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Bacteriostatic Effect of *Piper betle* and *Psidium guajava* Extracts on Dental Plaque Bacteria

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Abstract: In this study, the bacteriostatic effect of *Piper betle* and *Psidium guajava* extracts on selected early dental plaque bacteria was investigated based on changes in the doubling time (g) and specific growth rates (μ). *Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces* sp. were cultured in Brain Heart Infusion (BHI) in the presence and absence of the extracts. The growth of bacteria was monitored periodically every 15 min over a period of 9 h to allow for a complete growth cycle. Growth profiles of the bacteria in the presence of the extracts were compared to those in the absence and deviation in the g and μ were determined and analyzed. It was found that the g and μ were affected by both extracts. At 4 mg mL⁻¹ of *P. betle* the g-values for *S. sanguinis* and *S. mitis* were increased by 12.0- and 10.4-fold, respectively (p<0.05). At similar concentration *P. guajava* increased the g-value by 1.8- and 2.6 -fold, respectively (p<0.05). The effect on *Actinomyces* sp. was observed at a much lower magnitude. It appears that *P. betle* and *P. guajava* extracts have bacteriostatic effect on the plaque bacteria by creating a stressed environment that had suppressed the growth and propagation of the cells. Within the context of the dental plaque, this would ensure the attainment of thin and healthy plaque. Thus, decoctions of these plants would be suitable if used in the control of dental plaque.

Key words: *Piper betle*, *Psidium guajava*, bacteriostatic effect, dental plaque bacteria

INTRODUCTION

The extracts of the *Piper betle* (L.) and *Psidium guajava* (L.) plants have been reported to possess many biological activities that have contributed to their role in the development of therapeutic products (Nair and Chanda, 2008; Wirotasangthong *et al.*, 2007; Kamath *et al.*, 2008). *Piper betle* is popularly used in traditional medicine as it possesses antioxidant, antibacterial, antifungal, antidiabetic and radioprotective activities (Wirotasangthong *et al.*, 2007). *Psidium guajava* is often used as astringent for skin diseases (Ponglux *et al.*, 1987) and also showed antidiarrheal, hepatoprotective, hypoglycemic, lipid lowering, antibacterial and antioxidant activities (Kamath *et al.*, 2008). The methanolic extract of *P. betle* and *P. guajava* have been shown to exhibit antimicrobial effects on various Gram positive and negative food borne pathogens (Francis Parillon, 2006). While the effect of *P. betle* was on both the Gram-positive and Gram-negative bacteria, the effect of *P. guajava* was specific to the Gram-negatives. Membrane damage that causes loss of cell viability and leakage of intracellular constituents was suggested to be the main mechanism of action of these extracts.

In the perspective of oral health maintenance, the aqueous extracts of *P. betle* and *P. guajava* have showed positive antiplaque activities that act on dental plaque bacteria at the early phase of plaque formation. These extracts were reported to act by first reducing the adhering capacity of the acquired pellicle which forms on the tooth surface at the early phase of plaque formation, to receive and bind the bacteria (Fathilah and Rahim, 2003) and second by diminishing the cell-surface hydrophobicity of the bacteria which are required to assist the adherence process (Fathilah *et al.*, 2006). Study by Nalina and Rahim (2006) has also shown that the crude extract of *P. betle* inhibited the activity of glucosyltransferase (GTF) which is required for glucan synthesis by the cariogenic bacteria *Streptococcus mutans*. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of aqueous extracts of *P. betle* and *P. guajava* were reported within the range of 2.16-4.69 and 5.21-10.42 mg mL⁻¹, respectively (Fathilah and Rahim, 2003).

The objective of this study was to investigate the bacteriostatic effect of the aqueous crude extracts of *P. betle* and *P. guajava* on early dental plaque bacteria, *S. sanguinis*, *S. mitis* and *Actinomyces* sp. Bacteriostatic

effect will be determined based on deviations in the doubling time and specific growth rate of the growth profiles produced in the presence and absence of the extracts.

MATERIALS AND METHODS

Preparation of plant extracts: Fresh leaves of *P. betle* and *P. guajava* were obtained from the university's botanical garden. Aqueous extracts of *P. betle* and *P. guajava* were prepared by concentrating decoctions of the fresh leaves of the plants using a speed-vacuum concentrator (HETO/HS-1-110, Denmark). The dried extracts were then kept refrigerated at -80°C (Hetofrig, Denmark) prior to use in the experiment. The dried extracts of *P. betle* and *P. guajava* were weighed into sterile microfuge vials and prepared into stocks of 20 mg mL^{-1} using sterile distilled water as the diluents. The extracts were dissolved by sonicating the microfuge vials in a sonicator (Ultrasonic sonicator, Selecta CE95).

Preparation of bacterial suspensions: *Streptococcus sanguinis*, *S. mitis* and *Actinomyces* sp. used in the investigation were pure cultures 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 (Fathilah and Rahim, 2003). Each bacteria species was revived in Brain Heart Infusion (BHI, Oxoid) broth at 37°C overnight. Following incubation the bacteria cells were harvested by centrifugation at 10,000 rpm for 10 min. The cells were then resuspended in BHI broth and the concentration was standardized at 10^6 cells mL^{-1} [Optical Density (OD) of 0.014] by using a spectrophotometer read at 550 nm.

The effect of *P. betle* and *P. guajava* aqueous extracts on bacterial growth profiles: The antibacterial activity of *P. betle* and *P. guajava* was determined using an assay procedure which involved the changes in the optical absorbance as an indication of changes in the bacterial growth profile. Metal capped borosilicate glass tubes (13×75 mm) were sterilized and used as culture tubes in the experiments. The growth of *S. sanguinis*, *S. mitis* and *Actinomyces* sp. under four different conditions was monitored by measuring the increased in the OD of the growing cells every 15 min. The four growth conditions were: (a) in 5 mL BHI broth to represent the control, (b) in 5 mL BHI added with *P. betle* extract at 4 mg mL^{-1} , (c) in 5 mL BHI added with *P. guajava* extract at 4 mg mL^{-1} and (d) in 5 mL BHI added with chlorhexidine (CHX)-containing mouth rinse at neat concentration of 0.12 mg mL^{-1} . Each of the test tubes was then inoculated

with 50 μL of the respective bacterial cells suspension. The concentration of extracts was selected at 4 mg mL^{-1} to be within the range of the MIC. This would ensure that the addition of the extract would not kill the bacteria cells but instead would allow the growth of cells to be at its minimum. CHX-containing mouth rinse in the test was used to represent a positive control for the study as CHX is considered the standard antimicrobial agent in the dental and hospital arena (Jones, 1997; Kornman, 1986). All tests were carried out in triplicate and repeated three times for reproducibility of results.

The content of each culture tubes were mixed well using a vortex mixer. The OD readings of each tube were set to zero with a test tube containing everything else except for the bacterial cells. This was to accommodate differences in the OD of the mixture caused by the varying color intensities of the plant extracts and the CHX-containing mouth rinse. The cultures were then incubated at 37°C in a shaking water bath and changes in the OD readings of each tube were periodically monitored and recorded at every 15 min intervals over a period of 9 h. The growth curves of each bacterium under the four growth conditions were plotted and compared with the profile of the CHX-containing mouth rinse. The growth rate (μ) and doubling time (g) of *S. sanguinis*, *S. mitis* and *Actinomyces* sp. under the different growth conditions were then determined using the following equations (Gerhardt *et al.*, 1981; Cappuccino and Sherman, 2005):

$$\mu = [(\log_{10} N - \log_{10} N_0) (2.303 / (t - t_0))]]$$

$$g = (\log_{10} N - \log_{10} N_0) / \log_{10} 2$$

where, N is No. of cells at log phase, N_0 is No. of cells at zero time and t is time to reach, t_0 is zero time log phase.

Statistical analysis: The effect of the extracts on the growth of the bacteria was illustrated by comparative analysis of their growth profiles under the various growth conditions. Statistical analysis was carried out using the one way analysis of variance (ANOVA). The MINITAB 13 for Windows statistical program was used to determine the Mean, Standard Deviation and evaluate the significance of the data in the experiments. Results were expressed as Mean \pm SD from one nine determinations ($n = 9$) set at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Throughout the study, the concentration of *P. betle* and *P. guajava* was set within the sub-MIC concentration of 4 mg mL^{-1} and not exceeding the MBC concentration.

This is important as the aim of a good antiplaque agent is not to kill all but to allow some of the plaque bacteria especially those of the normal species, to grow at a minimal rate. Within the sub-MIC range the bacteria exist in a condition where the biological cell functions are not disrupted. The growth profiles in Fig. 1a-c strongly suggested that the antimicrobial activity of *P. betle* and *P. guajava* towards *S. sanguinis*, *S. mitis* and *Actinomyces* sp. was bacteriostatic and may have been targeted at the early lag phase of the growth cycle. *P. betle* and *P. guajava* extracts seem to have created a stressed environment for the cells to perform their normal biological functions. This explains for the extended g-values and reduction in the μ -values in Table 1. The attainment of minimal population size as the bacteria enters the stationary phase indicated the bacteriostatic activities of *P. betle* and *P. guajava* towards *S. sanguinis*, *S. mitis* and *Actinomyces* sp. Under the stressed growth environment the bacteria were unable to perform normal biological function and eventually ceased to propagate. Such growth inhibiting mechanism has also been reported when the requirement for nutrient was restricted for *S. sanguinis* growth (Fathilah *et al.*, 2007).

The search for new alternative agents to be used as adjuncts in oral health care products has spurred due to the unfavorable staining effect caused by the prolonged usage of CHX (Cummins, 1992; Baehni and Takeuchi, 2003). In this study CHX-containing mouth rinse at 0.12 mg mL⁻¹ was found to be bactericidal to *S. sanguinis*, *S. mitis* and *Actinomyces* sp. as no growth profile can be generated following the 9 h incubation period. This finding confirmed the reputation of CHX as the most effective antimicrobial agent for the oral microorganisms. Alternative to CHX, plant based bioactive compounds such as sanguinarine, gallotannins and catechins have been isolated using solvent extraction procedures from *Sanguinaria canadensis* (Kopczyk *et al.*, 1991; Harper *et al.*, 1990), *Melaphis chinensis* (Wu-Yuan *et al.*, 1988) and Japanese green tea (Hirasawa *et al.*, 2006; Otake *et al.*, 1991), respectively. Exhibiting significant antiplaque activities, these compounds have been incorporated as adjuncts in dental dentifrices. In other plants like *Azara chdita indica* (Neem) (Wollinsky *et al.*, 1996), *P. betle* (Fathilah and Rahim, 2003; Fathilah *et al.*, 2006; Nalina and Rahim, 2006, 2007) and *P. guajava* (Fathilah and Rahim, 2003; Fathilah *et al.*, 2006), aqueous extraction procedure were employed and the crude extracts have been used. The exploration of antimicrobial activities of these plants has been based on their effective used in folklore medicines which often make use of simple decoction using water. Aqueous extraction procedure was employed in this study as it is environment friendly and would also avoid any possibility of side effect that

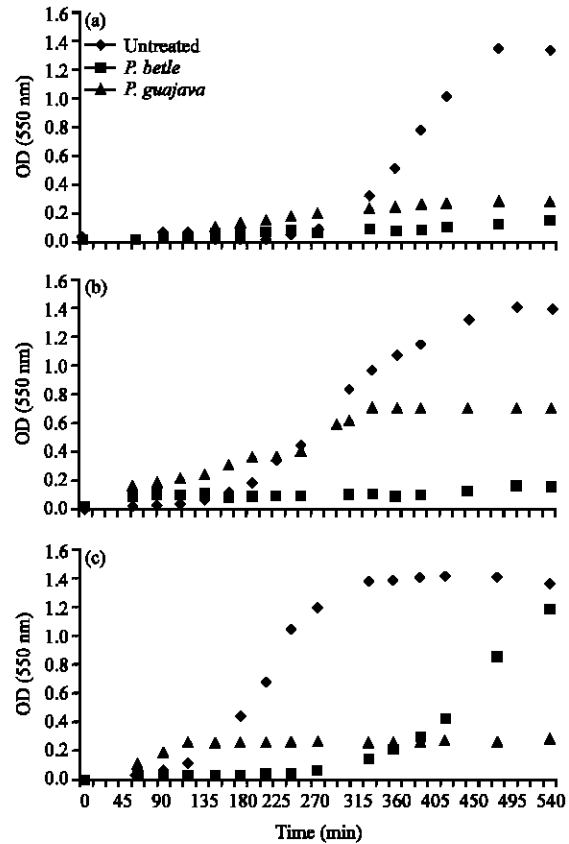


Fig. 1: The growth profiles of (a) *S. sanguinis*, (b) *S. mitis* and (c) *Actinomyces* sp. in the absence of extract, presence of *P. betle* and presence of *P. guajava*. Deviation of profiles from the untreated growth condition indicated the bacteriostatic effect of the extracts

Table 1: Changes in the generation times (g) and specific growth rates (μ) of *S. sanguinis*, *S. mitis* and *Actinomyces* sp. when cultured in the absence (untreated) and presence (extract treated) of *P. betle* and *P. guajava* were compared

Bacterias	Generation time (g) and growth rates (μ)	Growth conditions		
		Untreated	<i>P. betle</i>	<i>P. guajava</i>
<i>S. sanguinis</i>	g (h)	0.69±0.07	8.28±2.02	1.22±0.18
	μ (h ⁻¹)	1.01±0.1	0.09±0.02	0.58±0.09
	Increase in g	-	12-fold	1.8-fold
	Reduction in μ	-	91.1%	42.6%
<i>S. mitis</i>	g (h)	0.78±0.06	8.12±0.09	2.06±0.09
	μ (h ⁻¹)	0.89±0.07	0.09±0.01	0.34±0.02
	Increase in g	-	10.4-fold	2.6-fold
	Reduction in μ	-	89.9%	55%
<i>Actinomyces</i> sp.	g (h)	0.56±0.09	1.02±0.11	1.22±0.40
	μ (h ⁻¹)	1.25±0.18	0.69±0.08	0.61±0.21
	Increase in g	-	1.8-fold	2.2-fold
	Reduction in μ	-	44.8%	51.2%

p<0.05

could arise from the exposure to solvents if these extracts are to be used in the development of oral health care product.

CONCLUSION

The aqueous extracts of *P. betle* and *P. guajava* exhibited bacteriostatic effect on early dental plaque bacteria *S. sanguinis*, *S. mitis* and *Actinomyces* sp. under the stressed growth environment the bacteria appear to be unable to perform normal biological function and eventually ceased to propagate. Such events would ecologically control the development of dental plaque. Thus, both the plant extracts may have potential to be used as an active ingredient in the development of oral health care products.

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