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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

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Abstract: The aqueous extracts of *Piper betle* and *Psidium guajava* were prepared and tested for their anti-adherence effect on the adhesion of early plaque settlers (*Strep. mitis*, *Strep. sanguinis* and *Actinomyces* sp.). The saliva-coated glass surfaces were used to simulate the pellicle-coated enamel surface in the oral cavity. Our results showed that the anti-adherence activities of *Piper betle* and *Psidium guajava* extracts towards the bacteria were different between the bacterial species. *Psidium guajava* was shown to have a slightly greater anti-adherence effect on *Strep. sanguinis* by 5.5% and *Actinomyces* sp. by 10% and a significantly higher effect on *Strep. mitis* (70%) compared to *Piper betle*. The three bacterial species are known to be highly hydrophobic, and that hydrophobic bonding seemed to be an important factor in their adherence activities. It is therefore suggested that the plant extracts, in expressing their anti-adherence activities, could have altered the hydrophobic nature of the bonding between the bacteria and the saliva-coated glass surfaces. (J. Oral Sci. 45, 201-206, 2003)

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Introduction

Dental plaque when allowed to accumulate may lead to caries formation and discomfort due to the inflammation of the gingival area. Both conditions are direct consequences of poor oral hygiene measures of an individual. The initiation or progress of these common plaque-related diseases is very much affected by the level of oral hygiene of the individual. Effective plaque removal procedures are expected to prevent the development of these diseases. The use of dental products with marketed anti-plaque agents has been used as adjuncts to traditional cleaning methods (1,2). An anti-plaque agent is an agent that causes an effect on plaque which results in a reduction in caries and/or gingivitis (3). The response of an effective anti-plaque agent is manifested in both the reduction of the existing plaque and prevention of plaque accumulation (1,4,5). Plaque was formed through successive colonisation and coaggregation process. First, the adherence of the early plaque settlers to the tooth surface occurs, and subsequently secondary colonisers adhere to the surfaces of the already existing pioneer cells. This phenomenon has also been known as coaggregation. It is only logical that any interference with these adherence processes would disturb and impede the process of plaque formation. Thus, in investigating for an effective anti-plaque agent, the understanding of any anti-adherence activity would be an important criterion.

Decoctions of plants like *Piper betle* and *Psidium guajava* have long been used in folklore practices to maintain the hygiene of the oral cavity (6). It has been reported that their aqueous extracts have a positive

antibacterial effect on the growth of plaque bacteria *in vitro* (7). It was then suggested that the extracts may have potential as anti-plaque agents. Its efficacy as an anti-plaque agent could well be related to its anti-adherence effect on the adherence of the plaque bacteria on the pellicle-covered enamel surfaces. Thus the objective of this study was to determine *in vitro* the anti-adherence activities of the aqueous extracts of these plants using saliva-coated glass surfaces simulating the pellicle-covered enamel surfaces.

Materials and Methods

Materials

Brain Heart Infusion (Oxoid) was purchased from Basingtoke, Hampshire, England. Borosilicate culture tubes were purchased from Maple Leaf Brand, Canada. Aluminium caps for glass culture tubes were purchased from FARBLOS, Switzerland. Incubator used was of Memmert, Germany and autoclave was of Hirayama HVE-50, Japan. Sucrose was purchased from BDH Chemicals, Poole, England.

Preparation of crude aqueous extracts of *Piper betle* and *Psidium guajava*

Fresh leaves of *Piper betle* and *Psidium guajava* collected from a local farm were cleaned and dried using tissue paper. The leaves were then weighed and cut into small pieces before they were boiled in distilled water for several hours until the final volume is one third of the initial volume. The decoctions obtained were centrifuged at 10,000 rpm to eliminate sediments. The supernatant was aliquoted into microfuge tubes (1 ml/tube). Using the speed-vacuum concentrator (Heto LabEquipment), the aliquots were dried overnight. The dried extracts were collected and reweighed for use in the anti-adherence analysis.

Preparation of bacteria cultures and suspensions

Strep. sanguinis, *Strep. mitis* and *Actinomyces* sp. were isolated from 3-hour plaque samples using the method of Wollinsky et al (8). They were identified using the API Identification System (BioMerieux, France) and then kept in glycerol at -70°C as stocks. Prior to the experiment, these stocks were thawed, inoculated onto anaerobic agar plates and incubated at 37°C for 18-20 hours. The colonies were then harvested and dispersed into 30 ml Brain Heart Infusion (BHI, Oxoid) broth containing 5% (w/v) sucrose. The turbidity of the suspension was adjusted spectrophotometrically in cuvettes (OD 550 nm) to about 0.144 (equivalent of McFarland Standard # 0.5, BioMerieux, France) for use in the anti-adherence assay.

At this absorbance, the concentration of cell is standardised to about 10^8 cells/ml.

Collection of saliva

Whole saliva (WS) was collected into ice-chilled tubes from a single donor by expectoration after chewing a piece of rubber band. The WS was clarified by centrifugation ($17,000 \times g$, 30 min). The clarified WS was then used in the anti-adherence study (9-11).

Determination of the adherence affinities of *Strep. mitis*, *Strep. sanguinis* and *Actinomyces* sp. on the saliva-coated and water-coated glass surfaces

The determination of the adherence affinities of *Strep. mitis*, *Strep. sanguinis* and *Actinomyces* sp. was carried out according to the method described by Fathilah *et al* (12) which is a modification of method described by Ciardi *et al* (5). Saliva-coated glass culture tubes were prepared by treating the inner surface of 75×12 mm borosilicate glass culture tubes with 2 ml of clarified saliva for two minutes. The surface was then briefly rinsed with sterile distilled water. Water-coated glass culture tube was prepared by treating the inner surface of the culture tubes with sterile distilled water instead of saliva. These treated glass culture tubes were referred to as Tube 1.

To each of the saliva-coated and water-coated glass culture tubes, 2 ml of bacterial suspension was added (Tube 2) followed by an incubation period of 18-20 hours at 37°C. Bacterial suspensions used were *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. The total free and adherent bacterial cells were determined. Determination of the total free bacteria were carried out by transferring the growth suspension in Tube 2 into fresh glass culture tubes twice to ensure that all the unbound cells were collected and measured. The growth suspension in Tube 2 was transferred to Tube 3 and its turbidity was measured spectro-photometrically at 550 nm and referred to as the first reading. Following that, the now emptied tube was washed with another 2 ml of fresh sterile distilled water and the suspension collected into another fresh tube referred to as Tube 4. The turbidity of the bacterial suspension in Tube 4 was read spectrophotometrically at 550 nm and referred to as the second reading. The amount of the total unbound cells in each of the culture tubes corresponds to the sum of the turbidity from the first reading (Tube 3) and second reading (Tube 4).

Consequently, the then emptied Tube 2 contained the adherent cells. To this tube, 2 ml of fresh sterile distilled water was added. The tube was sonicated for 10 sec and the turbidity of the suspension containing the detached

bacteria was then read spectrophotometrically at 550 nm (Tube 5). This corresponds to the concentration of the adherent bacteria or amount of bound cells. The experiment was carried out within the laminar flow chamber to minimise contamination. A schematic diagram of the procedure is shown in Fig. 1.

The percentage of adherence was calculated by dividing the sum of the amount of the total unbound cells and bound cells with the amount of bound cells and multiplied by 100.

Determination of the anti-adherence effect of the aqueous extracts of *Piper betle* and *Psidium guajava* on the attachment of *Strep. mitis*, *Strep. sanguinis* and *Actinomyces* sp. on saliva-treated glass surfaces

Piper betle- and *Psidium guajava*-coated glass surfaces were prepared by re-treating the saliva-coated glass culture tubes with the respective plant extracts. Two ml of the plant extracts (equal concentration) was then added into the saliva-coated culture tubes and left for two minutes. After 2 minutes, the inner surfaces of the respective glass culture tubes were briefly rinsed with fresh sterile distilled water and the procedure as described in Fig. 1 was repeated. The percentage of the reduced adherence of cells in the presence of the extracts to that of in the absence, is a measure of the anti-adherence activities of *Piper betle* and *Psidium guajava* extracts and was calculated from the difference between the amount of adhered cells in the absence of plant extract and the amount of adhered cells in the presence of extract divided by the amount of adhered cells in the absence of plant extract and multiplied by 100. The amount of cells adhering to the saliva-coated glass surfaces in the absence of plant extract represented the total and maximum adhesion of *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp., and was regarded as an equivalent of 100% adherence.

Statistical analysis

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 tests. Results were expressed as mean ± standard deviation from one determination (n = 3) out of 3 reproduced experiments.

Results
Adherence affinities to water-coated glass surfaces and saliva-coated glass surfaces

Figure 2 shows the adherence affinities of the early plaque settlers towards water-coated and saliva-coated

glass surfaces. *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. showed different adhering capacity to both the water-coated and saliva-coated glass surface. *Strep. sanguinis* adhered with the highest capacity to the water-coated surface at 12.4 ± 1.2%, followed second by *Actinomyces* sp. at 5.3 ± 1.2% and last by *Strep. mitis* at 1.9 ± 0.5%. This order, however, was changed when the glass surface was coated with saliva. The adherence of *Actinomyces* sp. (32.4 ± 2.0%) was significantly higher than that of the two streptococci, *Strep. sanguinis* (22.1 ± 2.4%) and *Strep. mitis* (22.7 ± 2.0%) (P = 0.000). The presence of the saliva or experimental pellicle also has greatly enhanced the adhesion of the bacteria to the glass surface; *Actinomyces* sp. (84%), *Strep. mitis* (92%), and *Strep. sanguinis* (44%). The adherence affinities of the early plaque settlers to saliva-coated glass surface were found to be significantly higher compared to water-coated (P = 0.000).

Anti-adherence effect of *Piper betle* and *Psidium guajava* extracts

Figure 3 shows the percentage of reduced adherence of the bacteria to the saliva-coated glass surfaces by the plant extracts. The extract of both plants have shown positive anti-adherence effect on the adhesion of the microbes on
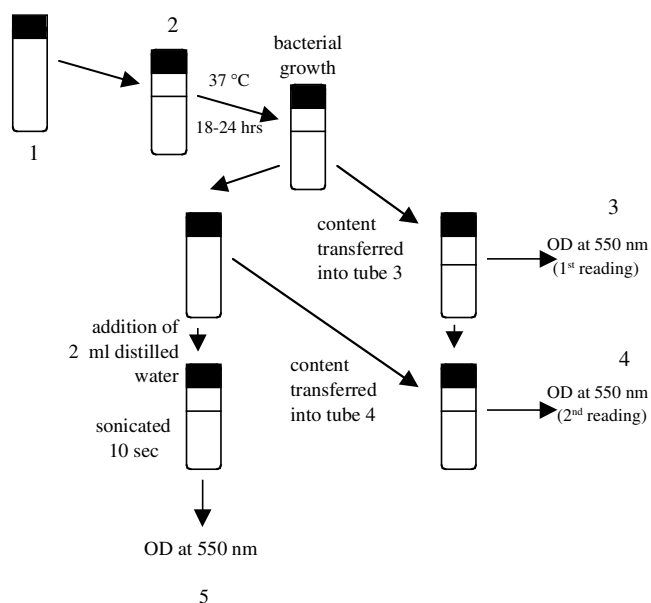

Fig. 1 *In vitro* assay method to measure the adherence of plaque bacteria to saliva-coated glass surfaces. 1) Saliva-coated glass tube; 2) Addition of 2 ml bacterial suspension; 3 & 4) The sum of both readings represent turbidity of free unbound cells; 5) Turbidity of adherent cells.

the saliva-coated glass surfaces. Variation in the degree of anti-adherence effect of the plant extracts was observed between the bacterial species. The aqueous extract of *Psidium guajava* was shown to inhibit the adherence of *Strep. sanguinis* by 28.1%, *Strep. mitis* by 48.7% and *Actinomyces* sp. by 40.4% whereas that of *Piper betle* was shown to reduce the adherence of *Strep. sanguinis* by 26.5%, *Strep. mitis* by 14.6% and *Actinomyces* sp. by 36.2%. The anti-adherence effect of the aqueous extract of *Psidium guajava* towards *Strep. mitis* was found to be significantly higher (> 3-fold) compared to the aqueous extract of *Piper betle* ($P = 0.000$). For *Actinomyces* sp., the anti-adherence effect of *Psidium guajava* was also found to be slightly higher compared to *Piper betle* ($P < 0.05$). There was not much difference in the strength of these activities shown by *Piper betle* and *Psidium guajava* towards *Strep. sanguinis* ($27.3 \pm 0.8\%$). In general, the anti-adherence effect of the aqueous extract of *Psidium guajava* towards the three bacteria was found to be higher compared to the aqueous extract of *Piper betle*.

Discussion

It was shown that water does have an influence on the adherence of bacterial species, especially that of the *Strep. sanguinis* (Fig. 2). However, the bacterial species adhered better in the presence of saliva. The highly increased number of cells adhering to the saliva-coated surface observed in this study is in agreement with what had been reported earlier (13-15) that saliva plays a role in the attachment of bacteria in the plaque formation. Saliva contains glycoprotein which has been widely reported to be involved in the formation of acquired pellicle. According to Lee *et al* (2), the adherence stage occurred very quickly (0-1 hour) as a result of the attachment of planktonic cells to the surface. The adhered cells will then begin to divide while more planktonic cells continuing to adhere to the surface. Cell growth following these two initial events contributes to the further development of plaque bio-film onto the surface.

In this *in vitro* study, glass surface was used to represent the hard surface of the tooth. The glass surface was exposed to the whole saliva for two minutes to allow for the deposition of salivary components to its surface. The coating of the surfaces with saliva would simulate the pellicle-covered tooth surfaces in the oral cavity. This experimental pellicle that was formed on the glass surface, like the acquired pellicle on tooth surfaces have a net negative charge due to its acidic protein components (15). It has also been shown to be equally satisfactory as adherence model compared to hydroxyapatite or an enamel surface (5). The presence of the artificial pellicle on the glass surface is important as it is a deposition of many salivary components which may act as receptors in the attachment of the early plaque settlers to the surface. This was clearly illustrated by the enhanced percentage of bound cells on the glass surfaces which have been coated with saliva compared to that of water-coated (Fig. 2). The prevalence of a bacterial strain to a surface correlates with the strain's inherent ability to recognise these binding receptors (16). The adsorbed components also impart an order of specificity for bacterial adherence, which is shown by the different binding affinities of the three bacteria to the saliva-coated glass surface (Fig. 2). *Actinomyces* sp. was shown to adhere with higher affinity to the saliva-coated surfaces compared to the streptococci. The equal ranges of adherence affinities shared by *Strep. sanguinis* and *Strep. mitis*, were also reported in an earlier study (17).

Strep. sanguinis, *Strep. mitis* and *Actinomyces* sp. possess external appendages to which many adhesions are associated with. External appendages of these early plaque settlers are crucial in the colonisation process as many hydrophobic domains consisting of non-polar amino acids are associated with those that confer the adhesion (18-20). Hydrophobic forces have been suggested to be involved in the adherence of a variety of bacteria to host tissues (21-23).

The cell-surface hydrophobicity in different species of streptococci is likely to result from the expression of different cell-surface molecules. *CshA* polypeptide which contributed a great extent to the hydrophobicity of the cell-surface is present in *Strep. gordonii* and *Strep. sanguinis*. It was shown that the presence of this polypeptide correlates well with the degree of cell-surface hydrophobicity. This protein is not detected on *Strep. mutans* and *Strep. parasanguinis*, the two streptococcus which are comparatively less hydrophobic than *Strep. gordonii* and *Strep. sanguinis* (23).

Gibbons and co-workers (22) have studied the association of fimbriae with the hydrophobic adherence of *Strep. sanguinis* to saliva-coated hydroxyapatite. The fimbriae were shown to be responsible for the hydrophobic properties (22,24). These fimbriae also contain protein adhesins (9) and/or sialic-binding lectin (25).

Results obtained from this study showed that the aqueous extracts of both *Piper betle* and *Psidium guajava* have caused a reduction in the adhesion of the early plaque settlers to the treated glass surface. The reduction in their adherence activities strongly suggests that the extracts have caused some sort of modification to their complementary binding sites in the experimental pellicle when the surface was being retreated with the extracts. The extent to which the adherence was affected by the
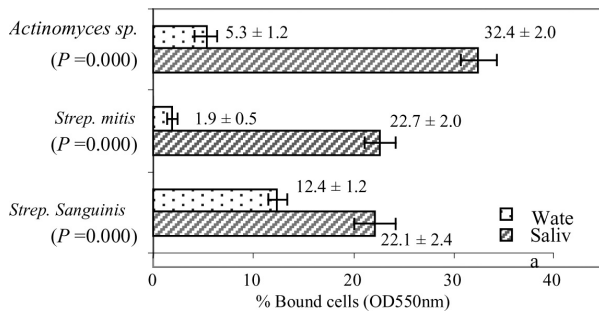
Fig. 2 Comparison of the adherence affinities of the early plaque bacteria to saliva-coated and water-coated glass surface. The percentages of bound cells were expressed as means ± standard deviation. The P values refer to the comparison of results of the adherence affinities of the early plaque bacteria to saliva-coated and water-coated glass surface. The one way analysis of variance (MINITAB 13) was used in the calculation of the mean, standard deviation and P-values (number of determinations = 3).

modification was however, not universal to all the three bacteria species. Treatment with *Piper betle* deactivated the adhesion of *Actinomyces* sp. (36.2%) more than it did on *Strep. sanguinis* (26.5%) and *Strep. mitis* (14.6%) (Fig. 3). The action of *Psidium guajava* on the other hand, was more on *Strep. mitis* (48.8%), followed by *Actinomyces* sp. (40.4%) and least by *Strep. sanguinis* (28.1%). The variation in the extent of the adherence effect exerted by the two extracts on *Strep. sanguinis*, *Strep. mitis* and *Actinomyces* sp. gives an indication of the specific target of the extracts.

The variations in the extent to which the adherence process was affected by *Piper betle* and *Psidium guajava* were different with each bacteria. Several suggestions can be drawn from these differences. *Strep. sanguinis* which responded equally to *Piper betle* and *Psidium guajava* may suggest that the extracts are affecting the same group of receptors provided in the experimental pellicle. Another explanation could be that one group of receptors are sensitive to *Piper betle* while the other are sensitive to *Psidium guajava*, and both have to be equally distributed in the experimental pellicle to give the equal responses (Fig. 3). The significant difference in the adhesion of *Strep. mitis* to the saliva-coated glass surfaces treated with the extracts of *Piper betle* and *Psidium guajava* strongly suggests the involvement of two different sets of receptors in the adhesion process. The first set which is sensitive to *Piper betle*, is less in number than the second set, which is sensitive to *Psidium guajava*. This may explain the reduction in the adherence affinity of *Strep. mitis* on glass surface treated with *Psidium guajava*. A similar effect although to a lesser extent was also shown by *Actinomyces* sp.

Conclusion

Oral bacteria adhere better to saliva-coated glass surfaces and with different affinities. The adherence of the early plaque settlers which include *Strep. mitis*, *Strep. sanguinis* and *Actinomyces* sp. was shown to be inhibited to a certain extent by *Piper betle* and *Psidium guajava* extracts. The results obtained in this study suggested that the mechanism of action of the anti-adherence effect of *Piper betle* and *Psidium guajava* may involve a modification of the hydrophobic bonding between the bacteria and the salivary components covering the tooth surfaces.
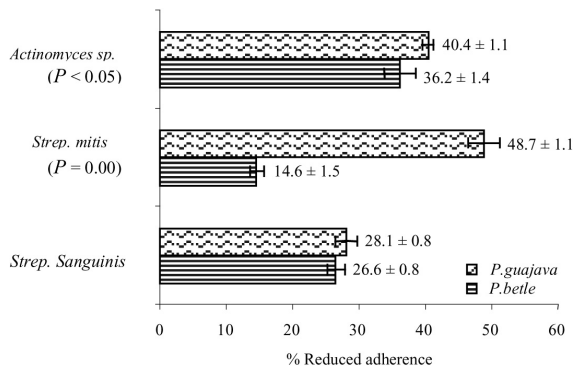
Acknowledgments

Fig. 3 Comparison of the anti-adherence effect of *Piper betle* and *Psidium guajava* on the binding of the early plaque settlers to saliva-coated glass surface. The anti-adherence effect was measured by the difference in % of bound cells on saliva-coated to the extract retreated saliva-coated surfaces. The percentage of reduced adherence showed in the chart were expressed as means ± standard deviation (n = 3). The P values refer to comparison of results between the effect of adherence effect of *Piper betle* and *Psidium guajava* on the binding of the early plaque settlers to saliva-coated glass surface The one way analysis of variance (MINITAB 13) was used in the calculation of the mean, standard deviation and P-values.

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