exposure time on biofilms of *Streptococcus mutans* at different phases of growth

Xanthorrhizol Exposure time (min)	Percentage of mean remaining cells of *S. mutans* biofilm after treatment with xanthorrhizol (% ± SD)†			
	Adherent phase (4 h)	Active accumulated phase (12 h)	Beginning plateau accumulated phase (20 h)	Plateau accumulated phase (24 h)
5 μmol l^{-1}				
1	0	77 ± 6	86 ± 6*	92 ± 8*
10	0	61 ± 9	77 ± 7	79 ± 9*
30	0	39 ± 2	60 ± 7	63 ± 1
60	0	23 ± 7	34 ± 4	36 ± 4
10 μmol l^{-1}				
1	0	71 ± 8	76 ± 4	84 ± 9
10	0	54 ± 3	57 ± 2	72 ± 2
30	0	13 ± 3	42 ± 9	59 ± 6
60	0	8 ± 2	28 ± 6	44 ± 5
50 μmol l^{-1}				
1	0	46 ± 7	68 ± 8	70 ± 6
10	0	10 ± 1	31 ± 9	54 ± 5
30	0	0	23 ± 1	39 ± 9
60	0	0	11 ± 4	24 ± 4

†Values are expressed as the percentage of absorbance (595 nm) of cells in treated wells compared with that in untreated wells (considered to be 100%), and SD is standard deviation (errors) of the mean percentage of absorbance (595 nm) of cells derived from four times of experiment and four replicates (wells) per experiment.

*Not significantly different from the control treatment. All entries in the table are significantly different ($P < 0.05$) from the controls unless otherwise indicated.

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Table 2 Contingency table for comparing the effectiveness of xanthorrhizol treatment on *Streptococcus mutans* biofilm growth

Biofilm growth Exposure time (min)	Xanthorrhizol (μmol l^{-1}) 10	50	*P*-value†
Active accumulated phase (12 h)			
1	71 ± 8	46 ± 7	0·635
10	54 ± 3	10 ± 1	0·003*
30	13 ± 3	0	0·0004*
60	8 ± 2	0	0·005*
Beginning plateau accumulated phase (20 h)			
1	76 ± 4	68 ± 8	0·724
10	57 ± 2	31 ± 9	0·264
30	42 ± 9	23 ± 1	0·071
60	28 ± 6	11 ± 4	0·013*
Plateau accumulated phase (24 h)			
1	84 ± 9	70 ± 6	0·705
10	72 ± 2	54 ± 5	0·359
30	59 ± 6	39 ± 9	0·138
60	44 ± 5	24 ± 4	0·036*

†*P*-values were computed using the Fisher's exact test.
*Significantly different ($P < 0·05$, Fisher's exact test).

Discussion

There are few reports concerning the susceptibility of oral bacterial biofilms to antimicrobial agents. Marsh and Bradshaw (1993) found that exposure of multi-species biofilms to triclosan (0·07 mmol l^{-1}) for 1 h killed approx. 40% of the bacteria present. In contrast, Wilson *et al.* (1998) reported that chlorhexidine gluconate killed approx. 40% of the bacteria at 2·23 mmol l^{-1}. In reality, simple comparisons are difficult because of differences in the biofilms compositions, the antimicrobial agents and their concentrations used. Our results demonstrated that exposure of *S. mutans* biofilm at plateau accumulated phase to XTZ (0·05 mmol l^{-1}) for 1 h removed 76% of biofilm quantity compared with that of biofilm quantity in the untreated experiment (Table 1). Our previous reports (Hwang *et al.* 2000a,b) have shown that XTZ has activity to kill *S. mutans* at planktonic growth. These results suggest that XTZ might kill *S. mutans* cells of the outer layer of biofilm as those cells have direct contact with XTZ. The dead cells could be degraded and released from the biofilm, resulting in the reduced biofilm quantity.

In this study, treatment with chlorhexidine showed a similar result to the XTZ treatment (data not shown). However, it is not safe to routinely use chlohexidine at high concentrations as part of daily oral hygiene (Jones 1997; Ernst *et al.* 1998). In contrast, XTZ, isolated from an edible plant (*C. xanthorrhiza* Roxb.), might be a good candidate to prevent and remove dental biofilms. To remove biofilm from teeth, an antibacterial with a relatively higher concentration and longer exposure time is needed, particularly in plateau accumulated or thick biofilm. However, this study is only a model and the actual activity of XTZ on dental surface has to be evaluated in further clinical tests.

Interest in the use of antimicrobial agents for the treatment of plaque-related diseases, such as caries and periodontitis, has generated a need for laboratory models for the evaluation of agents effective against oral bacterial biofilms (Wilson *et al.* 1998), but so far there are no reports on using antimicrobial agents isolated from edible plants in the treatment of oral bacterial biofilm. The results of this study showed that, although XTZ did not completely remove the bacteria at the plateau accumulated phase of biofilm growth, all bacteria were removed in the adherence and beginning active accumulated phase of biofilm growth. Thus, the later finding may account, in part, for the effectiveness of XTZ in the prevention of dental plaque.

In summary, to the best of our knowledge, this is the first report showing that XTZ has strong activity against *S. mutans* biofilm. The results suggest that XTZ can be developed as an antibacterial in the removal of oral bacterial biofilm.

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