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Effect of *Piper betle* L. Leaf Extract on the Virulence Activity of *Streptococcus mutans*-An *in vitro* Study

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Abstract: In this study the effect of crude aqueous extract of the leaves of *Piper betle* L. on the virulence properties of *Streptococcus mutans* ATCC 25175 was investigated. It was carried out based on the effect of the extract towards growth, cell surface hydrophobicity, adhering property and glucosyltransferase activity of the *S. mutans*. The concentration of crude aqueous extract of *Piper betle* L. used in the experiments above was between 0 to 20 mg mL⁻¹. Chlorhexidine (0.12%) and sterile deionised water was used as positive and blank control, respectively. The results obtained showed that the crude extract at a concentration as low as 2.5 mg mL⁻¹ exhibited reduced effect towards the growth (p<0.01), adhering ability (p<0.01), glucosyltransferase activity (p<0.05) and cell surface hydrophobicity (p<0.05) of *S. mutans* when compared with the blank control. This implies that the *Piper betle* L. extract may have anti-virulence property towards *S. mutans*.

Key words: *Streptococcus mutans*, *Piper betle* L., adherence, hydrophobicity, glucosyltransferase

INTRODUCTION

Streptococcus mutans, the secondary colonizer of dental plaque is generally regarded as one of the causative factors in the aetiology of dental caries (Loesche, 1986; Marsh and Bradshaw, 1995; Bowen, 2002). Its virulence activity may be determined by the ability to adhere to the oral surface, synthesize sticky glucan matrix and survive in the thick plaque environment.

In the development of dental caries three distinct processes may be involved. Firstly, the adherence of the mutans *streptococci* to the tooth surface. The adherence of bacteria to glass surfaces involved hydrophobic bonding and ionic interactions (Satou *et al.*, 1988) and cell-surface hydrophobicity is implicated in the process (Weiss *et al.*, 1982; Doyle *et al.*, 1990). Secondly, the formation of sticky glucan by the action of the bacterial enzyme glucosyltransferase using carbohydrates like sucrose as substrates and thirdly, the accumulation of biofilm (dental plaque) which create a suitable environment for continuous production of acid by the bacteria. The acid produced will then dissolve the hard tissues of the surfaces of teeth (Hamilton-Miller, 2001) and subsequently lead to caries formation.

There are many plants that have been reported to possess antibacterial activity towards oral bacteria (Nakahara *et al.*, 1993; Matsumoto *et al.*, 1999; Nostro *et al.*, 2004). Among them is *Piper betle* L. which

in folklore medicine has been used for oral hygiene (Ponglux *et al.*, 1987) and to control bad breath (Ong and Nordiana, 1999). It has been reported that it exhibits antibacterial activity towards *Streptococcus mitis*, *Streptococcus sanguis* and *Actinomyces viscosus*, some of the early colonisers of dental plaque (Razak and Rahim, 2003) and inhibits the adherence of these bacteria to saliva-coated glass surfaces (Razak and Rahim, 2003). The development of dental plaque may be influenced by factors that affect the growth of these early colonizers and their adherence to tooth surfaces. Hence the anti-growth and anti-adherence effect of *Piper betle* L. may implicate its potential anti-plaque property. This may also indicate potential anti-caries effect.

S. mutans is a cariogenic bacteria because it possesses caries inducing properties which include the ability to adhere and colonize on smooth surfaces and subsequently will lead to the development of dental plaque and in the presence of carbohydrates it will facilitate the development of dental caries (Mukasa and Slade, 1973; Loesche, 1986; Marsh, 1994; Marsh and Bradshaw, 1995). To date, there is little or no information on the effect *Piper betle* L. extract on the biological properties of *S. mutans*. Hence, the objective of this *in vitro* study was: i) to investigate the effect of the extract on the growth of *S. mutans*, ii) to determine the effect of the extract on the adhering property of *S. mutans* to smooth glass surface and iii) to examine the influence

of the extract on the cell surface hydrophobicity and glucosyltransferase activity of *S. mutans* which are said to be responsible for the adhering activity.

MATERIALS AND METHODS

Plant material: *Piper betle* L. leaves were obtained from one source in Mentakab, Pahang.

Preparation of bacterial suspension: The *Streptococcus mutans* ATCC 25175 was obtained commercially from American Type Culture Collection, USA. The stock of these bacteria was kept in glycerol at -70°C for further use. To revive the microorganism, the stock was thawed and dispersed in 30 mL Brain Heart Infusion (BHI) broth (OXOID) and incubated at 37°C for 18-20 h. To ensure that pure culture was used in the study, the revived bacteria were cultured on BHI agar plates containing 5% blood. This is done regularly to ensure the purity of the stock culture. The concentration of the bacteria in the suspension used in the study was adjusted using a spectrophotometer ($\text{OD}_{550\text{ nm}}$) to about 0.144.

Preparation of the crude aqueous extract of *Piper betle* L. by decoction: Fresh *Piper betle* L. leaves were cleaned, cut to small pieces and weighed and put in large conical flasks. 10% (w/v) of the leaves were put to boil in deionised distilled water and allowed to concentrate to about 90% (w/v). The leaves were sieved out and the crude aqueous extract obtained was filtered. One milliliter aliquots of the crude aqueous extract were dried overnight using the speed vacuum concentrator (Heto Lab Equipment). The dried pellets of the crude aqueous extract samples were kept refrigerated at -80°C until further use. The pellets were then weighed. Before use the pellets were dissolved and diluted to the required concentrations with deionised distilled water.

Determination of the *in vitro* effect on cell growth, cell adherence and cell surface hydrophobicity

Cell growth: Sterile crude aqueous *Piper betle* L. extract was prepared to the respective final concentrations of 2.5, 5, 10 mg mL^{-1} and dispensed into test tubes (13×150 mm) containing 20 mL of BHI broth. The seed culture (100 μL) of mutans *streptococci* was inoculated into the broth medium. Growth was measured periodically every hour for 6 h and at the 12th h by measuring the optical density of the culture at 550 nm using a UV160A spectrophotometer (Shidmadzu, Kyoto, Japan). Each assay was done in triplicates and repeated three times.

The influence of the extract on the rate of cellular growth of *S. mutans* was determined by calculating the mean doubling time in the presence and absence of crude aqueous *Piper betle* L. extract between 0.2 and 0.40 of

$\text{OD}_{550\text{ nm}}$. For blank control, the experiment was repeated devoid of the extract, using sterile deionised distilled water. For positive control, chlorhexidine gluconate (0.12%) was used in the place of the extract.

Cell adherence: Cell adherence was determined based on a method developed by Ooshima *et al.* (2000). Mutans *streptococci* were grown for 18 h at 37°C at an angle of 30° in a test tube with 3 mL of BHI broth containing 1% (w/v) sucrose and *Piper betle* L. extract (0-10 mg mL^{-1} ; final concentration). The percentage of adherent cells was determined turbidimetrically. The experiment was repeated using sterile deionised distilled water (blank control) and using chlorhexidine gluconate (0.12%) (positive control) in the place of the extract.

Cell hydrophobicity: Cell surface hydrophobicity was determined based on slight modification of the method described by Rosenberg *et al.* (1980). This method measured the adherence of bacteria to hexadecane. In this study, *S. mutans* revived as described above were cultured in 300 mL of BHI broth and resuspended in phosphate, urea and magnesium buffer to an optical density of 1.2 at 550 nm. Following that, aliquots of 1.5 mL of the bacterial suspension was mixed with an equal volume of crude aqueous *Piper betle* L. extract of different concentrations (0-10 mg mL^{-1} ; final concentration) in a test tube (13×100 mm). The mixture was allowed to stand for 10 min at room temperature before measuring the optical density at 550 nm. This will represent the initial optical density of the bacterial suspension before agitation (BS_i). Subsequently 400 μL of n-hexadecane (ICN Biomedicals) was added. The suspension was agitated uniformly on a vortex mixture for 1 min. After allowing 15 min for the hexadecane phase completely separate from the aqueous phase, the optical density of the bacterial suspension in the aqueous phase was measured at 550 nm (BS_f). The hydrophobicity was expressed as a percentage of the initial optical density of the bacterial suspension in the aqueous phase. To determine the effect of the extract on hydrophobicity, the experiment was repeated using sterile deionised distilled water (blank control) and chlorhexidine gluconate (0.12%) (positive control) in the place of the extract. Each experiment was carried out in triplicates and repeated three times.

Percentage of

hydrophobic cells = $\frac{[\text{Total number of cells in aqueous phase before agitation } (\text{BS}_i) - \text{Total number of cells in aqueous phase after agitation}] (\text{BS}_f)}{\text{Total number of cells in aqueous phase before agitation } (\text{BS}_i)} \times 100\%$

Preparation of the crude cell associated glucosyltransferase: *S. mutans* ATCC 25175 was grown in 1 L of BHI broth for 18 h and collected by centrifugation and washed three times with 10 mM potassium phosphate buffer, pH 6.0. The whole cell was treated with 8 M urea at 25°C for 1 h and the enzyme was then salted out with 60% saturated ammonium sulfate (MERCK). The salted solution was centrifuged at 19000 g. The precipitate collected was reconstituted in the phosphate buffer and then dialysed against 10 mM sodium phosphate (BDH) buffer (pH 6.0) and was referred as cell associated glucosyltransferase. The enzyme was stored at -20°C until further use (Ooshima *et al.*, 2000).

Enzyme assays: The glucan-synthesizing activities of the urea free cell associated glucosyl transferase were estimated by the modification of the colorimetric assay described by Fukushima *et al.* (1981). The activity of the enzyme was determined from the amount of glucan formed in 1 min. The amount of glucan formed is represented by the amount of glucose measured.

Analysis of the water insoluble glucan: The precipitate (water insoluble glucan) was washed three times with 50% ethanol. The washed polysaccharides were dissolved in 0.5N NaOH (MERCK) and the glucose content determined by phenol-sulphuric acid method (Dubois *et al.*, 1956). From the glucose content determined, the enzyme activities were calculated and expressed as μmol glucose/min. The concentration of crude aqueous *Piper betle* L. extract used was between 0-10 mg mL⁻¹. Sterile deionised distilled water was used as blank control and chlorhexidine gluconate (0.12%) was used as positive control.

Statistical analysis: Each experiment was carried out in triplicates and repeated three times. Thus the data in the present study was computed using means and standard deviations from nine determinations. Inter group difference was estimated by statistical analysis of variance (ANOVA) for factorial model.

RESULTS

Effect on the growth, adhering property, cell surface hydrophobicity and glucosyltransferase activity: It was shown that the growth rate of *S. mutans* was reduced by the presence of crude aqueous extract of the leaves of *Piper betle* L. (Fig. 1). The effect is concentration dependent. The doubling time of *S. mutans* in the absence of the extract (control) was 83 min (Table 1)

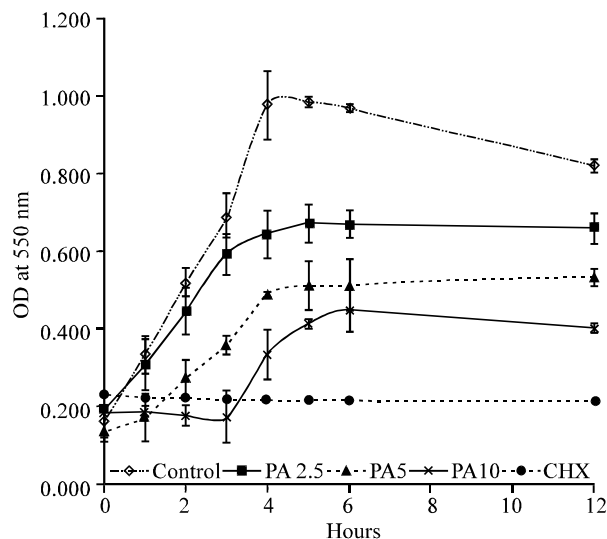


Fig. 1: Effect of different concentration of crude aqueous *Piper betle* L. extract on the growth of *S. mutans*. PA2.5-Crude aqueous *Piper betle* L. extract 2.5 mg mL⁻¹, PA5-Crude aqueous *Piper betle* L. extract 5 mg mL⁻¹, PA10-Crude aqueous *Piper betle* L. extract 10 mg mL⁻¹, CHX-Chlorhexidine 0.12% (v/v) (p<0.01) except at 0 h; intergroup differences were estimated by ANOVA). The values are expressed mean±SD from nine determinations

Table 1: Effect of crude aqueous *Piper betle* L. extract on the growth rate of mutans *Streptococci*

Concentration of crude aqueous <i>Piper betle</i> L. extract (mg mL ⁻¹)	Doubling time (min) ^a
0 (Negative Control)	83
2.5	105
5	203
10	285
Chlorhexidine 0.12% (Positive control)	0

^a Growth rate calculated by measuring mean doubling time (min) in the presence or absence of crude aqueous *Piper betle* L. extract between 0.2 and 0.4 of OD 550 nm

while in the presence of 2.5, 5 and 10 mg mL⁻¹ extract, the doubling time of *S. mutans* was 105, 203 and 285 min, respectively (p<0.01).

Extract of *Piper betle* L. inhibited the adherence of the *S. mutans* to smooth glass surface (Fig. 2). In the presence of 2 mg extract, the adherence was 67.2% of the control. Further reduction in the adherence was observed with increase in the concentration (p<0.01). It demonstrated maximum inhibitory effect (98.20%) at 10 mg mL⁻¹.

The cell surface hydrophobicity of *S. mutans* was affected by the presence of the extract. In the absence of the extract the cell surface hydrophobicity was 62.6%

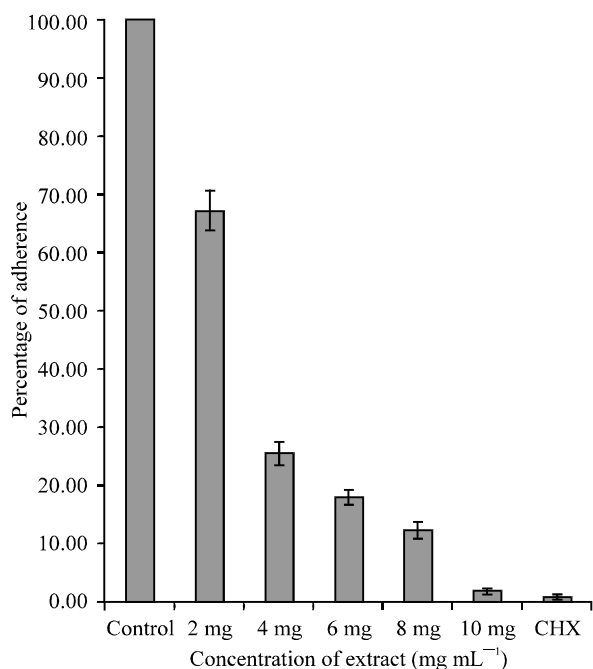


Fig. 2: Effect of different concentrations of crude aqueous *Piper betle* L. extract (2-10 mg mL⁻¹) on cell adhesion of mutans *Streptococci* to a smooth glass surface. Control-sterile deionised water; CHX-Chlorhexidine 0.12% (p<0.01; intergroup differences were estimated by ANOVA). The values are expressed as mean±SD from nine determinations

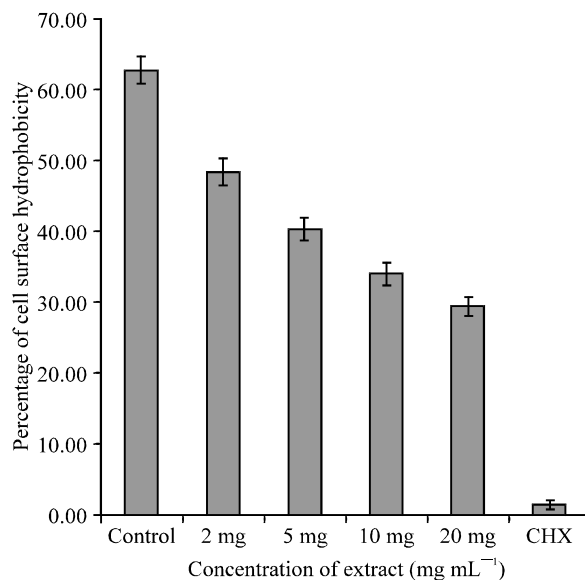


Fig. 3: Inhibitory effect of different concentrations of crude aqueous *Piper betle* L. extract (2.5-20 mg mL⁻¹) on cell surface hydrophobicity of *S. mutans*. Hydrophobicity was expressed as percentage of the initial absorbance of the aqueous suspension. Control-Sterile deionised water; CHX-Chlorhexidine 0.12% (p<0.05; Intergroup differences were estimated by ANOVA). The values are expressed as mean±SD from nine determinations

(Fig. 3). It was reduced by 22.6 and 52.9% in the presence of 2 mg mL⁻¹ and 20 mg mL⁻¹ extract, respectively (p<0.05).

The extract also inhibited the activity of the cell associated glucosyltransferase (Fig. 4). The activity was reduced by 18% in the presence of 2 mg mL⁻¹ extract. Further reduction in the activity was observed as the concentration was increased (p<0.05).

DISCUSSION

The inhibitory effect shown by the crude *Piper betle* L. extract on the growth of *S. mutans* may result in the reduction of its colony in dental plaque. The extract also reduced the adherence of bacteria to smooth glass surface. This therefore will affect the development of dental plaque. The cell adhering property of bacteria are influenced by cell surface hydrophobicity and the ability to form water insoluble glucans.

Cell surface hydrophobicity is one of the important initial factors for oral bacteria to adhere to tooth surface

(Weiss *et al.*, 1982; Doyle *et al.*, 1990). It has been shown that mutant strains of mutans *streptococci* which lost their cell surface hydrophobicity could not adhere to saliva-coated hydroxyapatite beads and these strains showed less colonizing ability compared to the common parent strains found in the oral cavity (Westergren and Olsson, 1983). It has been reported that the cell surface hydrophobicity is mainly attributed to the cell surface protein of the bacteria (McBride *et al.*, 1984). The observed reduction in the cell surface hydrophobicity of *S. mutans* in this study could be due to alteration of the cell surface protein of the bacteria by the presence of the extract. Nostro *et al.* (2004) has reported similar finding with the ethanolic extract of *Helichrysum italicum*. The decrease in cell surface hydrophobicity of *S. mutans* by the presence of the *Piper betle* L. extract as observed in the present study may reduce its adhering ability to the tooth surface. This will subsequently lead to a reduction in the population of cariogenic bacteria in the developing plaque.

Besides cell surface hydrophobicity, the water insoluble glucan formed in the plaque matrix may facilitate

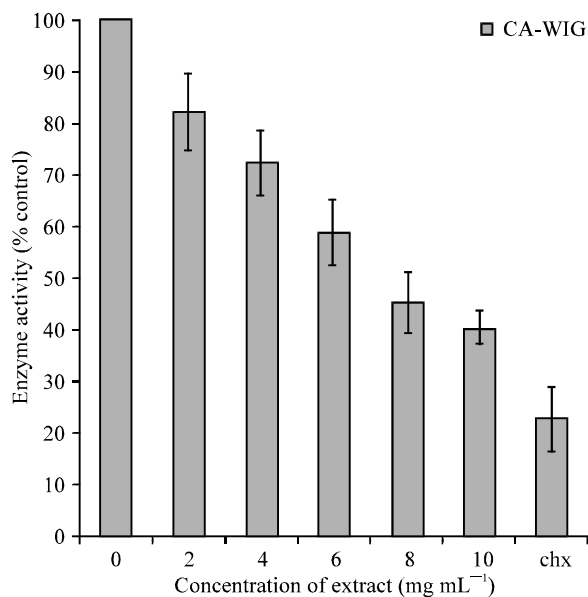


Fig. 4: Inhibitory effect of crude aqueous *Piper betle* L. extracts at different concentrations (0-10 mg mL⁻¹) on cell associated (CA) glucosyltransferase activity of *S. mutans* compared to control containing sterile deionised water. ($p < 0.05$; t-test assuming unequal variance compared to control containing sterile deionised water). The values are expressed as mean \pm SD from nine determinations. WIG-water insoluble glucan

the adherence and colonization of microbes in the plaque (Frostell *et al.*, 1967; Hamada and Slade, 1980). The synthesis of water-insoluble glucan is catalysed by glucosyltransferase and the presence of sucrose will enhance dental plaque formation by promoting the adherence and accumulation of large number of *S. mutans* and *S. sobrinus* on the teeth of animals and humans (Frostell *et al.*, 1967; Hamada and Slade, 1980). Water insoluble glucan is one of the main virulence factors for the caries induction in human and animals (Gibbons and van Houte, 1975; Slade, 1976; Hamada and Slade, 1980). There are at least two distinct types of glucosyltransferase (Gtf); Gtf-I, which produces water-insoluble glucan and Gtf-S, which produces water-soluble glucan (Hamada and Slade, 1980; Koo and Park, 1997). In this study, it was shown that the crude aqueous extract of *Piper betle* L. inhibits the synthesis of water insoluble glucan through the inhibition of GTF-I activity.

The inhibition of cellular growth and formation of water insoluble glucan may contribute to the reduction of sucrose-dependent adherence of *S. mutans* to glass surfaces. The multiple inhibitory effect of crude aqueous

extract of *Piper betle* L. on the virulence activity of *S. mutans* may affect the process of dental plaque formation and indirectly may prevent the development of dental caries.

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