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Inhibitory Effects of Endophytic *Streptomyces* sp. ST8 on the Growth, Adherence and Glucosyltransferase of *Streptococcus mutans*

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Abstract: The purpose of this study is to evaluate the inhibitory effects of crude extract of endophytic *Streptomyces* sp. ST8 against *Streptococcus mutans* that is one of the major causes of dental caries and oral diseases. The extract from culture filtrate of endophytic *Streptomyces* sp. ST8 by ethyl acetate has activity against *S. mutans* ATCC25175 and 104B. The extract at such concentrations (0.05-5 mg mL⁻¹) showed the inhibition of bacterial adherence on glass surfaces and saliva-coated hydroxyapatite. The crude extract also decreased the activity of glucosyltransferase and glucan-binding lectin from both strains. The very considerable increase in cellular permeability was observed from both strains. These results suggest that the crude extract of endophytic *Streptomyces* sp. ST8 may be a new potential source for pharmaceutical products used for prevention and/or treatment of dental caries and periodontal disease.

Key words: Adherence, cellular permeability, glucan-binding lectin, glucosyltransferase, endophytic *Streptomyces*, *Streptococcus mutans*

INTRODUCTION

Streptococcus mutans have been implicated as a primary causative microorganism of dental caries and periodontal disease, which comprise the most common oral disease (Law *et al.*, 2007). Microbial colonization of tooth and mucosal surfaces and the subsequent initiation of plaque formation are dependent on the adherence of bacteria to each other and to host cells (Seminario *et al.*, 2005). Glucosyltransferase (GTF) produced by *S. mutans* catalyze glucosyl transfer from sucrose to a glucan chain and synthesis of insoluble glucan with 93% of α -(1 \rightarrow 3) linkages and 7% of α -(1 \rightarrow 6) linkages from sucrose (Tsumori and Kuramitsu, 1997). Glucans mediate the adherence of *S. mutans* and other oral bacteria flora on tooth and mucosal surfaces contribute to the formation of plaque. Hence, the inhibition of this process would result in the prevention of dental caries and periodontitis (Tarsi *et al.*, 1997). Failure of conventional periodontal therapy may be related to an incomplete elimination of periodontopathic bacteria (Mombelli, 2003). Therefore, the microbial etiology of gingivitis and periodontitis provides the rationale for use of adjunctive antimicrobial agents in the prevention and treatment of oral diseases (Trombelli and Tatakis, 2003). The active agents should prevent biofilm formation without affecting the biological equilibrium within the oral cavity (Van der Weijden *et al.*,

1998). The use of discovery of new medicine and a number of natural extracts have been investigated recently (Limsong *et al.*, 2004; Koo *et al.*, 2002; Badria and Zidan, 2004).

We have recently isolated endophytic actinomycetes from 36 plant species. Some of the isolates showed strong antibacterial and antifungal activity (Taechowisan *et al.*, 2003). A search for specific endophytes that may produce antibiotics is not necessarily a random process. The first objective is to select one or more plants as a source of the endophyte. Usually this selection process is done on the basis of the environment, the age or the natural history of a given plant. Another approach is to select a plant on the basis of its ethnobotanical uses, which was the case in this study. In Thailand, various ancient Thais used the leaf of betel (*Piper betle*) to chew with areca nut (*Aareca catechu*) and calcium hydroxide for protection of dental caries and periodontal disease. Because of the native uses of this plant, it was selected as a source of endophytic microorganisms. Interest in this endophyte was further piqued because actinomycetes have not been reported to be endophytic on the betel leaf. Although streptomycetes in general have been an outstanding source of natural products used in medicine, generally they have been isolated from soil. The finding that some *Streptomyces* sp. have taken up residence in plants opens the possibility that this may be an entirely

untapped source of novel pharmaceuticals and agents for use in agriculture. Since the endophytic actinomycetes isolated from the betel leaf have inhibitory affected on the growth of *S. mutans*, it should be considered used in antiperiodontal pathogen. The objective of this study was to investigate *in vitro* effects of crude extracts on the growth and sucrose-dependent colonization of *S. mutans* as well as on its GTF activity and production.

MATERIALS AND METHODS

Organisms and growth conditions: *S. mutans* used in this study were *S. mutans* ATCC25175 and 104B (a local Thai strain isolated from dental caries patient). The culture was grown in Tryptic Soy Broth (TSB) (Merck, Germany) at 37°C in the presence of 5% CO₂. The stock organism was stored in TSB containing 50% glycerol at -80°C. Each cell suspension was adjusted spectrophotometrically to approximately 10⁶ cfu mL⁻¹ for MIC testing by agar diffusion and 10⁴ cfu mL⁻¹ for broth dilution. For the time-kill study, *S. mutans* suspension in pre-reduced phosphate buffered saline were adjusted to approximately 1×10⁷ cfu mL⁻¹. For each strain, the total microscopic count was first established relative to optical density at a wavelength of 600 nm. To radiolabel bacteria, the cells were grown in TSB containing 10 µCi mL⁻¹ of thymidine-[methyl-³H] (Sigma Chemical Co., St. Louis, MO, USA), washed with buffered KCl (0.05 M KCl, 1 mM potassium phosphate, 1 mM CaCl₂, 0.1 mM MgCl₂, pH 6.0) and suspended in the same buffer containing 5 mg mL⁻¹ bovine serum albumin (Sigma).

Streptomyces sp. ST8 was isolated from the leave tissues of *Piper betle* during the period of May-October, 2006 by the surface-sterilization technique (Taechowisan *et al.*, 2003). Identification of the isolate to species level was based on morphological, cultural, physiological and biochemical characteristics and also 16S rDNA gene sequencing as described by Taechowisan and Lumyong (2003). Solid medium for sporulation used in this study was International *Streptomyces* Project Medium 4 (ISP-4) and the liquid medium used for fermentation was ISP-2 (Shirling and Gottlieb, 1966). The large scale fermentation and crude extraction by difference organic solvents, was carried on the methods of Taechowisan *et al.* (2005).

Inhibitory effect on growth of *S. mutans*: The inhibitory effect of the crude extracts on the growth of *S. mutans* was determined by the disk diffusion tests according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 1999). All samples were dissolved in ethyl acetate just prior to performance

of the assays. *S. mutans* (0.5 McFarland turbidity) was spread onto tryptic soy agar plate. Disks (diameter, 6 mm, Whatman, USA) were impregnated into solutions of tested materials, dried under sterile condition and placed on the inoculated agar surface. The plates were inverted and incubated for 18 h in 5% CO₂ at 37°C before the results were determined. Antimicrobial activity was recorded by measuring the diameter of the clear inhibition zones around each disk. The negative control test consisted of the same plate with solvent alone (no tested material). The positive control test consisted of the same plate with 0.1% chlorhexidine gluconate (v/v) disk. All assays were performed in triplicated.

Bacterial adherence to glass surface assay: Glass surface adherence assay was performed by means of a modification of the method described by Hamada *et al.* (1981). The bacteria were grown for 6 h at 37°C with 5% CO₂ at an angle of 30° in a glass test tube with 1 mL of TSB containing 1% (w/v) sucrose and difference concentration of crude extract. After incubation, the attached cells were removed by 0.25% trypsin (Sigma), suspended in 0.5 M phosphate buffer, pH 6.8 and quantified by reading the optical density at 550 nm.

Bacterial adherence to hydroxyapatite assay: The assay was based on the method of Gibbons *et al.* (1986) with slight modifications. Human whole saliva was collected from an adult donor and clarified by centrifugation. Five milligrams of hydroxyapatite beads (Bio-Rad Laboratories, USA) were equilibrated in 120 µL of buffered KCl at room temperature overnight. The beads were treated with 120 µL of clarified human whole saliva, rotated at 5 rpm for 1 h and then washed three times with buffered KCl. The saliva-coated hydroxyapatite (S-HA) beads were further treated for 30 min with buffered KCl containing 5 mg mL⁻¹ bovine serum albumin and then rewashed. A mixture of a suspension of 60 µL radiolabeled bacterial suspension, 30 µL of 4% sucrose solution and difference concentration of crude extract was incubated with S-HA beads for 1 h at 37°C. After washing three times with buffered KCl, the number of streptococcal cells attached to S-HA beads was determined by direct scintillation counting.

Glucan-binding lectin activity assay: GBL activity was examined by means of centrifugation-aggregation assay as described by Drake *et al.* (1988). *S. mutans* was suspended in glycine-NaOH buffer (25 mM glycine, pH 8.6) and difference concentration of crude extract was added. After incubation for min at 37°C, glucan T2000 (Sigma) was added and the suspension was

subsequently centrifuged. The bacteria were suspended in the buffer, smeared on glass slides, stained with crystal violet and examined under a microscope.

Inhibitory effect glucosyltransferase activity: *S. mutans* was grown for 24 h at 37°C in the TSB. Culture supernatant was obtained by centrifugation at 8,000 x g for 30 min at 4°C. The supernatant was concentrated by 50% saturated ammonium sulphate precipitation. After centrifugation at 14,000 x g for 30 min, the precipitates were used as the crude glucosyltransferase (GTase). 0.025 mL of GTase and 0.175 mL of crude extract samples (concentration 0.001-0.5 mg mL⁻¹) were added to 0.8 mL of 0.0625 mol L⁻¹ potassium phosphate buffer (pH 6.5) containing 12.5 µg L⁻¹ sucrose and 0.25 µg L⁻¹ sodium azide. To measure GTase activity, the reaction mixture was incubated for 24 h at 37°C, in a total volume of 1 mL. Then, the water-insoluble glucan was sedimented and washed with 3.0 mL of distilled water. The sediment suspended in 3.0 mL of distilled water was ultrasonicated for 5 sec (Ultrasonic generator US-300, Nissei, Japan). For the analysis of the glucan, the absorbance of the suspension was measured at 550 nm (UV/Visible Spectrophotometer Ultrospec 2000, Pharmacia Biotec) against the corresponding blank (Koo *et al.*, 2000).

Cellular permeability assay and scanning electron microscopic observation: Whole culture broth of *S. mutans* containing tested samples at difference concentration was incubated for 20 min at 37°C and centrifuged at 10,000 x g for 20 min at 4°C. After the supernatant was removed, the harvested cells were resuspended in 0.1 M phosphate buffer (pH 7.0) and kept for 10 min at 37°C. The absorption was determined at 260 nm (Koga *et al.*, 1996). The harvested cells were washed several times using 0.1 M phosphate buffer and fixed overnight in 2.5% glutaraldehyde at 4°C. They were then dehydrated in a graded alcohol series (30-95%) followed by air-dried in a desiccator and mounted on stubs, sputter-coated with gold and viewed by means of the scanning electron microscopy (SEM, JEOL-JSM840A SEM, Tokyo, Japan) at an accelerating voltage of 20KV. Photomicrographs were recorded on Kodak VP200 film (New York, USA).

Minimal inhibitory (MIC) and minimal bactericidal concentration (MBC) determination: The crude extract was serially diluted in sterile TSB in microtitre wells and each inoculated with 25 µL of standardised cell suspension. Wells were incubated at 37°C overnight and the highest dilution where there was no growth was recorded as the MIC. For MBC testing, aliquots (10 µL) of broth from wells containing no growth were plated onto

tryptic soy agar and again incubated overnight at 37°C. The highest dilution where there were no survivors was recorded as the MBC. In both the above methods, controls for each organism were performed using sterile PBS in place of the crude extract and purity of the cultures was confirmed by plating growth form wells.

Time-kill curve: *S. mutans* (1×10⁷ cfu mL⁻¹) were incubated in TSB with the crude extract at a dilution equivalent to their MBC. Control suspensions with buffer in place of the crude extract were incubated in parallel. At timed intervals, aliquots were removed, serially diluted and the number of survivors estimated in tryptic soy agar using the Galvin technique (Galvin *et al.*, 1999), which involves spotting 5 µL aliquots of serial dilutions onto agar plates and counting the colonies in each inoculated spot.

Data analysis: Data are reported as mean±SEM values of three independent determinations. All experiments were done at least three times. Each time with three or more independent observations. Statistical analysis was performed by students t-test. A one-way ANOVA was performed for comparison of multiple means.

RESULTS

Among the crude extracts tested, culture filtrate extracts were showed inhibitory effects against *S. mutans* ATCC25175. In mycelium extracts, any significant change in the growth inhibition activity were not observed versus the negative control, so they were excluded from further study. The extract of the culture filtrate by hexane showed weak activity (d<10 mm) against *S. mutans* ATCC25175. However, a significant growth inhibitions of *S. mutans* was found in their ethyl acetate extractions (final concentrations ranged from 90 to 500 µg mL⁻¹). They showed strong antimicrobial activities (d>15 mm) on *S. mutans* ATCC25175, similar to that of chlorhexidine gluconate (100 µg mL⁻¹) used as positive control. Chlorhexidine gluconate showed 21.35±2.50 (Table 1).

Inhibitory effect on bacterial adherence to glass surface: The adherence of both strains of *S. mutans* to glass surface was significantly reduced in a dose-dependent manner of crude extract treatments. No significant difference in adherence between the two strains was found in solvent control and no crude extract experiment (p>0.01) (Fig. 1).

Inhibitory effect on bacterial adherence to hydroxyapatite: Significant difference on adherence of both strains of *S. mutans* was observed. Comparing

Table 1: Antimicrobial activity of difference crude extracts on *Streptococcus mutans* ATCC25175

Tested materials	Concentration ($\mu\text{g mL}^{-1}$)	Inhibition zone (mm)	
		Ethyl acetate extraction	Hexane extraction
Control		0.00 \pm 0.00	0.00 \pm 0.00
Mycelium extract	90	0.00 \pm 0.00	0.00 \pm 0.00
	125	0.00 \pm 0.00	0.00 \pm 0.00
	200	<0.50*	0.00 \pm 0.00
	500	<0.50	0.00 \pm 0.00
Culture filtrate extract	90	15.70 \pm 2.34*	5.00 \pm 0.00*
	125	16.24 \pm 2.25*	5.00 \pm 0.27*
	200	19.38 \pm 1.58*	8.55 \pm 0.25*
	500	21.76 \pm 0.53*	9.80 \pm 0.32*

Results are mean \pm SD (n = 3). Control group were not exposed to the crude extracts. Chlorhexidine gluconate (100 mg mL⁻¹) used as positive control showed 21.35 \pm 2.50. *: p<0.05, significant to the control group. *: No effect: <0.50 mm

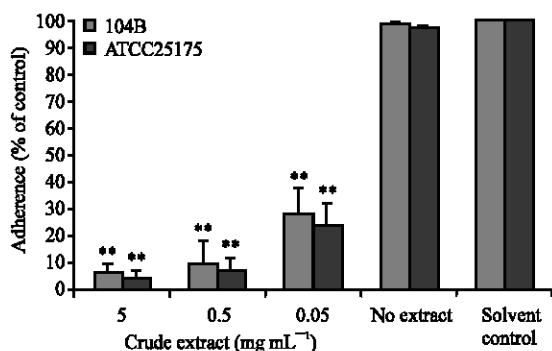


Fig. 1: Inhibitory effect of crude extract at difference concentration on adherence to glass surface of *S. mutans*. The bacteria was grown for 6 h at 37°C with 5% CO₂ at an angle of 30° in a glass tube containing TSB with (1%) sucrose and difference concentration of crude extract. The attached cells were removed by 0.25% trypsin and suspended in phosphate buffer. The quantity of attached cells was determined turbidimetrically at 550 nm. The assay was done in triplicate and the values are expressed as the mean. **: p<0.01, unpaired t-test compared to ethanol control

S. mutans ATCC25175 to 104B, it was found that crude extract showed higher inhibition on *S. mutans* ATCC25175 (p<0.05) (Fig. 2).

To determine the response trend and the lowest concentration that give at least 50% inhibition, various concentrations of the crude extracts were tested (Fig. 3). It was found that both strains of *S. mutans* responded to the crude extract in a dose-dependent manner. The lowest concentrations that could inhibit the adherence of *S. mutans* ATCC25175 and 104B at least 50% were 0.025 and 0.045 mg mL⁻¹ of crude extract, respectively.

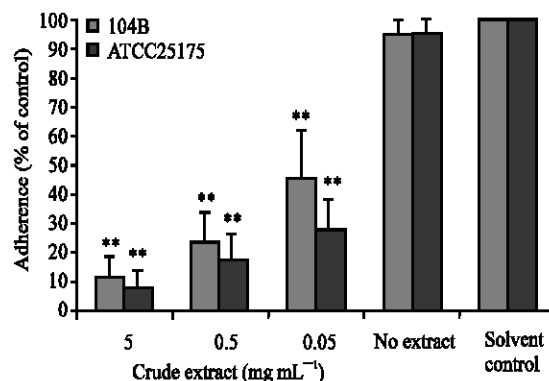


Fig. 2: Inhibitory effect of crude extract at difference concentration on adherence to S-HA of *S. mutans*. Mixtures of radiolabeled bacterial suspension, 4% sucrose solution and herbal extract were incubated with saliva-coated hydroxyapatite beads for 1 h at 37°C. The quantity of attached cells to hydroxyapatite beads was determined by direct scintillation counting. The assay was done in triplicate and the values are expressed as the mean. *: p<0.05, **: p<0.01, unpaired t-test compared to ethanol control

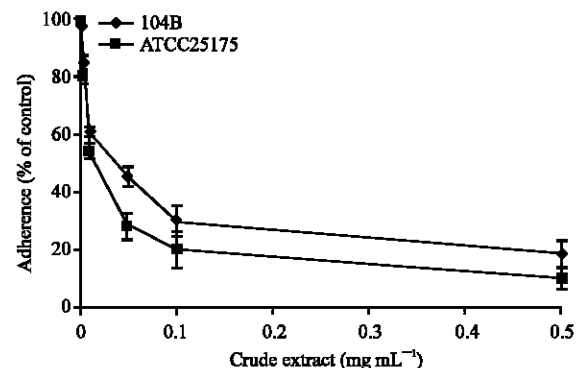


Fig. 3: Dose-response curve of crude extract on adherence to S-HA of *S. mutans*. Experiments were performed as in Fig. 2, with difference concentrations of crude extract. The assay was done in triplicate and the values are expressed as the mean

Inhibitory effect on glucan-binding lectin: In order to study the effect of the crude extract at the difference concentrations (the concentrations that could inhibit the adherence of *S. mutans* at 0.05-5 mg mL⁻¹) on GBL activity were examined. The crude extract could decrease the activity of GBL of both strains, resulting in reduced bacterial aggregation (Fig. 4, Table 2).

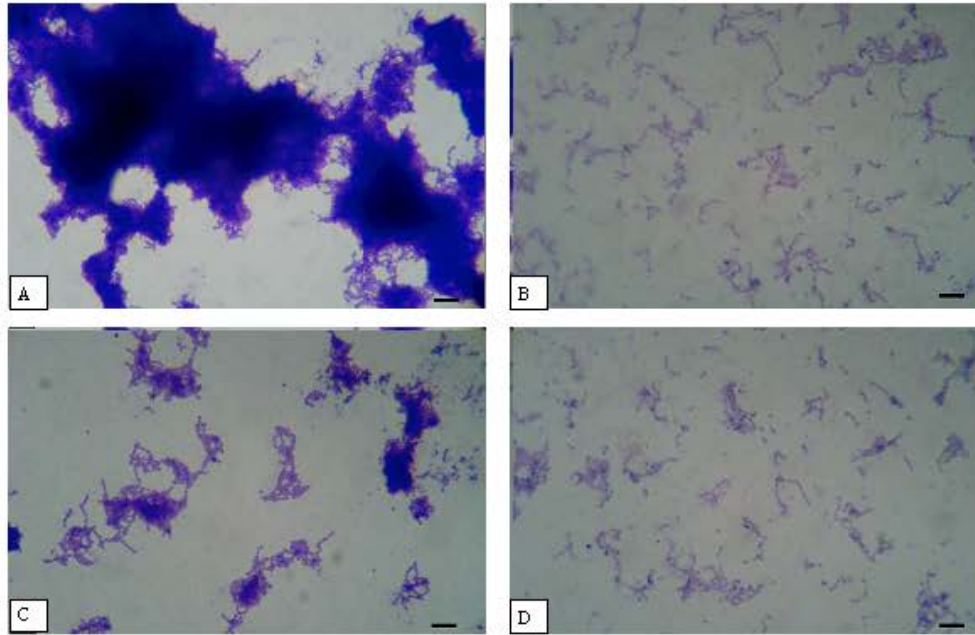


Fig. 4: GBL activity assay under microscope. Bacterial suspension was prepared in glycine-NaOH buffer. The crude extract and glukan T2000 were added. Smears were prepared on glass slides and stained with crystal violet. (A) Large bacterial aggregation of *S. mutans* ATCC25175 under effect of GBL when glukan was added (positive control group), (B) No bacterial aggregation of *S. mutans* ATCC25175 when glukan was omitted (negative control group), (C) Small bacterial aggregation of *S. mutans* ATCC25175 (test group; 0.05 mg mL⁻¹ of crude extract) and (D) No bacterial aggregation of *S. mutans* ATCC25175 (test group; 5 mg mL⁻¹ of crude extract). The assay was done in triplicate (bar = 10 µm). The GBL activity of *S. mutans* 104B gave the similar results

Table 2: Effect of various concentrations of crude extract on GBL activity

Concentration of crude extract (mg mL ⁻¹)	Bacterial cell aggregation	
	<i>S. mutans</i> ATCC25175	<i>S. mutans</i> 104B
5	-	-
0.5	+	+
0.05	+	++

++: Large bacterial cell aggregation, +: Small bacterial cell aggregation. -: No bacterial cell aggregation

Inhibitory effect on glucosyltransferase activity: The inhibitory effects of crude extract on glukan synthesis by crude GTase obtained from both strains of *S. mutans* were examined as a function of its concentrations and are shown in Fig. 5. The inhibitory effects of the samples were graphically expressed as the relative inhibition rate produced at a certain sample concentration as compared to the amount produced in the absence of any sample (the control test). All of the tested samples showed the inhibitory activity ranging from 8.5 to 96.82% at the tested concentration in a concentration-dependent manner. As showing in Fig. 5, the crude extract at a concentration of 0.5 mg mL⁻¹ suppressed glukan synthesis at 96.82 and 89.75% on *S. mutans* ATCC25175 and *S. mutans* 104B, respectively. A significant inhibition of glukan synthesis

by GTase was found at concentrations of more than 0.005 mg mL⁻¹ on both strain of *S. mutans* (p<0.05), when compared to the negative control by Student's t-test. Furthermore, they exhibited IC₅₀ values (concentration needed for 50% inhibition of glukan synthesis), which were estimated at 0.065 mg mL⁻¹ on *S. mutans* ATCC25175 and 0.07 mg mL⁻¹ on *S. mutans* 104B. However, the crude extract exhibited slightly lower effects on *S. mutans* ATCC25175 than *S. mutans* 104B.

Effect on cellular permeability of *S. mutans*: The cellular permeability of *S. mutans* exposed to the crude extract at difference concentration was established by the plot of absorbance (260 nm) of the culture versus concentration (0.1-50 mg mL⁻¹ final concentration), as illustrated in Fig. 6. All samples increased the cellular permeability of *S. mutans* significantly (p<0.05) and the effects were dependent on the concentrations in the reaction mixture. Moreover, the values of the samples treated at 50 mg mL⁻¹ concentration were comparable to that of control, about five times more. The absorbance of the control culture (not exposed to samples) was 0.382±0.014 and 0.352±0.020, for *S. mutans* ATCC25175

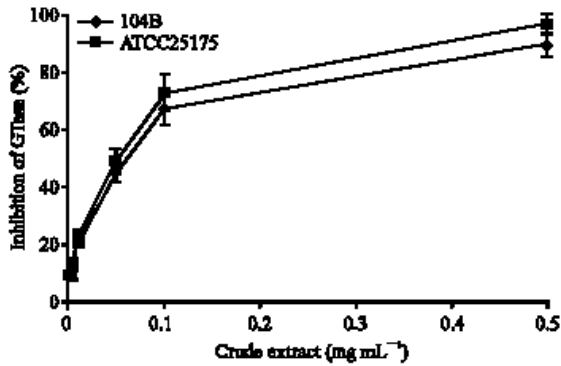


Fig. 5: Inhibitory effects of crude extract at difference concentration on glucan synthesis by crude glucosyltransferase of *S. mutans*. The mixture of GTase, crude extract and phosphate buffer containing sucrose was added and incubated for 24 h at 37°C. The precipitated glucan was collected and measured at 550 nm. The percent inhibition of glucan synthesis means the relative amount (%) of glucan produced at a certain sample concentration as compared to the amount produced in the control test. The assay was done in triplicate and the values are expressed as the mean. *: p<0.05, **: p<0.01, unpaired t-test compared to control

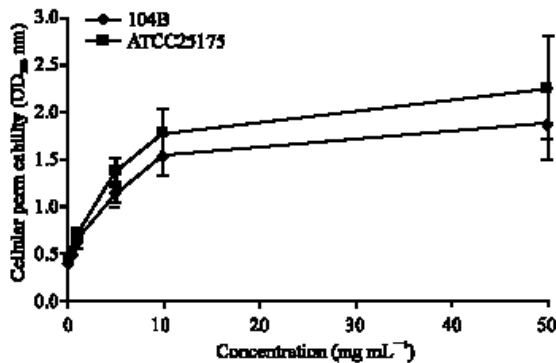


Fig. 6: Effects of crude extract at difference concentration on cellular permeability of *S. mutans*. The mixture of crude extract and *S. mutans* was added and incubated for 20 min at 37°C then centrifuged. The cells were resuspended in phosphate buffer. The cellular permeability was measured at 260 nm. The assay was done in triplicate and the values are expressed as the mean. *: p<0.05, unpaired t-test compared to control

and *S. mutans* 104B, respectively. As shown in Fig. 6, treatments with crude extract at the same concentrations

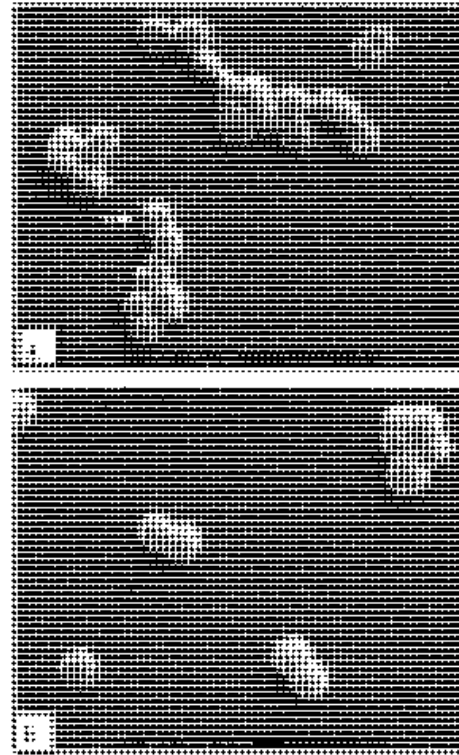


Fig. 7: Scanning electron micrographs of *S. mutans* ATCC25175 treated with crude extract at 0.5 mg mL⁻¹ final concentration (A) and control (B). The cell surface was broken when treated with the crude extract (arrowhead). The cell surface of *S. mutans* 104B gave the similar results

Table 3: Inhibitory effect (MIC and MBC) of crude extract on *Streptococcus mutans*

Strains	MIC	MBC
Crude concentration (mg mL ⁻¹)		
<i>S. mutans</i> ATCC25175	2.0	16.0
<i>S. mutans</i> 104B	4.0	16.0

Controls were cell suspensions added to broth without crude extract

exhibited stronger effect on *S. mutans* ATCC25175 than *S. mutans* 104B. However, the close absorbance value for cellular permeability of *S. mutans* ATCC25175 and *S. mutans* 104B was observed, these treatments were found to be significantly different from each other at the tested concentration (p<0.05). The highest absorbance values reached by crude extract at 50 mg mL⁻¹ final concentration-treated cultures were 2.255±0.550 and 1.882±0.384 on *S. mutans* ATCC25175 and *S. mutans* 104B, respectively.

The pathogenesis on cell surface was observed on *S. mutans* ATCC25175 treated with crude extract at 0.5 mg mL⁻¹ final concentration by scanning electron microscope (Fig. 7).

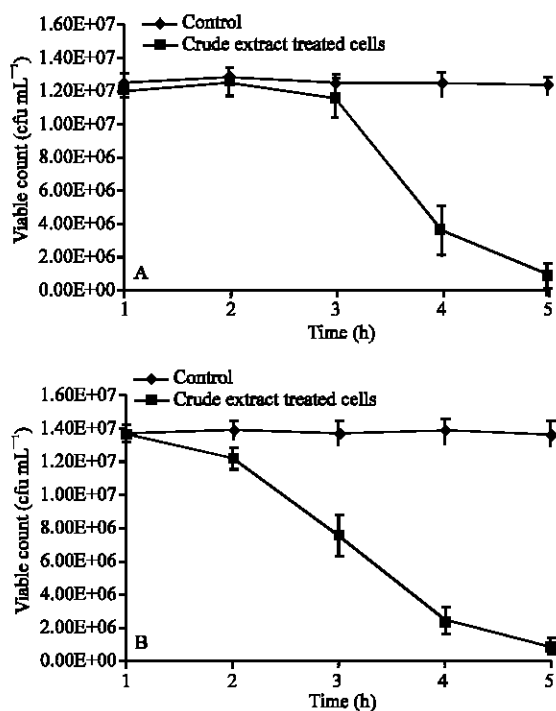


Fig. 8: Time-kill curves in the presence of crude extract. (A) *S. mutans* 104B and (B) *S. mutans* ATCC25175. Cell suspensions were exposed to crude extract at the MBC (16.0 mg mL⁻¹). Control cell suspensions lacking crude extract were incubated in parallel. Data are expressed as cfu mL⁻¹ recovered on tryptic soy agar at each time point

Effect of crude extract on growth and survival: The crude extract inhibited the growth of both strains tested and Table 3 shows the MIC and MBC values obtained, expressed in terms of the crude extract concentration.

Time-kill curve: Time kill curve was performed for both strains of *S. mutans* in the presence of crude extract at its respective MBC (Fig. 8). Killing of *S. mutans* 104B and was not apparent over the first 2 h of incubation but a greater than 2 log drop in viability occurred over the following 3-5 h. In contrast, killing of *S. mutans* 104B ATCC25175 began almost immediately. Control cell suspensions without crude extract showed no drop in viability over the same period.

DISCUSSION

Besides the purpose of prevention and treatment of dental caries and periodontitis, *Streptomyces* sp. ST8 was isolates from the leaf of betel, a preliminary screening

showed that endophytic *Streptomyces* sp. ST8 has the potential for inhibiting the growth of *S. mutans*, so endophytic *Streptomyces* sp. ST8 was selected to study the inhibitory effects on the growth, adherence and glucosyltransferase of *S. mutans*.

Based on these findings, it would be supposed that the inhibitory effect on glucan synthesis might be due to the antimicrobial activity against *S. mutans*. Therefore, the antimicrobial activity of the crude extract against *S. mutans* was assessed using the disk diffusion test. The crude extract from culture filtrate exhibited very strong inhibitory activity on the growth of *S. mutans* but the crude extract from mycelium did not showed any activity. It is apparent from this study that the secondary metabolites secreted by endophytic *Streptomyces* sp. ST8 has antimicrobial activity against *S. mutans*. Moreover, the active culture filtrate of endophytic *Streptomyces* sp. ST8 was extracted with ethyl acetate and hexane and comparisons of the antimicrobial activity of the extracts indicated that the major active metabolites were ethyl acetate-extractable.

There are several model systems for the study of streptococcal adhesion to saliva-coated surfaces. Some of the model systems include metal slabs or wires, glass, hydroxyapatite, pulverized enamel and sections of teeth (Schilling and Doyle, 1995).

The model system chosen in this study is hydroxyapatite, which is one of various forms of calcium phosphate. Hydroxyapatite seems to bind the same proteins of saliva as enamel surfaces and this form of calcium phosphate is dense and can be conveniently separated from suspended bacteria by its rapid rate of sedimentation (Schilling and Doyle, 1995) and it is simple to use. Bacterial adherence to glass surface is the model system chosen because the adherence is mediated by glucan as well as the *in vivo* situation (Law *et al.*, 2007) and the glass adherence assay is still used in some recent studies (Mattos-Graner *et al.*, 2000; Carter *et al.*, 2001; Tao and Tanzer, 2002; Limsong *et al.*, 2004). The crude extract exhibited more inhibitory effects on adherence of *S. mutans* ATCC25175 than the adherence of *S. mutans* 104B. These observations indicated that a difference in bacterial strain have an effect on response to substance or drug. It could be suggested that different strains attach to difference receptors in the pellicles, which may lead to the different patterns in response to salivary glycoproteins (Napimoga *et al.*, 2005).

Comparing the glass surface to the S-HA study, the crude extract showed less effective inhibition in the S-HA study, suggesting that the salivary glycoproteins play an important role in bacterial surface interaction, leading to the stronger adherence to S-HA than to the glass surface.

Therefore the crude extract can affect the S-HA adherence less than the glass surface one. Furthermore, the time needed in the eye-detected glass surface adherence is 6 h, so the data received is the adherence of the growing cells. In contrast, the radiolabeled bacteria method used in the S-HA adherence assay required only 1 h for detection, so the data received is the adherence of existing cells. These factors may account for the different outcome in the two assays.

Studies of the mechanism of action revealed that GTase activity was decreased by the crude extract in dose-dependent manner, suggesting that the crude extract could interfere in glucan synthesis. Considering the large effect of the crude extract on S-HA adherence and GTase activity from *S. mutans*, it may be indicative that adherence inhibition by the crude extract was mainly a result of GTase inhibition. Most of the work done to date on identifying inhibitors of GTase, as well as this study, has focused mainly on enzymatic activity in solution, not enzyme adsorbed to the pellicle on the tooth surface. Recent data showed that the pellicle-adsorbed enzyme has more resistance to some agents than that in solution (Wunder and Bowen, 1999). It may be possible that the GTase mechanism in bound form may differ from another.

Apart from GTase activity assay, another mechanism of action tested in this study was GBL activity. Although the role of aggregation in cariogenicity has been less emphasized than the sucrose-induced adherence, it nevertheless contributes to the accumulation of oral streptococci on tooth surfaces (Law *et al.*, 2007). The results revealed that the crude extract could decrease the activity of GBL of both strains. Many plant extract for example *Juglandaceae regia* (Jagtap and Karkera, 2000), *Andrographis paniculata* (Limsong *et al.*, 2004), has been reported to prevent glucan-induced aggregation in *S. mutans* and another study indicated that approximately 1.5 mM fluoride was required for a 50% reduction in GBL activity (Luengpailin *et al.*, 2000).

Promotion of the cellular permeability is another antimicrobial factor of *S. mutans*. Most bacteria have a delicate cell membrane, which contains a cytoplasm enclosed within the membrane. The cell membrane is selectively permeable and capable of transporting nutrients inward and wastes outward. Therefore, the effect of crude extract on the cellular permeability of *S. mutans* was evaluated and at the tested concentration caused significant increase of the cellular permeability leading to the leakage of cytosolic components. This study confirmed by the pathogenesis on cell surface of *S. mutans* which observed by scanning electron microscope. Furthermore, this result is agreement with data of GTase inhibition. Collectively, these findings

suggested that the crude extract of endophytic *Streptomyces* sp. ST8 could inhibit adherence of *S. mutans* via at least three mechanisms, i.e., GTase, GBL activity inhibition and cellular permeability.

These biological activities such as antimicrobial, GTase inhibitory activities of endophytic *Streptomyces* sp. ST8 and observed in the present study support its traditional use. In conclusion, our results suggest that the crude extract of endophytic *Streptomyces* sp. ST8 could be successfully incorporated into pharmaceutical products employed in prevention and/or treatment of dental caries and periodontal disease.

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