

THE EFFECT OF *PSIDIUM GUAJAVA* AND *PIPER BETLE* EXTRACTS ON THE MORPHOLOGY OF DENTAL PLAQUE BACTERIA

A.R. Fathilah¹, M. Yusoff², Z.H.A. Rahim³

ABSTRACT

Objective: It has been reported that the aqueous extracts of *Psidium guajava* and *Piper betle* leaves showed anti-plaque activities during the early stages of dental plaque formation. The aim of the study was to elucidate if such anti-plaque activities involve any ultra-structural changes to the morphology of three early dental plaque bacteria, *Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces* sp.

Methodology: Pure cultures of the bacteria were suspended in BHI medium and treated with the test herbal extracts at the sub-lethal concentrations. The growth mixtures were incubated at 37°C. At the logarithmic growth phase (t_1), aliquots of 1 ml of the growth mixtures were fixed and used in the preparation of specimens for SEM studies. Ultra-structural alterations to the morphology of the treated cells noted were compared to those of the cells cultured under untreated conditions.

Results: Following exposure of the bacteria to the two test herbal extracts, profound ultra-structural changes to their morphology were observed. The observed structural or morphological alterations could attribute to the bacteria being less active in performing normal physiological metabolic functions and thus rendering them less efficient to multiply. The changes noted included (i) reduced sizes of the bacteria, and (ii) majority cells at the non-dividing state as compared to those cultured under controlled conditions.

Conclusions: This study has shown anti-plaque effects of aqueous extract of both *P. betle* and *P. guajava*.

KEY WORDS: *Psidium guajava*, *Piper betle*, Bacterial ultra-structural alteration, Anti-plaque activity.

Pak J Med Sci October - December 2009 (Part-II) Vol. 25 No. 6 928-933

How to cite this article:

Fathilah AR, Yusoff M, Rahim ZHA. The Effect of *Psidium guajava* and *Piper betle* extracts on the Morphology of Dental Plaque Bacteria. Pak J Med Sci 2009;25(6):928-933.

Correspondence:

Dr. Fathilah Abdul Razak, PhD,
Associate Professor,
Department of Oral Biology,
Faculty of Dentistry,
University of Malaya,
50603 Kuala Lumpur,
Malaysia.
E-mail: fathilah@um.edu.my

- * Received for Publication: May 23, 2009
- * Revision Received: September 19, 2009
- * Accepted: September 23, 2009

INTRODUCTION

Plaque control measures have become the basic principle in the prevention of plaque-related oral diseases such as caries, gingivitis and periodontitis.¹ Dental plaque becomes thick and unhealthy when its microbial population multiplies and produces extra-cellular polysaccharides that contribute to the increase in its matrix.² An effective plaque control strategy is to target at ways to disrupt the accumulation of plaque on the tooth surface favouring the

formation of thin and loosely bound plaque which are more readily dislodged.

It has been reported that the extracts of *Psidium guajava* and *Piper betle* plants exhibit therapeutic properties and are popularly used in the formulation of traditional medicines.³⁻⁵ Aqueous extract of the leaves of both the plants have been well studied for their anti-microbial, anti-plaque and anti-caries activities. Their use has been recommended with possible potential for further development and incorporation as active agents in oral health care products.⁶⁻⁹

Bacteria cells undergo several morphological changes during its growth cycle. The largest cell size corresponds closely to the period when rapid division is taking place during the exponential growth phase. At the lag and approaching the death phases, cells tend to be smaller in size.¹⁰ Attaining optimal cell size is important for a bacterium to be able to perform normal metabolic function to grow and flourish.¹¹ Alterations in cell morphology may render the cell unable to multiply and hence to remain bacteriostatic. In this study, the direct effect of *P. guajava* and *P. betle* extracts on the ultra-structure of selected plaque bacteria was assessed using the scanning electron microscope (SEM). Results obtained may help elucidate the mechanism of action of *P. guajava* and *P. betle* extracts as anti-plaque agents.

METHODOLOGY

Preparation of plant extracts: Aqueous extracts of *P. betle* and *P. guajava* were prepared by boiling small pieces of the fresh leaves of the plants in distilled water for several hours until the final volume was one third of the initial volume. Following this, the decoction was centrifuged at 10,000 rpm to eliminate sediments. The supernatant was then divided, into one ml aliquots, in micro-fuge tubes. They were then concentrated using a speed-vacuum concentrator (HETO/HS-1-110, Denmark). The concentrated extracts were weighed into sterile micro-fuge vials and prepared into stocks of 20 mg/ml using sterile distilled water as diluent.⁶

Preparation of bacterial suspensions: *Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces* sp.

were representatives of dental plaque bacteria used in this investigation. Pure test bacterial cultures were obtained from frozen (-80 °C) stocks isolated from dental plaque specimens collected from volunteers visiting the Dental Clinic at the Faculty of Dentistry, University of Malaya. Identification of the bacterial isolates was done using conventional microbiological methods and the API Identification System (BioMereux, France). Each bacterial species was revived in Brain Heart Infusion (BHI, Oxoid) broth at 37 °C overnight. Following incubation, bacterial cells were harvested by centrifugation at 10,000 rpm for 10 minutes. The cells were then re-suspended in BHI broth and the concentration was standardised at 10⁸ cells / ml [optical density (OD) of 0.144] by using a spectrophotometer read at 550 nm.

Determination of the mid logarithmic phase (t₁): 100 ml of BHI broth in separate sterile conical flasks were seeded with 100 µl of *S. sanguinis*, *S. mitis* and *Actinomyces* sp. suspensions and allowed to grow in a shaking water bath at 37 °C. The changes in the OD of each bacterial culture were periodically monitored and recorded at every 15 minutes intervals over a period of 9 hours. A growth curve of time versus OD was then plotted for each bacteria and the time at which the mid of the logarithmic growth phase (t₁) was reached were determined.

Effect of P. betle and P. guajava extracts on bacterial morphology: Similar culturing procedure was repeated but the bacteria were instead allowed to grown under three different conditions: (a) with addition of *P. betle* extract, (b) in addition of *P. guajava* extract, and (c) with addition of chlorhexidine (CHX)-containing mouth-rinse. The concentration of extracts in (a) and (b) was standardised to a sub-lethal concentration of 4 mg / ml to allow for minimal cell growth. The sub-lethal concentration refers to a concentration that falls between the minimal inhibition and minimal bactericidal concentrations (MIC and MBC) of the extracts.¹² The concentration of CHX-containing mouth-rinse in the test was set at 0.12 % which is the concentration of CHX used in many commercially available mouth-rinses such as Oral B™. This was to represent a

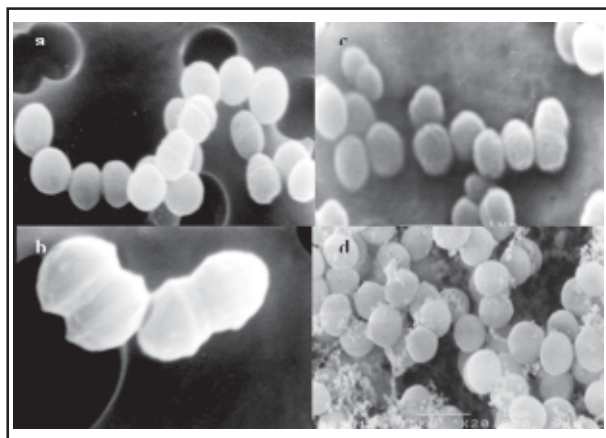


Fig-1: SEM micrograph of *S. sanguinis*. (a) Majority of the cells cultured under the untreated condition were at the dividing state (90,000x). (b) Higher magnification of dividing cells showing the groove where the cell would split into two identical cells (200,000x). (c) Cells cultured following exposure to *P. betle* extract were of variable sizes and majority existed as single, non-dividing cells (90,000x). (d) Under the influence of *P. guajava*, the size of the cells were more uniform and were clumped together in extracellular matrices (90,000x).

positive control for the study as CHX is considered the standard anti-microbial agent in the dental and hospital arena.¹³ All tests were carried out in triplicate and repeated three times for reproducibility of results.

The culture flasks were placed in a shaking water bath and the bacteria were allowed to grow at 37° C. When the t_1 was reached, the flasks were removed and 100 μ l of bacteria cells suspension were removed and captured on Nucleopore™ membrane discs (pore size 1 μ m). The membrane was then carefully picked and transferred into glass vials containing one μ l of glutaraldehyde (4%). The following morning, the membrane was then washed by displacing the fixative using a Pasteur pipette and replacing it with one ml of sodium cocadylate buffer (0.1 M). The washed membrane was then fixed in osmium tetra-oxide (1%) and left overnight in tightly capped vials at 4° C. The osmium tetra-oxide was then gently displaced with sodium cocadylate buffer and later washed three times by gently displacing the buffer with fresh buffer each time. The membrane discs were then treated with ascending percentage

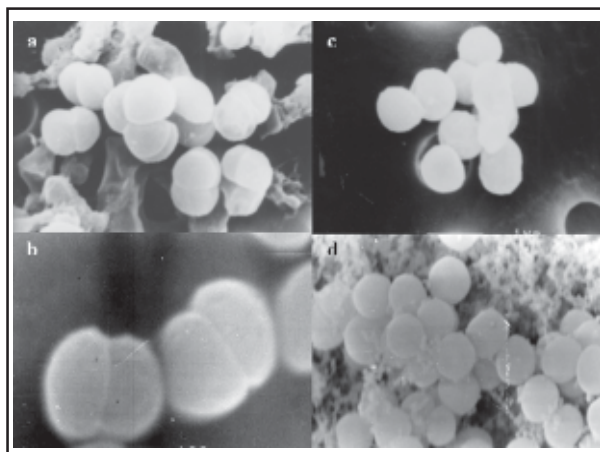


Fig-2: SEM micrographs of *S. mitis*. (a) Majority of the cells cultured under the untreated growth condition were in the dividing state (90,000x). (b) The dividing cells at higher magnification (200,000x). (c) Under the influence of *P. betle*, cell were observed to be of uniform sizes and existed as single non-dividing cells (90,000x). (d) Cells cultured in the presence of *P. guajava* were also of uniform sizes & aggregated together in heavy extracellular matrices (90,000x).

(10% to 100%) of ethanol (15 minutes each) to dehydrate the cells. Finally the discs were mounted onto aluminium stubs and coated with gold before it was ready for SEM (JOEL, Japan) studies.

RESULTS

Figures-1, 2 and 3 represented SEM micrographs of *S. sanguinis*, *S. mitis* and *Actinomyces* sp., respectively at the mid of their logarithmic growth phases. Ultra-structural observations of bacterial cells were summarised and tabulated in Table I. In the absence of the extracts, the cells of *S. sanguinis* (Fig-1a & b), *S. mitis* (Fig-2a & b) and *Actinomyces* sp. (Fig-3a & b) were observed to have attained optimum cell sizes of (0.98 x 0.80) μ m to (1.00 x 0.77) μ m, (0.64 x 0.56) μ m to (0.76 x 0.72) mm and (2.66 x 0.89) mm to (14.93 x 0.87) μ m, respectively. Most of these cells were also shown to be at the actively dividing state.

Comparatively however, in the presence of the plant extracts the bacteria cells were observed to be smaller in size and many have resumed the non-dividing state (Fig-1c & d, 2c & d and 3c & d) (Table-I). In addition to that, the number of bacteria cells was observed to be

denser in the presence of *P. guajava* as compared to cells in the presence of *P. betle* (Fig-1c & d and 2c & d). Heavy mesh-like matrices were observed to form around the cells in the presence of *P. guajava* that tend to aggregate them together in large clumps (Fig-1d, 2d & 3d). The growth of *S. sanguinis*, *S. mitis* and *Actinomyces* sp. was found to be totally inhibited in the presence of CHX and none was captured on the nudeogore membrane. The growth medium appeared clear, an indication of the bactericidal effect of the CHX-containing mouth-rinse. This was to be expected as CHX has been accepted by many researchers to exhibit maximum antimicrobial effect on oral micro-organisms.¹⁴

DISCUSSION

For cells to perpetuate themselves they must grow, that is increase in mass and size in an orderly fashion before they can reproduce or divide.¹⁵ With respect to the oral environment, once bacteria are attached to the tooth surface they will continue to perform their normal biological functions and reproduce by binary fission so that the population will increase geometrically.^{11,16} In addition to biochemical nutrients that are needed to provide energy to support cell growth, it is also equally important that appropriate biophysical environment is provided to ensure that the cells can propagate normally at a specific growth rate within a specific generation-time. Many factors such as temperature, pH and nutrients have been known to play important role to ensure the completion of a growth cycle.¹¹ Studies have shown that the physiological growth of a bacterial cell is often accompanied by changes in its morphological characteristics. *Campylobacter jejuni* for example was shown to exhibit gross morphological changes with time when grown in a liquid medium. The cells were the typical short spirals at the exponential phase, became twice the length at the mid-stationary phase, but eventually were observed as coccus at the end of the growth cycle.¹⁷ Such changes in cell morphology during a growth cycle were suggested to be attributed to the differential expression of genes that control the timing of cell division.

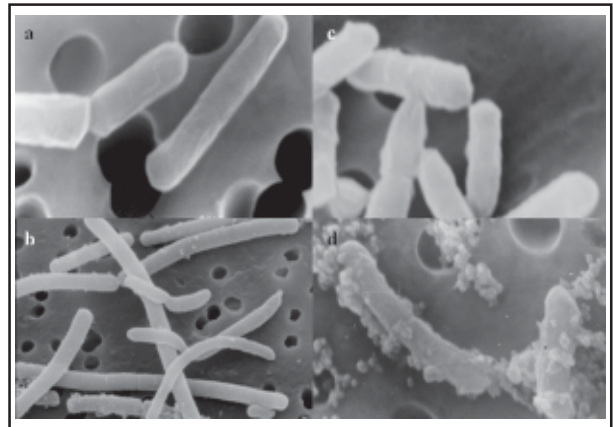


Fig-3: SEM micrographs of *Actinomyces* sp. (a) Cells resumed rod shape with smooth surface under the untreated growth condition (90,000x). (b) The varying length resumed by the cells under the untreated condition was obvious at lower magnification (30,000x). (c) Cells cultured under the influence of *P. betle* display tapering ends and irregular surfaces (90,000x). (d) In the presence of *P. guajava* extracts, the cells were observed to be covered in extracellular matrices (90,000x).

Based on the examination of the micrographs, the treatment of *S. sanguinis*, *S. mitis* and *Actinomyces* sp. with either *P. betle* or *P. guajava* extracts have in a way suppressed the morphological development of the bacteria cells. The extracts appeared to have slowed down cell growth as most cells at t_1 were small and have not attained the optimal cell size as observed in the absence of the herbal extracts (Table-I). The introduction of the extracts into the growth environment has created an unfavourable condition for the bacteria to grow. Therefore longer time was required for the bacteria to adapt and synthesize new inducible enzymes that are necessary to metabolise the substrates introduced into the growth medium. This was an indication of the disruption of the normal physiological activities of the cells and hence, accounted for the extension of lag phases of the growth cycles. The retarding effect of these plant extracts on the growth profiles of dental plaque bacteria has been reported.^{18,19}

In the immature condition, the underdeveloped bacterial cells may not be able to perform their biological function efficiently and as such, metabolic energy becomes insufficient to allow

Table I. Ultra-structural features of *S. sanguinis*, *S. mitis* and *Actinomyces* sp. cells under ideal and test herbal extract-treated growth environments. Observations were based on SEM examinations.

Bacteria	Morphological features of cells cultured under normal physiological condition	Morphological features of cells grown under the influence of plant extract	
		<i>P. betle</i>	<i>P. guajava</i>
<i>S. sanguinis</i>	<i>Fig. 1a:</i> Cocco-bacilli of (0.80x0.98) to (1.00x0.77) μ m individual & in chain. Many cells were at the dividing stage; (0.90x0.73) to (1.17 0.58) μ m (<i>Fig. 1b</i>). <i>Inference</i> <i>Cells attained optimum size.</i>	<i>Fig. 1c:</i> Cocco-bacilli of (0.64x0.44) to (0.91 x0.62) μ m. Individual & in chain. Most cells were at the non-dividing stage. <i>Cells sizes were relatively smaller and elongated. Cell population was greatly reduced.</i>	<i>Fig. 1d:</i> Cocco-bacilli of (0.64x0.56) to (0.69 x0.58) μ m. Cells were aggregated & embedded in extra-cellular matrix. <i>Cells sizes were more uniform & relatively smaller. Dense extra-cellular matrix was observed to have aggregated the cells together.</i>
<i>S. mitis</i>	<i>Fig. 2a:</i> Cocco-bacilli of (0.64x0.56) to (0.76x0.72) μ m. Cells existed as individual & in chain. Most cells were at the dividing stage; (0.68x0.61) to (0.78x0.58) μ m (<i>Fig. 2b</i>). <i>Inference</i> <i>Cells attained optimum sizes & were relatively smaller than S. sanguinis.</i>	<i>Fig. 2c:</i> Cocco-bacilli of (0.77x0.68) μ m. Cells existed as individual & in chain. Most cells were at the non-dividing stage. <i>Cell population was greatly reduced but cell sizes were maintained at optimum.</i>	<i>Fig. 2d:</i> Cocco-bacilli of (0.65x0.61) to (0.70 x0.65) μ m. Cells were aggregated & embedded in extra-cellular matrix. <i>Cells maintain optimum sizes. Dense extra-cellular matrix was observed to aggregate the cells together.</i>
<i>Actinomyces</i> sp.	<i>Fig. 3a:</i> Rods of various lengths; (2.66x0.89) to (14.93x0.87) μ m. Cells existed as individual short & long rods/ filaments. Some cells were at the dividing stage. <i>Inference</i> <i>Cells attained various lengths under optimum condition (Fig. 3b).</i>	<i>Fig. 3c:</i> Uniform short rods of (1.61x0.52) to (1.67x0.48) μ m. Cells were elongated & tapered at the end. Cells were at the non-dividing stage. <i>Cells' lengths were more homogenous & slimmer with greatly reduced width. Cells population was reduced greatly.</i>	<i>Fig 3d:</i> Rods of (1.46x0.46) to (1.9x0.48) μ m. Cells were surrounded in extracellular matrix. Cells lengths were not uniform but they were relatively slim with greatly reduced width. Dense extra-cellular matrix clumped the cells together.

for growth activities such as cell division and multiplication.²⁰ The formation of heavy matrix with the introduction of *P. guajava* in the growth environment also poses an aggregative effect to the bacterial cells. In the aggregated form, the

growth performance of bacteria cells may also be affected.

With respect to the dental plaque, the suppression of bacterial growth may slow down the process of plaque formation and minimize the

accumulation of plaque on the tooth surface. Thin plaque is healthy as it is loosely bound to the tooth surface and can easily be dislodged by tooth brushing.²¹ Thus, the incorporation of either *P. guajava* or *P. betle* extract in a mouth-rinse formulation may be considered for use in oral plaque control. Such attempt however, requires further investigation to ensure that the herbal preparations would not incur any adverse effect on the mucosal cells when exposed to the oral surfaces. Although preliminary studies have shown that both extracts showed no toxic effect on *Artemia salina* (unpublished result), investigating the responses of the mucosal cells to the extracts using tissue culture technique is necessary to ensure the safe use of the extracts before it can be developed into commercial oral products. In addition to that, *in vivo* trials of the products on study groups would also be relevant. This would avoid any biases that may arise due to the limitation of *in vitro* toxicity testing.

CONCLUSION

The aqueous extracts of *P. betle* and *P. guajava* were found to have profound effect on the ultrastructure of selected dental plaque bacteria. The extracts appeared to have interfered with the normal growth cycle and development of the bacterial cells rendering them less efficient to divide and multiply. Hence, the development of dental plaque may be slowed down and controlled. This property indicates the antiplaque activity of *P. betle* and *P. guajava* extracts.

ACKNOWLEDGEMENT

This research was financially supported by the IRPA 09-02-03-0197-EA197 Grant from the Malaysian Government and Vote F0392/2004B from the University of Malaya.

REFERENCES

- Scheie AA. Mechanisms of dental plaque formation. *Adv Dent Res* 1994;8:246-53.
- Samanarayake LP. *Essential Microbiology for Dentistry*. Churchill Livingstone 2006.
- Hobert I, Tietze HW. *Guava as Medicine: A Safe and Cheap Form of Food Therapy*. Pelanduk Publications, Selangor, Malaysia 1998.
- Indu BJ, Ng LT. *Herbs: The green pharmacy of Malaysia*, Malaysian Book Publishers Association, CT Book Makers Sdn Bhd, Malaysia 2000.
- Ponglux D, Wong S, Phadungcharoen T, Ruangrungrsi N, Likhitwitayawuid K. *Medicinal Plants*. Victory Power Point Corp Ltd, Bangkok, Thailand 1987.
- Fathilah AR, Rahim ZHA. The anti-adherence effect of *Piper betle* and *Psidium guajava* extracts on the adhesion of early settlers in dental plaque to saliva-coated glass surfaces. *J Oral Sci* 2003;45(4):201-06.
- Fathilah AR, Othman Y, Rahim ZHA. The effect of *Piper betle* and *Psidium guajava* extracts on the cell-surface hydrophobicity of selected early settlers of dental plaque. *J Oral Sc* 2006;48(2):71-75.
- Nalina T, Rahim ZHA. Effect of *Piper betle* L. leaf extract on the virulence activity of *Streptococcus mutans*-An *in vitro* study. *Pak J Biol Sci* 2006;9(8):1470-75.
- Nalina T, Rahim ZHA. The crude aqueous extract of *Piper betle* and its antibacterial effect towards *Streptococcus mutans*. *Am J Biotech Biochem* 2007;3(1):10-15.
- Clark PF, Ruehl WH. Morphological changes during the growth of bacteria. *J Bacteriol* 1919;4(6):615-29.
- McCall D. *Introduction to Microbiology: 11th Hour*. Blackwell Science Inc 2001;37-50.
- Fathilah AR. An *in vitro* study on the potential antiplaque effect of *Piper betle* and *Psidium guajava*. PhD Thesis, University of Malaya 2005.
- Jones CG. Chlorhexidine: Is it still the gold standard? *Periodontol* 2000. 1997;15:55-62.
- Fathilah AR, Othman Y, Rahim ZHA. An *in vitro* study on the anti-adherence properties of chlorhexidine gluconate and hexetidine mouthrinses. *Annals Dent* 1999;6:17-20.
- Fathilah AR, Aishah A, Zarina MZ. The effect of environmental stress on the growth of plaque bacteria. *Res J Microbiol* 2007;2(4):381-86.
- Gerhardt P, Murray RGE, Costlow RN, Nester EW, Wood WA, Krieg NR, Phillips GB, eds. *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, DC 1981.
- Griffiths PL. Morphological changes of *Camphylobacter jejuni* growing in liquid culture. *Lett Appl Microbiol* 2008;17(4):152-55.
- Todar K. *Todar's Online Textbook of Bacteriology*. http://www.twxtbookofbacteriology.net/growth_3.html
- Fathilah AR, Yusoff M, Othman Y, Rahim ZHA. The bacteriostatic effect of *Psidium guajava* and *Piper betle* on dental plaque bacteria. *Pak J Biol Sci* 2009;12(6):518-21.
- Nisengard RJ, Newman MG, eds. *Oral Microbiology and Immunology Second Edn*. WB Saunders Company, USA 1994.
- Marsh PD, Bradshaw DJ. Microbial community aspects of dental plaque. In: *Dental plaque revisited*. Newman HN, Wilson M eds. University College London 1999;237-54.