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Plant Extracts of *Psidium guajava, Mangifera* and *Mentha* sp. inhibit the Growth of the Population of Single-species Oral Biofilm

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Abstract

The use of oral gargle has gained popularity recently. Despite being either alcohol-based or plant-based, the main purpose for the usage of oral gargle is to prevent the accumulation of heavy plaque or oral malodour, that usually caused by the growth of oral biofilm. In this study, the antimicrobial effect of *Psidium guajava, Mangifera* sp. and *Mentha* sp. was tested against the single-species biofilm consisting of *Streptococcus sanguinis* and *Streptococcus mitis*. The biofilms were allowed to grow on the saliva-coated glass beads in Nordini's Artificial Mouth (NAM) model to represent the oral cavity for 24 hours. The saliva would form the experimental pellicle on the glass beads. The results obtained showed that biofilm of *Streptococcus mitis* displayed a maximum adherence (11.53%) compared to *Streptococcus sanguinis* (1.83%) on the untreated experimental pellicle. When the aqueous extraction of plant extract was applied on the experimental pellicle, the bacterial adherence was significantly reduced to 1.54% (*Streptococcus mitis*) and 0.21% (*Streptococcus sanguinis*). This result indicates that the selected plant extracts can be used to inhibit the heavy growth of oral biofilm.

Keywords: Plant extracts; Oral biofilm; *Streptococcus mitis; Streptococcus sanguinis*; Bacterial adherence 2

Introduction

Psidium guajava (guava), *Mangifera* sp. (mango) and *Mentha* sp. (mint) have a long history of traditional uses [1,2] much of which has been validated by scientific research. The guava leaves extract which comes from the family *Myrtaceae,* was reported to be very effective in inhibiting the growth of *Staphylococcus aureus* [3] while the guava bark methanolic extract showed a positive antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* [4]. In addition, the leaves were also reported to have an anti-adherence effect towards the oral bacteria, especially the early colonizers of dental plaque [5,6]. Mangifera sp. or popularly known as mango, comes from the family *Anacardiaceae*. The mango tree is not only grown naturally, but is also cultivated mainly in tropical and subtropical regions. The decoction of the leaves functions as antihelmintic and also can be used to gargle in the prevention of halitosis [1]. It was also reported that an ethanolic extract of mango seed kernel possessed an antimicrobial activity against food-borne pathogenic bacteria [7]. In the fourteenth century, mint (*Mentha* sp.) which comes from the family *Labiatae*, was used for whitening the teeth, and its distilled oil is still used to flavour tooth-pastes and chewing gum.

Streptococcus sanguinis (*Strep. sanguinis*) or previously known as *Streptococcus sanguis* [8] which belongs to the mitis-group [9], is one of the early colonizers of dental plaque [5,10]. Once a tooth erupts into the oral cavity, *Strep. sanguinis* colonizes its surface. The colonization of *Strep. sanguinis* to the tooth surfaces begins at the age of nine months in infants and their population was shown to increase with the age of the infants [11,12]. *Streptococcus mitis* (*Strep. mitis*) is a common species in the mouth and frequently predominates with Strep. sanguinis during the initial colonization of the tooth surface [10]. Strep. mitis is commonly found on the soft tissues of the cheeks, lips and the ventral surface of the tongue as they tend to adhere to non-keratinized mucosa in the mouth [9]. In an oral cavity, these microbes tend to grow in the form of biofilm. They are arranged in micro colonies and surrounded by protective matrix [13] consisting of extracellular 3 polymers that form a thick, continuous, hydrated, charged layer around the cells [14,15]. The matrix protects the biofilm from host defences [14], desiccation and the action of antimicrobial agents [16]. Throughout the years, many researchers have attempted to study the mechanism of action of plant extracts as antibacterial agents [5,17-23]. However, their research involved mainly microbes growing under the planktonic state, which refers to the bacteria that live as floating organisms in the test tube or flask cultures in the laboratory. Under such condition, the microbes may not best represent those found in dental plaque. Investigation of oral biofilm in vivo however is often made difficult by its heterogeneity, limited access, the existence of variable and uncontrollable oral environments as well as ethical problems [24]. Therefore, there is a need to investigate the effect of the chosen plant extracts on the single-species oral biofilm *in vitro.*

Materials and Methods

Preparation of plant extracts

Leaves of mango (*Mangifera* sp.) (Figure 1) were obtained from Puchong, Selangor and leaves of guava (*Psidium* sp.) (Figure 2) were obtained from Kota Bharu, Kelantan. Leaves of mint (*Mentha* sp.) (Figure 3) grown in Cameron Highlands, Pahang were purchased from the local market in Kuala Lumpur. The leaves were oven-dried at 60°C for 48 hours until no changes in the weight were observed. The dried leaves were then grounded into powder and used in the preparation of the aqueous extract. One hundred gram of powdered samples prepared from the leaves of Mangifera sp., *Mentha* sp. and *Psidium* sp. were

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Figure 1: The leaves of *Psidium* sp.

Figure 2: The leaves of *Mangifera* sp.

Figure 3: The leaves of *Mentha* sp.

weighed and put into a large beaker. One thousand ml of deionised distilled water was added and the mixture was allowed to boil until the final volume became one tenth (1:10) of the original. The debris was then filtered out using filter paper (Whatman No.1, diameter 24 cm) with the aid of a suction pump (SPARMAX, Taiwan). One ml of the clear crude extracts was dispensed into micro centrifuge tubes (1 ml/tube) and dried using the speed vacuum concentrator (HETO) until no further changes in weight were observed. The dried extracts were then stored at -20°C until further use [25]. Prior to use, all three extracts were diluted with sterile deionised distilled water and mixed to a final concentration of 0.5 mg/ml. All three extracts were combined together before used in the experiment.

NAM model

The Nordini's Artificial Mouth (NAM) model was used in the study to represent the oral cavity. The model was developed according to the method described earlier [26]. Basically, the model consists of a glass chamber, glass beads, saliva reservoir, bacterial reservoir and peristaltic pump (Figure 4).

Preparation of the glass beads as substratum

In the study, the glass beads (3 mm diameter) were used to represent the tooth enamel onto which the experimental pellicle and single-species biofilm will develop. The glass beads were cleaned and sterilized by autoclaving at 121°C (15 p.s.i) for 20 minutes. The sterilized glass beads were kept in a sterilized bottle before use.

Preparation of sterile saliva

Undiluted sterile saliva was prepared according to the method described by De Jong and Van der Hoeven [27]. Approximately 25 ml of stimulated whole saliva (SWS) was collected everyday from a single volunteer to minimize any variations that may arise. The volunteer was asked to chew on a sugar-free gum to stimulate saliva production. The collection of SWS was done using ice-chilled test tubes. The aggregation of protein in the SWS samples was minimized by adding 1,4-Dithio-D,L-threitol (DTT) to a concentration of 2.5 mM. Upon the addition of DTT, the saliva was stirred slowly for 10 mins before it was centrifuged at 864g for 30 mins. The supernatant obtained was then filter-sterilized through a disposable 0.2 µm (Supor® Membrane) low protein-binding filter (Acrodisc® Syringe Filters, Pall Corp, USA) into sterile test tubes. The sterile SWS was then stored at -20°C. Prior to use, the SWS was thawed and centrifuged once again to remove any precipitate.

Preparation of bacterial suspension

The stock culture of oral bacteria (*Strep. mitis* and *Strept. Sanguinis*) were obtained from the Department of Oral Biology, Faculty of Dentistry, University of Malaya. The stock which was kept at -80°C was thawed at room temperature. Each of the thawed stock culture was then inoculated into sterilized BHI broth (1:100 v/v) and incubated at 37°C for 18-24 hours. The bacterial suspension was adjusted spectrophotometrically at 550 nm to 0.144 absorbance which is equivalent to108 cells/ml [5]. This procedure is important to standardize the number of cells in the suspension to be used in the study.

The development of experimental pellicle and single-species biofilm in NAM model

Experimental pellicle: negative-control: The development of experimental pellicle and single-species biofilm (*Strep. sanguinis* and *Strep. mitis*) in NAM model was carried out according to the method described by Wan Nordini Hasnor et al. [28]. Ten glass beads were placed in the glass chamber to represent the tooth in the mouth. The glass chamber was then placed in water bath which serves as an incubator to maintain temperature that mimics the human body temperature.

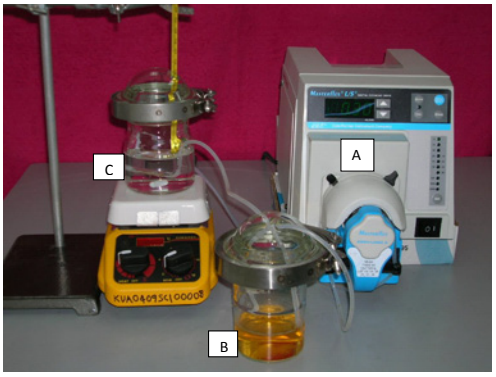


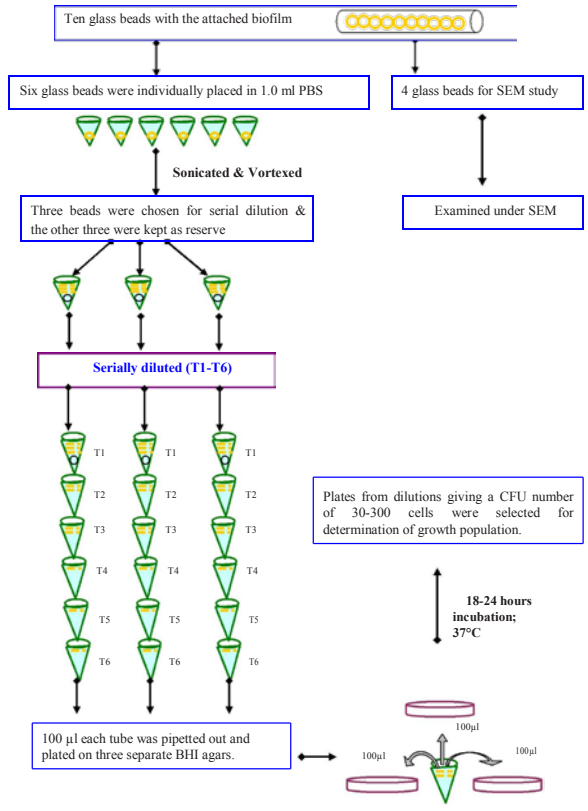
Figure 4: Nordini's Artificial Mouth (NAM) model. This model consists of (A) peristaltic pump; (B) bacterial reservoir; (C) water bath system, which was connecting via rubber tubings. The water bath system houses the glass beads inside the glass chamber. Hot-plate was used to maintain the temperature at 37°C.

A sterilized saliva reservoir with a capacity of 50 ml was connected to a peristaltic pump and the NAM model via sterilized rubber tubing. Sterilized saliva was pumped into the NAM model for two minutes at a flow rate of 0.3 ml/min to coat the glass beads. This was followed by a flow of sterile distilled water to rinse off the excess saliva that remains on the glass beads. The experimental pellicle was now ready to receive bacterial inoculum. Bacterial reservoir which consisted of single-species inoculum (*Strep. sanguinis* and *Strep. mitis*) (108 CFU/ml) was pumped into the model at a rate of 0.3 ml/min and was allowed to form on the glass beads for 24 hours to form a 24 hours biofilm.

Treated experimental pellicle: The steps in the above step i.e. experimental pellicle: negative-control was repeated. After rinsing off the excess saliva on the glass beads with sterile distilled water, a 50 ml aqueous solution consisted of mixture plant extracts (0.5 mg/ml) was allowed to flow into the NAM model for two minutes at a rate of 0.3 ml/min. This was followed by a flow of sterile distilled water once again to rinse off the excess extracts on the glass beads. Subsequently, the bacterial inoculum (108 CFU/ml) was pumped into the model over a period of 24 hours to allow for the formation of a 24 hours single-species biofilm on the glass beads. This procedure was repeated by using 0.12% chlorhexidine (CHX) in place of the plant extracts to serve as a positive-control. The experiment was carried out in triplicates.

The harvesting of the single-species biofilm on the glass beads and determination of bacterial population

The procedures employed in the harvesting of the single-species biofilm were carried out according to the chart in figure 5. After 24 hours, each of the six glass beads with the biofilm formed on them was carefully taken out and placed in the respective micro centrifuge tubes (1.5 ml) containing 1.0 ml of phosphate-buffered saline (PBS). Each of the tubes was sonicated (10 seconds) and vortexed (1 minute) to dislodge any attached bacteria on the glass bead. Each of the tube subsequently containing the bacterial suspension. Out of the six tubes, three were chosen for serial dilution while the other three were kept as reserve. Six serial dilutions of the bacterial suspensions were carried out for each of the tubes. The first serial dilution was referred to as tube 1 (T1) and the sixth serial dilution as tube 6 (T6). A 100 µL of each of the serially diluted bacterial suspensions (T1 to T6) was pipetted out and plated on three separate Brain Heart Infusion (BHI) agars. The plates were incubated aerobically at 37°C for 18-24 hours. From the plated results, the plates with the Colony Forming Unit (CFU) number between 30-300 were used in the determination of adhered bacteria [29]. In this study, the fourth serial dilution tube (T4) was found to correspond to this CFU numbers.



*The experiment was carried out in duplicate. This will give total number of determination (N) =18

Figure 5: Schematic chart of the procedures employed in harvesting biofilms from the glass beads.

Preparation of samples for scanning electron microscope (SEM) viewing

The preparation of samples for SEM viewing was carried out according to the method described by Lagacé et al. [30]. The glass beads with the biofilm formed on them were fixed in glutaraldehyde (4%) in glass vials for one hour at room temperature. The glutaraldehyde was discarded and the glass beads were rinsed once with distilled water. The washed glass beads were then fixed in osmium tetraoxide (1%) and left overnight (14 hours) in tightly capped vials at 4°C in the refrigerator. On the next day, the vials were taken out from refrigerator and left for 30 minutes at room temperature. The osmium tetraoxide (1%) was gently pipetted out and the samples were washed with distilled water for 15 minutes. The dehydration process was carried out by treating the samples with the ascending percentages of ethanol (10%-100%). The samples were immersed in the different concentrations of ethanol for 15 minutes. The samples were then immersed in 100% ethanol twice to ensure that most of the water in the samples was eliminated. Gradual displacement of ethanol with acetone was then carried out (20 minutes each) using the following ratios (v/v): Ethanol: Acetone 3:1 1:1 1:3 Following that, the samples were immersed in 100% acetone for four times, 20 minutes each time, followed by Critical Point Dessication (CPD) process. The samples were then mounted on metal stubs and coated with gold. After gold-coating process, the samples were ready for SEM viewing.

Statistical Analysis

All data obtained in the study were analysed using t test of SPSS software. The values were expressed as mean ± SD.

Results

From 100% bacterial inoculum pumped into the model, a single-species biofilm of *Strep. mitis* showed the maximum adherence (11.53%) on the untreated experimental pellicle over a period of 24 hours, whereas only 1.83% of *Strep. sanguinis* adhered (Figure 6). The differences in the adherence capacity of these bacteria in the formation of the single-species biofilms were statistically significant ($p<0.05$). When the experimental pellicle was treated with plant extracts, the populations were significantly reduced for both streptococci. On the experimental pellicle treated with 0.12% chlorhexidine (CHX), it was observed that there was no adherence of *Strep. sanguinis*, while *Strep. mitis* showed slight adherence at 1.13%. The population of Strep. sanguinis on the experimental pellicle treated with CHX was observed

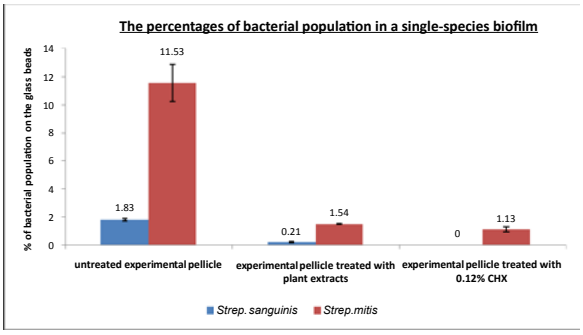


Figure 6: The histogram summarizing the bacterial population in the single-species biofilm of *Strep. sanguinis* and *Strep. mitis* on the (i) untreated experimental pellicle (negative-control), (ii) experimental pellicle treated with plant extracts, and (iii) experimental pellicle treated with 0.12% CHX (positive-control). The values represent cells population adhering to the experimental pellicle. The values were expressed as means ± SD (N=18).

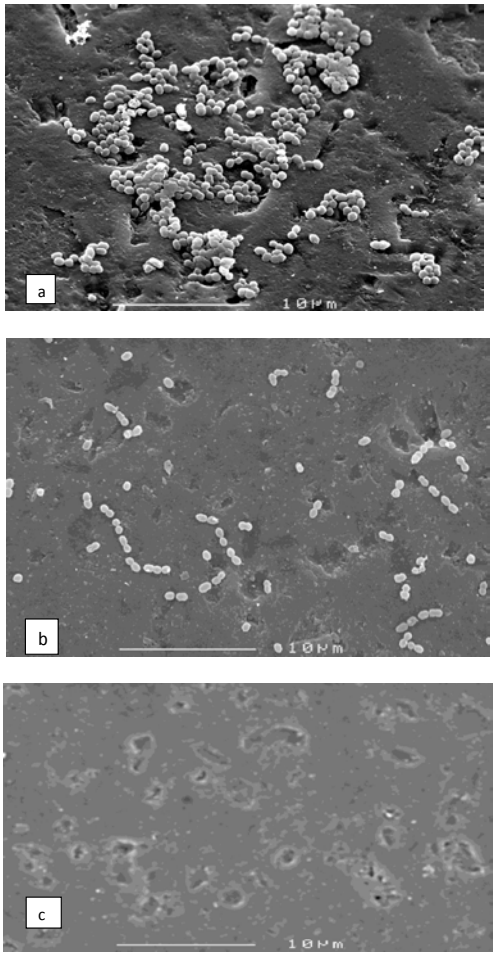


Figure 7: SEM micrographs showing populations of *Strep. sanguinis* in 24 hours biofilm on (a) untreated experimental pellicle, (b) experimental pellicle treated with plant extracts, and (c) experimental pellicle treated with 0.12% CHX. (3500X).

to be significantly less than their populations on the experimental pellicle treated with plant extracts ($p<0.05$). The population of *Strep. mitis* adhering on the experimental pellicle treated with CHX and plant extracts however showed no difference ($p>0.05$). This result was further confirmed by SEM viewing (Figures 7 and 8). It was clearly shown that population of *Strep. sanguinis* showed a moderate number of cells on the untreated experimental pellicle (Figure 7a). The cells tended to clump to each other. However, the population of *Strep. sanguinis* was decreased on the experimental pellicle treated with plant extracts (Figure 7b). The cells were also arranged in colonies of short chain consisted of two to three cells. On the experimental pellicle treated with 0.12% CHX, no cells were detected on the glass beads (Figure 7c).

The population of *Strep. mitis* showed a large number of cocci cells when they were allowed to grow on the untreated experimental pellicle (Figure 8a). The cells were clumped together. The numbers of cells were much reduced when the experimental pellicle was treated with plant extracts. Similar to *Strep. sanguinis*, the cells of *Strep. mitis* were also arranged in chains of colonies consisting of four to seven cells per chain (Figure 8b). On the experimental pellicle treated with 0.12% CHX, the number of cells adhered were almost similar to the cells that adhered on the experimental pellicle treated with plant extracts. However, only short chains were observed which consisted of two to four cells per chain (Figure 8c).

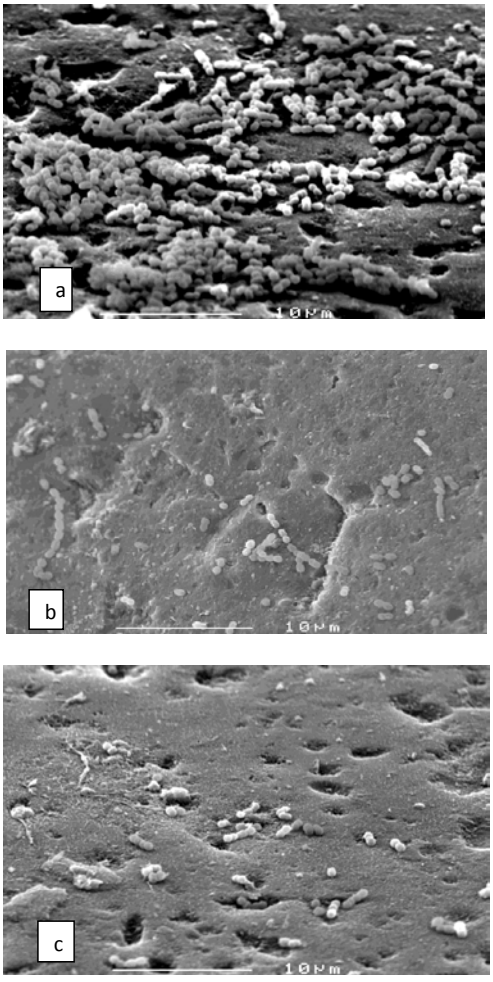


Figure 8: SEM micrographs showing populations of *Strep. mitis* in 24 hours biofilm on (a) untreated experimental pellicle, (b) experimental pellicle treated with plant extracts, and (c) experimental pellicle treated with 0.12% CHX. (3500X).

Discussion

Currently, there is an increasing interest to investigate the effect of natural compounds, especially plants extracts, on the residence of the oral cavity. Many of the investigations have been focused on the ability of the compound to either promote the growth of beneficial organisms or inhibit the growth and metabolism of oral bacteria associated with certain diseases. It has been reported that *Morus alba* [21], *Andrographis paniculata* and Chinese black tea [31], *cranberry* [32] and *Mikania* sp. [33] exhibited potentially useful antibacterial properties towards some oral pathogens. An alkaloid extract sanguinarine obtained from the plant *Sanguinaria canadensis* (bloodroot) is one example of natural based antimicrobials. The combined usage of mouthrinse and dentifrice containing sanguinarine has been shown to demonstrate the antiplaque effect [34-37] and also can be used to cure gingivitis [36,37].

Our study has shown that the extract of the leaves of *Psidium* sp., *Mentha* sp. and *Mangifera* sp. exhibited antimicrobial activities. The selection of these plants was based on findings that their extracts exhibited antimicrobial activities against oral microbes grown under the planktonic state [5]. The planktonic state refers to the condition where the bacteria were allowed to grow as suspension in the test tubes. It has been demonstrated in their studies that pre-treatment of experimental pellicle on saliva-coated glass beads with either *P. guajava* or *Piper betle* can significantly disrupt the adhesion of the early plaque colonizers to the pellicle. This subsequently will interfere with the initial stage of biofilm development. Similar observations have also been reported by Percival et al. [38] who strongly acknowledged the importance of the salivary pellicle during the initial stages of biofilm formation. The property of the pellicle can be altered in the presence of certain plant extracts. In a study carried out by Prashant et al. [39], positive antimicrobial activity of mango chewing stick was detected against oral *Streptococci*. The many positive antimicrobial activities on oral bacteria exhibited by plant extracts provide great support in the promotion of such extracts as oral healthcare agents. Their use may help to moderate the development of dental plaque so that its texture is always thin and porous. Besides, mouthwash sold in pharmacy stores or local supermarkets are either contains many chemicals or alcohol-based which may cause unwanted side effects to the consumers. On the untreated experimental pellicle, the adhesion affinity of the single-species biofilm of *Strep. mitis* is the highest (11.53%) compared to those of *Strep. sanguinis* (1.83%). The data obtained in this study however contradicted the findings reported by Fathilah [40] who reported that *Strep. mitis* and *Strep. sanguinis,* both showed almost similar percentage of adhesion affinities (22%). The discrepancy in the results obtained in this study and Fathilah [40] might have been due to the state of cells used in the experiment. In our study, the bacteria cells and the nutrients were continuously supplied to the growth system of the artificial mouth (NAM) model. On the contrary in the planktonic state, bacteria cells were grown under a static phase [40]. In a condition when there was no flow of nutrients involved, the cells in the planktonic state may have the ability to adhere at a greater extent than cells growing in a continuous system [41]. Under this state, cells were more exposed to the clearing effect of the flowing liquid. Significant differences with respect to the growth of cells while under the planktonic and biofilm condition have also been reported by Black et al. [42] and Ceri et al. [43].

When the experimental pellicle was treated with plant extracts, it was clearly shown in the results that the number of adhering cells in the single-species biofilm was reduced. This is because the binding of bacterial cells to the acquired pellicle in the mouth or to the experimental pellicle *in vitro* is much influenced by the adherence capacity of the pellicle. Once the experimental pellicle was altered by plant extracts or any other antimicrobial agent, it will affect the binding affinity of the bacteria. Our results are consistent with the study carried out by Oliveira et al. [44], which confirmed the antimicrobial effects of plant's components to the single-species oral biofilm. Throughout the studies, CHX was chosen as a positive-control as it is the most widely used chemotherapeutic agent and known for its ability to inhibit plaque and gingivitis [44-48]. CHX has been vastly used in the prevention of dental caries especially in patients following radiation therapy who often have difficulty in performing tooth brushing [49,50]. The mechanism of action of CHX has been associated with its effect on pellicle formation [51], bacterial adherence mechanisms as well as modification of the bacterial cell wall properties which ultimately will cause lysis of the cell [52]. From SEM results, we are suggesting that the mechanism of action of mixed-plant extracts used in this study might be towards the formation of experimental pellicle. Once this pellicle was disrupted, the pioneer bacteria in the oral cavity like *Strep. mitis* and *Strep. sanguinis* could not adhere to the tooth surfaces. However, further study need to be done to investigate more on this matter.

Conclusion

The plant extract applied to the saliva-coated glass beads appeared to have altered the experimental pellicle and subsequently reduced the adhesion affinity of the bacteria in the biofilms. This may suggest that the extracts of the plants have potential as anti-plaque agents.

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