

The Crude Aqueous Extract of *Piper betle* L. and its Antibacterial Effect Towards *Streptococcus mutans*

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Abstract: In this study, the antimicrobial influence of crude aqueous extract of *Piper betle* L. on *Streptococcus mutans* (*S. mutans*) was investigated. The focus of the antimicrobial effects includes the ultrastructure and acid producing properties of *S. mutans*. Transmission electron microscopy (TEM) was used to determine the effect of the extract on the ultrastructure of *S. mutans*. Analysis of the effect on the acid producing properties was analyzed by pH drop assay. The investigation was further carried out to determine the possible chemical components of the extract using thin layer chromatography (TLC), bioautography and gas chromatography mass spectrometry (GCMS). From the micrographs of the transmission electron, it was found that the crude extract of *Piper betle* L. leaves causes plasma cell membrane damage and coagulation of the nucleoid. The extract was found to significantly reduce acid producing properties of the bacteria. Chemical analysis of the extract showed that hydroxychavicol, fatty acids (stearic and palmitic) and hydroxy fatty acid esters (stearic, palmitic and myristic) as the main components. It was suggested from the results obtained that the crude extract of *Piper betle* L. leaves may exert anticariogenic activities that are related to decrease in acid production and changes to the ultrastructure of *S. mutans*. Further study will be carried out to determine if the effect observed is attributed to the presence of hydroxychavicol, fatty acids and hydroxy fatty acid esters in the extract.

Key words: *S. mutans*, *Piper betle* L., antimicrobial, bioautography, acid production

INTRODUCTION

In the South East Asia region, *Piper betle* L. (sireh) are among the plants that have been associated with the control of caries and periodontal diseases^[1] and to the control of bad breath^[2]. Fathilah *et al.*^[3] have reported that the crude aqueous extract of *Piper betle* L. leaves exhibits antibacterial activity towards *Streptococcus mitis*, *Streptococcus sanguis* and *Actinomyces viscosus*, some of the early colonizers of dental plaque.

The microbial etiology of caries involves oral pathogens and ecological shifts in dental plaque in response to increased sugar challenge or decreased salivary flow in the oral cavity^[4]. The bacteria that are most closely associated with caries development are the mutans streptococci, which are known to possess high acidogenic and aciduric properties, together with their ability to synthesize extracellular glucans from sucrose catalysed by GTFs. Their virulence is related directly to their capacity to adhere and produce acids from glycolysis in dental plaque at pH values of 4.0 or even lower^[5]. There are many ways of chemotherapeutic avenues other than eliminating *S. mutans* selectively; this includes reduction of glucan formation by GTFs

and reduction in the ability of the organism to produce acids.

Most of the studies on plant extracts^[6-9] have demonstrated their antibacterial activities towards *S. mutans*. The antibacterial activities may be attributed to reduced growth, reduced adhering ability and reduced glucosyl transferase activity. The adherence of the early colonizers prepares the environment conducive for the colonization of the secondary colonizers like *Strep. mutans*^[4]. Razak and Rahim^[10] have reported that the aqueous extract of *Piper betle* L. inhibits adherence of early plaque settlers, which include *Streptococcus mitis*, *Streptococcus sanguinis* and *Actinomyces* sp. to saliva-coated glass surfaces. In this way, extract of *Piper betle* L. leaves indirectly inhibits the adherence of *S. mutans* by making the environment not conducive for *S. mutans* to adhere. The extract also exhibited potent inhibitory properties against the growth of *S. mutans* and activity of glucosyltransferase (GTF)^[11]. The inhibition of glucosyl transferase will affect the formation of glucan which subsequently will make the environment less conducive for the growth of *S. mutans*.

Thus, it has been shown that the crude extract of *Piper betle* L. leaves exhibits antibacterial activity

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towards *S. mutans* by affecting the adhering property, growth and formation of glucan^[11]. In this study, the objective was to investigate the effect of the extract on ultrastructure and acid production of *S. mutans*. In addition, the chemical components of the extract were also determined.

MATERIALS AND METHODS

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

Preparation of bacterial suspension: The *S. mutans* ATCC 25175 was obtained commercially from the American Type Culture Collection, USA. The stock of these bacteria was kept in glycerol at -70 °C for further use. The bacterial suspension was prepared according to the method previously described by Nalina and Rahim^[11].

Preparation of crude aqueous extract of *Piper betle* L. by decoction: The crude aqueous extract was prepared according to the procedure that have been reported^[11].

Analysis of acid production: Sterile crude aqueous *Piper betle* L. extract (final concentration of 1, 2, 5, 10 mg mL⁻¹) was added to 13 x 150mm tubes containing 20ml of BHI broth and 1% glucose. 100µl of the seed culture of mutans streptococci was then inoculated into the broth medium. During the incubation at 37°C, 1 mL of the culture was periodically scooped up and its pH was measured with a pH meter (Model pH 213, Hanna Instruments) according to the method described by Ooshima *et al.*^[12].

Cell ultrastructure study: Prior to the transmission electron microscopy (TEM) study, the bacteria culture was prepared according to the procedure described below.

Bacterial cultures and growth: *Streptococcus mutans* ATCC 25175 primary cultures were grown in brain heart infusion broth to the late log phase of growth (optical density of 1.20 at 550nm). Cell pellets were washed three times in ice-cold distilled water and suspended in broth to an optical density of 0.144 at 550 nm immediately before assays were performed.

Treatment of the bacteria with the plant extract: Cell suspension (10 mL) was preincubated at 37°C for 1h and then treated with the respective 1 mg mL⁻¹ and 2 mg mL⁻¹ of crude *P. betle* L. leaves extract. The

mixture (final volume; 100 mL) was left to incubate for 2 hrs. Mouthrinse containing chlorhexidine (0.12%) and sterile deionised water was used as positive and negative control, respectively. After the 2 hrs incubation, the treated bacterial cells were precipitated by centrifugation and processed for electron microscopy.

Transmission electron microscopy (TEM) study: The control and the *P. betle* L. leaves extract treated cells were fixed in 4% glutaraldehyde and later post-fixed in 1% osmium tetroxide (0.1M cacodylate buffer, pH 7.4) at room temperature. After eliminating the remaining osmium tetroxide, the samples were dehydrated in a graduated cold ethanol series (35 to 100%). Each step was performed for about 10 to 15 mins at room temperature. The fixed cells were embedded with Epon and the small blocks of bacteria were cut with an ultramicrotome (Ultracut, Reichert). The ultra thin sections were then analyzed using TEM (Libra 120, Leo).

Analysis of the chemical components of the extract: The crude aqueous extract of *Piper betle* L (10mg) was analyzed by TLC on Silica Gel G plates (20 x 10 cm) (Macherey-Nagel) using solvent system chloroform (Mallinckrodt) – methanol (Fisher Scientific) (90:10). Three sets of TLC plates (A, B, C) were prepared. Plate A, the reference chromatogram was used to determine the spots as visualized by UV irradiation (254 and 366nm), plate B was used for bioautography and plate C was used to identify spots with phenolic compound. 25% Folin Ciocalteu phenol reagent (Sigma Chemical Co.) was used to identify spots with phenolic compound. To plate C, tannic acid (Sigma Chemical Co.) was added and used as reference for phenolic compound. R_f values of the spots separated on the TLC plates were determined. The R_f value for a substance is the ratio of the distance that the substance travels to the distance that the solvent travels up the plate.

Bioautography: Chromatogram developed as described above was placed in a sterile bioassay petri dish (NUNC) and an inoculum of *Strep. mutans* containing 10⁶ CFU mL⁻¹ in molten BHI agar (OXOID) was distributed over the plates. After solidification of the medium, the sterile bioassay petri dish with the TLC plate was incubated at 37 °C for 24 hrs. The R_f of the inhibition zones on plate B was compared with the R_f of reference chromatogram (plate A). The corresponding spots that showed antibacterial activity were scraped and collected and used in the gas chromatography-mass spectrometry study.

Analysis of the chemical components of the extract by Gas chromatography-mass spectrometry (GCMS):

The scrapings of spots with the antibacterial activity were then dissolved in ethanol and centrifuged to remove silica. The supernatant collected were then filtered with a 0.45 μm filter and evaporated to dryness. The trimethylsilyl (TMS) derivatives of the extract were prepared using *N, O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA, Aldrich, St Louis, MO). About 3mg of the extract was derivatized in 300 μL of BSTFA by heating for 60min at 60°C and analyzed using GCMS. The analyses were obtained in gas chromatography (Agilent GC 5973N) equipment with mass selective detector (Agilent G2589A) in the electron impact ionization mode, capillary column SGE BPX5 (30m x 0.25mm x 0.25 μm). The instrumentation procedure was according to the method described by Uzel *et al.*^[13].

The percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area. The GCMS peaks were identified by comparison with several data reported by other researchers^[14-16] and the profiles from the Wiley 7n and Nist 98 libraries.

Statistical analysis: Each experiment was carried out in triplicates and repeated three times. Thus the data in this study was computed and expressed as mean \pm standard deviation from nine determinations. Inter group difference was estimated by statistical analysis of variance (ANOVA) for factorial model.

RESULTS AND DISCUSSION

Figure 1 shows the profile of the effect of crude aqueous extract of *Piper betle* L. towards *S. mutans*. 1mg mL⁻¹ of the extract reduced the rate of acid production to 93.53%. Increasing the concentration further to 2, 5 and 10 mg mL⁻¹ reduced the rate of acid production to 88.20, 40.51 and 23.56% respectively.

Figures 2A to 2F are the electron micrographs showing cytological effects of the *Piper betle* L. leaves extract towards *S. mutans*. Figure 2A is the electron micrograph of the blank control and it was observed that the cells have well-defined outer cell and dense

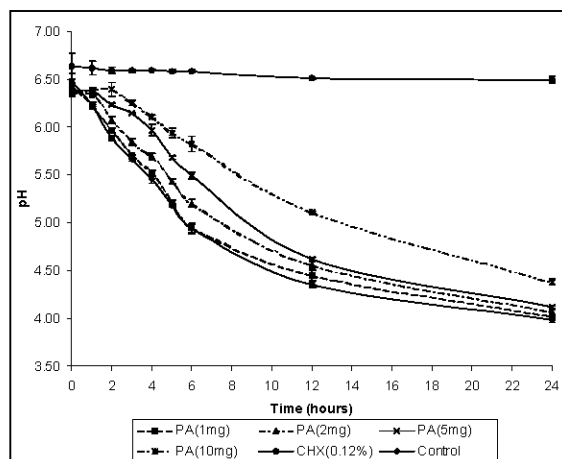


Fig. 1: Effect of different concentrations of crude aqueous extract Piper betle L. leaves on the acid production of *S. mutans*. PA(1mg) - Crude aqueous Piper betle L. extract 1mg mL⁻¹, PA(2mg) - Crude aqueous Piper betle L. extract 2mg mL⁻¹, PA(5mg) - Crude aqueous Piper betle L. leaves extract 5mg mL⁻¹, PA(10mg) - Crude aqueous Piper betle L. leaves extract 10mg mL⁻¹, Control - Sterile deionised water indicated by the lowest graph; CHX - Chlorhexidine 0.12% (v/v) indicated by the top most graph, (p<0.01 except at 0 hour; Intergroup differences were estimated by ANOVA). The pH values are expressed as the mean \pm standard deviation from nine determinations.

inner cell walls and intact plasma cell membranes. Figure 2B shows the electron micrograph of *S. mutans* treated with mouth-rinse containing chlorhexidine (0.12%). This serves as a positive control. It was observed that the nucleoid and plasma cell membrane disintegrate and the cytoplasm leaches out from the cells while the cell remains intact. This indicates that lysis is occurring at an early stage.

It was observed that upon exposure to the crude extract of the *Piper betle* L. leaves, the ultrastructure of *S. mutans* was affected (Figures. 2C-2F). The effect of the treatment with 1 mg mL⁻¹ of the extract is shown in Figures 2C and 2D and that of 2 mg mL⁻¹ is shown in Figures 2 E and F. All of the micrographs showed cells with nucleoid material coagulated into thick electron-dense filaments and destruction of the plasma cell membrane and inner cell wall. The effect is more significant with the higher concentration of the extract.

Figure 3 shows the chemical components of the crude extract of *Piper betle* L. leaves as separated using TLC. Plate A is the chromatogram showing separation of the components on TLC. The components of the extract separated into one big spot and two or more smaller spots. Plate B is the chromatogram treated with bacterial inoculum to determine the spots with antibacterial zone. Of the number of spots observed, the bigger spot exhibited the antibacterial activity with

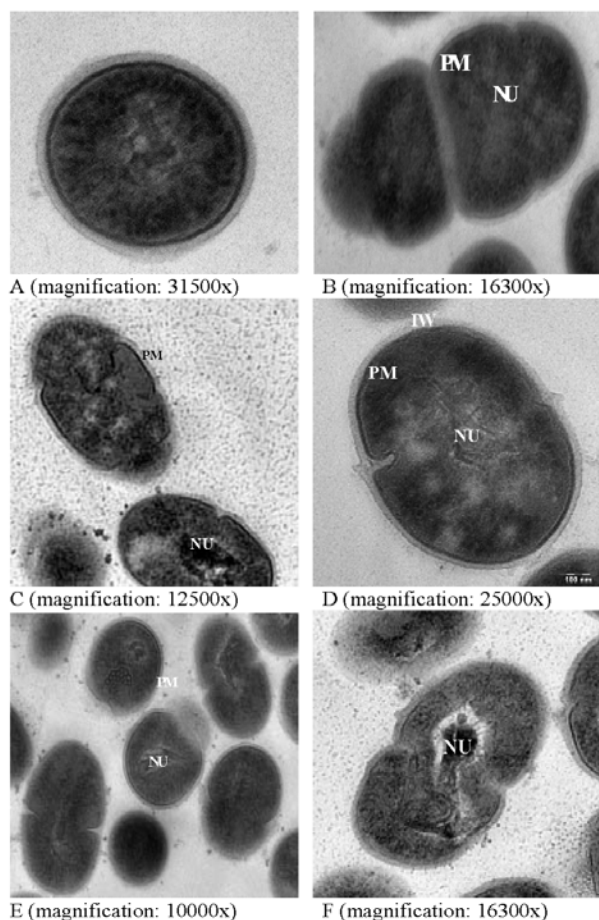


Fig. 2: Cytopathological effects of *Piper betle* L. leaves extract on *S. mutans*. A shows control (treated with sterile deionised water) and B is cells treated with mouthrinse containing chlorhexidine (0.12%). C and D shows electron micrograph of *S. mutans* treated with crude *P. betle* L. leaves extract (1mg mL⁻¹) and E and F shows *S. mutans* treated with crude *P. betle* L. leaves extract (2mg mL⁻¹). NU – nucleoid, PM-plasma cell membrane, IW - inner cell wall, OW - outer cell wall

diameter of the inhibition zone measuring 30 ± 0.86 mm (mean \pm Standard deviation from nine determinations). Plate C is the chromatogram sprayed with 25% Folin-Ciocalteu phenol reagent to locate the spots with phenolic compound. All of the spots stained positive for phenolic component. The bigger spot which has an R_f values between 0.82 to 0.91 corresponds to the inhibition zone on the plate B. No inhibition zone was observed corresponding to the spots with the R_f values below 0.82.

The chromatogram of GCMS of the *Piper betle* L. leaves extract is shown in Fig. 4 and the relative percentage of the identified compounds is summarized in Table 1. It was found that the main constituents of

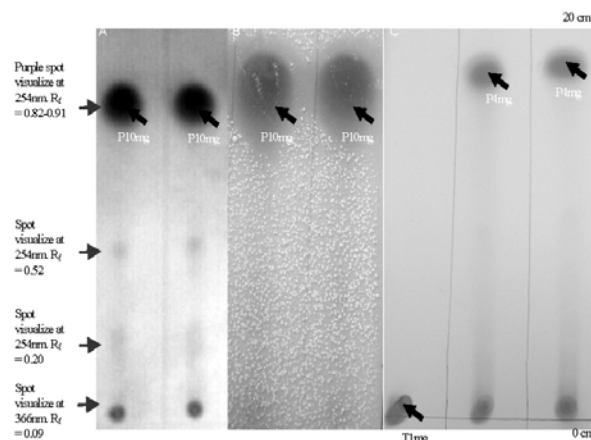


Fig. 3: Thin layer chromatography plates were run in triplicates and one set was visualized as above (plate A), the other set was used for bioautography with *Streptococcus mutans* (plate B) as described in the methodology and the third set (plate C) was sprayed with 25% Folin Ciocalteu phenol reagent to identify spots with phenolic compound. Arrow indicates antibacterial activity at R_f values between 0.82 to 0.91. Spots were visualized by UV irradiation (254 and 366nm). T1 – Tannic acid (1mg/ml) as control for phenolic content, P4mg - Crude aqueous *Piper betle* L. leaves extract 4 mg mL⁻¹, P10mg – Crude aqueous *Piper betle* L. extract 10mg mL⁻¹

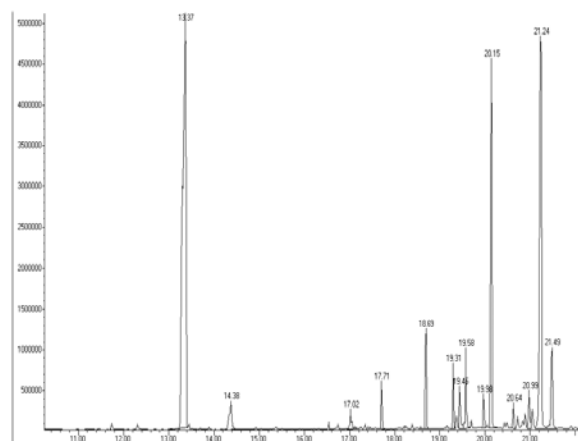


Fig. 4: Chromatogram of *Piper betle* L. leaves extract

the extract correspond to hydroxychavicol (39.31%), fatty acids [stearic (3.77%) and palmitic (1.60%)], hydroxybenzeneacetic acid (3.96%) and hydroxy esters of fatty acids [stearic (24.49%), palmitic (14.71%) and myristic (1.58%)].

Natural products are in great demand due to its extensive biological properties and providing source for the discovery of many types of effective bioactive compounds. *Piper betle* L. has been recognized for their many pharmacological activities such as its antioxidant [17,18] and antibacterial properties [3,6-9,11,15].

Table 1: GCMS data (EI mode) for TMS derivatives of some components in *Piper betle* L. leaves extract.

No.	Compounds	TMS groups	Ions selected (m/z)	Retention time (min)	Area (%)
1	Hydroxychavicol*	2	294(M ⁺), 205, 73	13.37	39.31
2	Benzenecetic acid, alpha-hydroxyphenyl	2	284(M ⁺), 269, 240, 73	17.02	0.95
3	Hexadecanoic acid	1	328(M ⁺), 313, 145, 117, 73	17.71	1.60
4	Octadecanoic acid	1	356(M ⁺), 341, 201, 145, 117, 73	18.69	3.77
5	Myristic acid,2,3-bis(hydroxy)propyl ester	2	446(M ⁺),431, 343, 211, 147, 103, 73	19.31	1.58
6	Hexadecanamide	0	255(M ⁺), 86, 59	19.46	1.46
7	n.d.			19.98	1.06
8	Hexadecanoic acid,2,3-bis(hydroxy)propyl ester	2	474(M ⁺), 459, 371, 313, 239, 147, 103, 73	20.15	14.71
9	n.d.			20.64	0.88
10	2-Monopalmitin	2	474(M ⁺), 313, 218, 191, 129, 103, 73	20.99	1.56
11	Octadecanoic acid,2,3-bis(hydroxy)propyl ester	2	502(M ⁺), 487, 399, 341, 267, 205, 147, 103, 73	21.24	24.49
12	Benzenecetic acid, alpha-hydroxy	2	296(M ⁺), 281, 179, 105, 73	21.49	3.96

Hexadecanoic acid – palmitic acid

Octadecanoic acid – stearic acid

Hexadecanoic acid,2,3-bis(hydroxy)propyl ester - palmitic acid,2,3-bis(hydroxy)propyl ester

Octadecanoic acid,2,3-bis(hydroxy)propyl ester – stearic acid,2,3-bis(hydroxy)propyl ester

* values obtained by calculation of mass.

n.d. – not determined

It was found that the extract of *Piper betle* L. leaves contains fatty acids (stearic acid and palmitic acid) and hydroxy fatty acid esters (hydroxy esters of stearic, palmitic and myristic acids) and hydroxychavicol, with the latter as main component. This is in accordance with what have been reported^[14-16]. The hydroxychavicol is said to exhibit antibacterial activity^[19]. Fatty acids can act as anionic surfactants and have antibacterial and antifungal properties at low pH^[20], in addition to being selective against Gram-positive organisms^[21] by targeting the structure and function of bacterial cell walls and membranes. These components may be the contributing factors to the effect demonstrated by the crude extract of *Piper betle* L., reducing the acid producing activity and affecting the ultrastructure of *S. mutans*.

The inhibition of acid production is related to the energy producing process or glycolysis. It was found that the reducing effect on acid production is comparable to the reduction in its growth rate, in the presence of the extract at similar concentrations^[11]. The fatty acids present in the extract could act at the glycolytic enzymes which may be one of the attributing factors to its antibacterial activity as have been reported^[22-25].

The effect of the crude extract on the ultrastructure of *S. mutans* observed in this study could be due to the fatty acids and hydroxy fatty acid ester components present. The hydrophobic parts of the compound may enable them to partition the lipids of the bacterial cell

membrane, thereby disturbing the structures and rendering them more permeable. When the membrane is more permeable, other components present in the extract could make its way into the bacterium and coagulate the nucleoid while maintaining the cell intact. However, further studies need to be carried out to ascertain the mechanism involved in the biological activities of these compounds.

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