Effects of *Streblus asper* leaf extract on the biofilm formation of subgingival pathogens

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A B S T R A C T

*Streblus asper* leaf extract (SAE) possesses antibacterial activity towards *Streptococcus mutans* and periodontal pathogens, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. However, there is no report on inhibitory effects of SAE against subgingival biofilm formation. The purpose of this study was to investigate the inhibitory effects of SAE on subgingival biofilm formation using an in vitro model. Subgingival plaque samples from 5 periodontitis patients were cultivated in saliva-coated 96-well microtiter plates in the presence of SAE at concentrations 0.5–90 mg/ml under anaerobic atmosphere at 37 °C for 2, 4 and 8 days by replacing every 2 days with fresh medium alone or SAE-containing medium. Biofilm formation was determined quantitatively by crystal violet staining. TaqMan-based real-time polymerase chain reaction was used to quantify the numbers of *P. gingivalis*, *A. actinomycetemcomitans* and total bacteria in the grown subgingival biofilm. The results showed that SAE exhibited more than 70% anti-biofilm activity at 90 mg/ml. The reduction in cell numbers of *P. gingivalis*, *A. actinomycetemcomitans* and total bacteria in subgingival biofilm of test groups were observed compared to untreated control. These results revealed that SAE is able to inhibit in vitro subgingival biofilm formation and reduce the numbers of *P. gingivalis*, *A. actinomycetemcomitans* and total bacteria.

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1. Introduction

The oral cavity is an ideal environment for biofilm development. Most common infections of the oral cavity, e.g. caries, gingivitis and periodontitis result from the accumulation of biofilms. Bacteria that are sessile (attached to a surface) express different genes and, so, behave differently from free-floating or planktonic bacteria. Notable among these differences is up to a 1000-fold increase in resistance to antimicrobial agents for a species in a mature biofilm relative to that same species grown planktonically (Gilbert et al., 1997).

Medicinal plants as new sources of antimicrobial agents have attracted considerable research interest. A wide variety of plant extracts has been reported to have antimicrobial effects and anti-inflammatory properties and, in fact, several herbal extracts have been added to some cosmetics and healthcare preparations (Aburaji and Natshel, 2003; Uprety et al., 2012; Fatima et al., 2013; Hassan-Abdallah et al., 2013). *Streblus asper* Lour, Moraceae, is a medicinal plant which has been used as a remedy for relief of fever, dysentery and toothache, and as an antingvitis agent (Gaiteone et al., 1964; Rastogi et al., 2006). In the Indian traditional system of medicine, *S. asper* is prescribed for the treatment of diabetes mellitus. Recently, α-amyrin acetate isolated from *S. asper* and the petroleum ether extract of *S. asper* stem bark were reported to have antidiabetic activity in streptozotocin-induced diabetic rats (Karan et al., 2013). Anti-hepatitis B virus constituents from *S. asper* were also demonstrated (J. Li et al., 2012; L. Li et al., 2012; Li et al., 2013). In addition, *S. asper* has been used as an antiseptic and astringent (Mukherjee and Roy, 1983; Rastogi et al., 2006). Our previous studies reported the antibacterial action of *Streblus asper* leaf extract (SAE) against *Streptococcus mutans* both in vitro and in vivo (Taweechaisupapong et al., 2006b; Wongkhram et al., 2001). We also found that SAE possesses antibacterial activity against endodontic and periodontal pathogens, e.g., *Porphyromonas gingivalis, Prevotella intermedia, Actinomyces naeslundii*, and *Aggregatibacter actinomycetemcomitans* (Taweechaisupapong et al., 2000a, 2002a). Moreover, rinsing with SAE mouthrinse and subgingival irrigation with SAE solution as an adjunct to scaling and root planning also have a clinically measurable effect on gingival health (Taweechaisupapong et al., 2002b, 2006a). Although SAE at concentration ≤ 250 mg/ml has no fungicidal activity towards *Candida albicans*, the sublethal concentrations of SAE can block the adherence of *Candida* to human buccal epithelial cells and denture acrylic in vitro (Taweechaisupapong et al., 2005, 2006b). In order to provide more information about SAE for its potential development as a new potential therapeutic agent which may help in the treatment of periodontitis, we evaluated the inhibitory
effects of SAE on biofilm formation in vitro using subgingival biofilm model.

2. Materials and methods

2.1. Preparation of Streblus asper leaf-extract

Leaves of S. asper were locally collected in Khon Kaen province and identified by Dr. Pranom Chantaranonthai, Department of Biology, Faculty of Science, Khon Kaen University, Thailand (Berg et al., 2011). The voucher specimens (4883; Sopit Wongkhram) were deposited at the KKU herbarium, Thailand. The leaves (1500 g) were washed, air-dried, pulverized and soaked in 750 ml of 50% (v/v) redistilled ethanol for 7 days with occasional stirring. After passing though several layers of cheesecloth, the ethanol in the extract was evaporated under reduced pressure at 60 °C. The remaining material was freeze-dried and kept in a desiccator at room temperature until used. (Taweechaisupapong et al., 2000b). Approximately 5 g of dark brown sticky extract was obtained from 100 g of dried–pulverized leaves. The extract was dissolved in distilled water to a final concentration of 500 mg/ml, centrifuged at 10,000 rpm (9410 × g) at 4 °C for 20 min, then passed through a 0.2 μm filter (Acrodisc®, PF, Gelman Sciences, USA). The filtrate was used as the starting material for subsequent studies.

2.2. Subjects and sampling of subgingival plaque

Subjects were a convenient sample of apparently healthy Thai adults who sought periodontal treatment at the Faculty of Dentistry, Khon Kaen University, Khon Kaen, Thailand. The criteria used for selection included: subjects between the ages of 35 and 45 years with no known systemic diseases, who had not received antibiotics within the previous 3 months, and who were not taking medications that might influence the subgingival microbiota. The study protocol was approved by the Khon Kaen University ethical review committee. Informed consent was obtained from all volunteers. Then subgingival plaque samples were collected from five individuals with adult periodontitis. The sampling site had pocket probing depths ranging from 4 to 5 mm and clinical attachment loss ≥ 3 mm. The subgingival plaque was collected by inserting a sterile absorbent paper point to the sulcus depth and moving it laterally along the tooth surface and the sulcular epithelial lining. The paper point sample was immediately placed into 1-ml thioglycollate medium and gently sonicated to disperse the bacterial suspension. The bacterial DNAs from biofilm samples were extracted using subgingival biofilm samples were extracted using a 0.2 μm filter. The bacterial DNA was extracted using a protocol described (Walker and Sedlacek, 2007). In brief, each saliva sample was divided (1:10) with pre-reduced, anaerobically-sterilized Ringer solution, containing 0.05% cysteine (Sigma Chemical Co, St Louis, MO) as a reducing agent, and centrifuged at 2000 × g for 10 min to remove any particulate matter. The supernatant was passed through 0.45 μm sterile syringe filter. 

2.3. Saliva collection and processing

Unstimulated saliva was obtained in 5-ml samples from the same subjects who had donated subgingival plaque and processed as previously described (Walker and Sedlacek, 2007). In brief, each saliva sample was diluted (1:10) with pre-reduced, anaerobically-sterilized Ringer solution, containing 0.05% cysteine (Sigma Chemical Co, St Louis, MO) as a reducing agent, and centrifuged at 2000 × g for 10 min to remove any particulate matter. The supernatant was passed through 0.45 μm sterile syringe filter. 

2.4. Inhibitory effects of Streblus asper leaf extract on subgingival biofilm formation

To determine the effects of SAE in inhibition of biofilm formation in test groups, subgingival plaque sample from each periodontitis patient was cultivated in saliva-coated 96-well microtiter plate in the presence of SAE at concentrations 0.5–90 mg/ml under anaerobic atmosphere at 37 °C for 2, 4, and 8 days (Sedlacek and Walker, 2007). A bacterial suspension in the wells that contained only medium without SAE served as a control group. The initial count of total bacteria, which were inserted into each well, was 1 × 10^6 CFU/ml. For model 1, the supernatant was removed at 48-h intervals and the wells were added with fresh medium without SAE. For model 2, the supernatant was removed and the wells were added with medium containing 0.5–90 mg/ml SAE at 48-h intervals. At the indicated times (2, 4, and 8 days), the supernatant in each well was removed and the wells were washed with 200 μl of sterile phosphate buffered saline. The attached bacteria were stained for 15 min with 150 μl of 1% crystal violet. Excess stain was removed with running tap water. The plates were air-dried and the dye bound to the adherent cells was solubilized with 150 μl of 33% (v/v) glacial acetic acid per well. After transferring the dye solution to a new plate, the optical density (OD) of each well was measured at 595 nm using microplate reader (Varioskan Flash, Thermo Fisher Scientific Inc., USA). Percentage of biofilm formation inhibition of SAE was calculated using the formula [1 − (OD595 sample / OD595 control)] × 100%. Concentrations of SAE exhibiting 50% inhibition (IC50) of subgingival biofilm formation in each model were determined. All experiments were repeated on three separate occasions, with triplicate determinations in each experiment.

2.5. DNA extraction

The bacterial DNAs from biofilm in 96-well microtiter plate were extracted by using InstaGene Matrix (Bio-Rad Lab., CA, USA) according to the manufacturer’s instructions. Dilutions of known amounts of reference strains DNA (10–10^8 cells) were used to determine the standard curve for real-time quantification.

2.6. Real-time PCR

Primers and probes used in this study are shown in Table 1. TaqMan probes were labeled with the reporter dye 6-carboxyfluorescein (FAM) at the 5’ end and with non-fluorescent quencher (NFQ) at the 3’ end. Amplification and detection of bacterial DNA by real-time PCR were performed using Applied Biosystem 7500 Fast Instrument (Applied Biosystems, USA). Samples were assayed in a 20 μl reaction mixture containing 2 μl of template DNA, 10 μl of 2 × TaqMan® Universal PCR Master Mix (Applied Biosystem), 1 μl of 20× assay mix containing probe, forward and reverse primers (Applied Biosystem). The cycling conditions used were as follows: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min each. All data were analyzed using the Applied Biosystem 7500 Fast software.

To determine the validity of using the universal probe and primers set to estimate the total number of bacteria in a mixed culture, three bacteria, P. gingivalis, A. actinomycetemcomitans and S. mutans, were grown separately in vitro and equal volumes of the three cultures mixed together. The number of P. gingivalis, A. actinomycetemcomitans

<table>
<thead>
<tr>
<th>Primers and Probes</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. gingivalis</td>
<td>Forward 5′-AGGATCGCTGACAGACGCATA-3′ Reverse 5′-TCGCCGGGAAGAAGTCTTCA-3′</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Probe 5′-FAM-TGCCGGGAAGAAGTCTTCA-NFQ-3′</td>
<td></td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>Forward 5′-AGCCACACAGCTGATGTAC-3′ Reverse 5′-GCTTCCACAGGTTATATTACGTT-3′</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Probe 5′-FAM-TACCCTGCTTATGTTACGTT-NFQ-3′</td>
<td></td>
</tr>
<tr>
<td>Total bacteria</td>
<td>Forward 5′-GCTTACCCCGCAGGTAGTGTC-3′</td>
<td>466</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GCTTACCCCGCAGGTAGTGTC-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe 5′-FAM-TATTCCCCGCAGGTAGTGTC-NFQ-3′</td>
<td></td>
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</tbody>
</table>
and S. mutans in CFU was determined by serial dilution on agar plates and compared with the relative bacterial load determined by real-time PCR using the universal probe and primers set (Nadkarni et al., 2002).

2.7. Statistical analyses

Kruskal–Willis analysis and Dunn’s multiple comparison tests were carried out for comparison of biofilm formation and bacterial number between the test and control groups in both models. P values <0.05 were considered statistically significant.

3. Results

The results showed that SAE exhibited more than 70% anti-biofilm activity at concentration 90 mg/ml on days 2, 4, and 8 of both models (Fig. 1). The 50% inhibition concentrations (IC50) of SAE in each model were showed in Table 2. It was clear that older biofilm showed less susceptible to SAE. However, SAE at concentration 90 mg/ml was able to kill all A. actinomycetemcomitans in subgingival biofilm on day 2, 4, and 8 of both models (Fig. 2B), while the bactericidal activity of SAE against P. gingivalis in subgingival biofilm was observed on day 4 of model 2 and day 8 of both models (Fig. 2A). On day 2 and day 4 of model 1, although lower numbers of P. gingivalis in subgingival biofilm of test groups were found, they were not significantly different compared with those of untreated control (Fig. 2A). In addition, total bacterial numbers in subgingival biofilm of test groups were not significantly different compared with those of untreated control in both models (Fig. 2C).

4. Discussion

The subgingival biofilm model used in this study as described by Walker and Sedlacek was an in vitro multi-species biofilm that closely mimics the composition of the in vivo state (Walker and Sedlacek, 2007). Therefore, this model was useful for investigating the effect of antimicrobial agents against biofilm-grown bacteria. It is well accepted that bacteria growing in a biofilm are more recalcitrant to the action of antibiotics than cells growing in a planktonic state (Hoiby et al., 2010). From our previous studies, we found that SAE possessed antibacterial activity towards periodontal pathogens growing in a planktonic state (Taweechaisupapong et al., 2002a). In addition, mouthrinses containing SAE or subgingival irrigation with SAE solution as an adjunct to scaling and root planning also have a clinically measurable effect on gingival health (Taweechaisupapong et al., 2002b, 2006a).

In the present study, we further investigated the effects of SAE on biofilm formation and biofilm-grown bacteria. Our data demonstrated that SAE possessed in vitro activity in inhibiting biofilm formation. Although adherent populations were not completely eradicated by treatment with SAE, a >70% reduction in biofilm formation was detected at SAE concentration 90 mg/ml (Fig. 1). It has been also demonstrated that SAE was able to reduce the number of A. actinomycetemcomitans and P. gingivalis in an in vitro subgingival biofilm model. These results support the antimicrobial properties of SAE against A. actinomycetemcomitans and P. gingivalis that were reported from our previous study (Taweechaisupapong et al., 2002a). However, total numbers of bacteria in subgingival biofilm of test groups of both models were not significantly different compared with those of untreated control. A probable explanation for this may be that the concentration of SAE used in the present study does not reach minimum bactericidal concentration towards other bacteria in subgingival biofilm. These findings indicated an advantage of using SAE for periodontal disease treatment since it can reduce the number of periodontal pathogens

![Fig. 1. Effects of different concentrations of Streblus asper leaf extract (SAE) on biofilm formation. The presented percentages of inhibition were calculated from the formula \[
\left[1 - \frac{OD_{sample}}{OD_{control}}\right] \times 100\%
\] Results are from 5 periodontitis patients (N = 5). The upper and lower box margins represent 75th percentile and 25th percentile, respectively. The horizontal line inside each box indicates the median (50th percentile).](image-url)

<table>
<thead>
<tr>
<th>Day</th>
<th>2</th>
<th>4 Model 1</th>
<th>4 Model 2</th>
<th>8 Model 1</th>
<th>8 Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (mg/ml)</td>
<td>8.4</td>
<td>30.3</td>
<td>33.4</td>
<td>34.3</td>
<td>31.9</td>
</tr>
<tr>
<td>95% Confidence intervals</td>
<td>7.2–9.8</td>
<td>17.5–52.5</td>
<td>22.1–50.4</td>
<td>24.5–50.0</td>
<td>5.5–186.1</td>
</tr>
</tbody>
</table>
without changing the subgingival ecology in an in vitro model. However, further in vivo (clinical) tests will be necessary to determine whether the results in vivo will be validated.

Ideally, supragingival and subgingival plaque control should prevent periodontal tissue inflammation and breakdown. However, since complete plaque removal is unrealistic, prevention may be achieved by (1) reducing the quantity of plaque below the individual’s threshold for disease or (2) changing the quality of plaque to a more tissue friendly composition (Kornman, 1986). In the present study, we found that SAE possessed in vitro activity in inhibiting biofilm formation. Moreover, qualitative changes of plaque may have occurred since a lower number of A. actinomycetemcomitans and P. gingivalis in subgingival biofilm was found in tested groups compared with those of untreated control. Therefore, it would be possible to use SAE for periodontal disease treatment as a subgingival irrigant or developing SAE as a gel for local application at periodontitis sites.

The age of the biofilm also affects its susceptibility to SAE as shown in Table 2. We found that older (4- or 8-day-old) biofilms were more resistant to SAE than were younger (2-day-old) biofilms. These results are in accordance with those reported by several other groups of investigators (Anwar et al., 1992; Amorena et al., 1999; Donlan and Costerton, 2002; Sedlacek and Walker, 2007).

In conclusion, the results obtained in this study demonstrated that SAE possesses inhibitory effects on subgingival biofilm formation and is able to inhibit P. gingivalis and A. actinomycetemcomitans in subgingival biofilm in vitro. This information, together with our previous studies which reported that SAE possessed antibacterial activity towards caries associated bacteria (Taweechaisupapong et al., 2000b), endodontic pathogens (Taweechaisupapong et al., 2000a) and interferes with the in vitro adherence of Candida to human buccal epithelial cells and acrylic surface (Taweechaisupapong et al., 2005, 2006b), indicate the multiple advantages of SAE and suggest a potential for developing SAE as a natural oral hygiene product against oral infection in people.

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References


