INTRODUCTION
Dental biofilm, more commonly known as dental plaque, is an intricate composition of multiform, well-organized and mutually benefitting community of microorganisms that forms naturally on tooth surfaces. Accumulation of dental plaque, if not under control can give rise to detrimental effects on teeth, gums and oral supporting tissues which manifests in the form of dental caries, gingivitis and periodontitis respectively. Its development involves a number of stages: (i) formation of acquired pellicle on tooth surface; (ii) successful adherence of bacteria to this pellicle; (iii) successful colonization of the pioneer bacteria that finally leads to the formation of dental plaque; (iv) co-adhesion of secondary colonizers; (v) maturation of plaque and (vi) detachment of bacteria. Dental plaque agglomerates preferentially at stagnant sites that afford protection from the vigorous removal forces that apply in the mouth. Many commercially available mouthrinses have been claimed to be efficient in controlling the development of dental plaque in the stagnation areas. Among them is the mouthrinse that incorporates chlorhexidine as the antibacterial ingredient. However, chlorhexidine is expensive and has unwanted side effects such as extrinsic tooth staining, alteration of the taste sensation, irritation of the oral mucosa and oral hypersensitivity reactions. Combinations with other antibacterial agents such as tetracycline mouthrinses is also available. However, tetracycline been reported to alter the composition of dental plaques, thereby favouring bacteria resistant to tetracycline and other unrelated agents to predominate. In addition, most of the commercially available mouthrinses contain alcohol. McCullough and Farah reported that using alcohol-containing mouthrinses may promote the development of oral cancer. It has been reported that extracts from plants such as Piper betle and Psidium guajava, Syzygium aromaticum, Mangifera indica and Mentha piperita have antibacterial effect towards oral bacteria. This leads to the suggestion of incorporating the extracts as antibacterial agent in mouthrinses. Leaves of Psidium guajava in the form of paste have been used in folklore practices as toothpaste to maintain the oral hygiene. The extracts of Piper betle and Psidium guajava were also known for their anti-adhering activities towards oral bacteria and the effect varies with the bacterial species. In previous studies, the antibacterial effect of the extracts were determined in planktonic conditions.
The interest to search for a Plant Extracts Mixture (PEM) which would have potential antiplaque activity as well as possessing natural preservative and flavours, drove the authors to use a mixture of the extracts of Mangifera sp., Mentha sp. and Psidium sp. as the test material in the investigation for potential antiplaque activity. In this study, single-species biofilm developing in a dynamic environment using an artificial mouth (NAM) model was used in the determination of the effect of this Plant Extracts Mixture (PEM) on the adhering capacity of the bacteria to experimental pellicle and the retention of bacterial population in the biofilm. The antiplaque activity of the Thymol (0.064%) and PEM, we focus on the anti-adhering activities of the Thymol (0.064%) and PEM towards bacteria on the simulated pellicle. The anticariogenic activity is demonstrated by the bacterial population in the biofilm upon treatment with the Thymol and PEM.

**METHODOLOGY**

This experimental study was conducted at the Department of Oral Biology, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia, from December 2009 to December 2011.

*Streptococcus mutans* (S. mutans) ATCC 25175 was purchased from American Type Culture Collection (ATCC) resource center. The bacterial suspension was prepared according to Nalina and Rahim's methods. Streptococcus sanguinis (S. sanguinis) and *Streptococcus mitis* (S. mitis) used in this study were clinical isolates available at the Department of Oral Biology, Faculty of Dentistry, University of Malaya. These two microbes were identified using the API Identification system (Biomereux, France). Leaves of respective plants (*Mangifera sp.*, *Psidium sp.*, *Mentha sp.*, *Streptococcus sanguinis* (S. sanguinis) and *Streptococcus mitis* (S. mitis)) grown in Cameron Highlands, Pahang (Malaysia) were purchased from same source at the local market in Kuala Lumpur. Leaves of respective plants (*Mangifera sp.*, *Psidium sp.* and *Mentha sp.*) used in the study were treated in a similar manner to eliminate variations due to preparation procedure. The leaves were initially washed with running tap water followed by deionized distilled water and oven-dried at 60°C for 48 hours. The drying continued until no further changes in weight were observed. The dried leaves were then grounded into powder and used in the preparation of the aqueous extract. One hundred (100) gm of the powdered leaves of the respective plants were separately boiled in 1 liter of deionized distilled water until the final volume became one-tenth of the original volume. The concentrated extract was then filtered using a filter paper (Whatman No1, diameter 24 cm) with the aid of a suction pump (SPARMAN, Taiwan). Aliquots of 1 mL filtered extract were then dispensed into microcentrifuge tubes and dried using the speed vacuum concentrator (HETO, Denmark) until no further changes in weight were observed. The dried extracts were then stored at -20°C until further use. Prior to use, the extracts were dissolved in sterile deionized distilled water and mixed. The resulting concentration of each of the extract in the mixture was 0.5 mg/mL. In this study, the mixture of extracts was subsequently referred to as Plant Extracts Mixture (PEM).

The respective bacterial stocks of *S. mitis*, *S. sanguinis* and *S. mutans* ATCC 25175 which were kept frozen at -80°C were thawed at room temperature. The cultures were respectively sub-cultured on Brain Heart Infusion (BHI) agar and incubated at 37°C for 24 hours.

A loopful of the growth colonies of each culture was transferred respectively into BHI broth. The turbidity of the respective bacterial suspension was measured at 550 nm using a spectrophotometer (Shimadzu UV-VIS1800, Japan) and standardized to an absorbance of 0.144, an absorbance which is equivalent to 10^6 cells mL^{-1}. The working bacterial suspension was standardized throughout the experiment. Sterile saliva was prepared according to the method described by De Jong and Van der Hoeven. Approximately 25 ml of Stimulated Whole Saliva (SWS) was collected everyday from a single volunteer. The volunteer was asked to chew a sugar-free gum to stimulate saliva production. The SWS was collected in ice chilled test tubes. Saliva composition may vary between individuals and in order to minimize the variations due to this, the saliva was collected from a single volunteer. The aggregation of protein in SWS can be minimized by the addition of 1, 4-Dithio-D L-threitol (DTT) to a concentration of 2.5 mM. After stirring slowly for 10 mins, the saliva was centrifuged at 864 x g for 30 minutes. The supernatant was then collected and filter-sterilized through a disposable 0.2 µm (Sopor Membrane) low protein-binding into sterile test tubes and stored at -20°C until further use. Prior to use, the sterile SWS was thawed and recentrifuged to remove any precipitate. It was later used to coat glass beads (3 mm in diameter, Merck Germany) in an artificial mouth model forming a layer that mimics the acquire pellicle on the tooth surface and thus referred as the Experimental Pellicle (EP).

The toxicity of the PEM was determined using the brine shrimp (Artemia salina) bioassay. The doses causing a 50% death (LD_{50}) was analysed using dead counts of shrimps at five different PEM concentrations; 200, 400, 600, 800 and 1000 ppm (1 to 5 mg/mL). Dead shrimps were counted after 24 hours. ED_{50} values were determined using tissue culture technique.
Sterile SWS was allowed to flow for two minutes at a rate of 0.3 mL min⁻¹ into an artificial mouth model (NAM model)⁹ which contained five glass beads. The saliva flowing onto the glass surface would form experimental pellicle mimicking the acquired pellicle that covers the tooth surface in the oral cavity.¹⁵ Excess saliva was removed by pumping in deionized distilled water into the model for two minutes. Subsequently, bacterial inoculation was carried out by pumping the respective bacterial species (10⁶ cells mL⁻¹) (S. mitis, S. sanguinis and S. mutans) separately into the model at 0.3 mL min⁻¹ for 24 hours to allow for the development of 24 hours single-species biofilms. The biofilms were then harvested as described later. The bacterial population in the deionized distilled water (negative control) treated biofilms represents the maximum adhering capacity (100%) for the respective bacteria. The experiments were carried out in duplicates and the determination of the CFU per bead was carried out in triplicates. Hence a total number (n) of 30 determinations were carried out.

The above experiment was repeated using PEM instead of the deionized water. The PEM was allowed to flow into the NAM model for two minutes at a rate of 0.3 mL min⁻¹ to treat the experimental pellicle. Excess PEM was removed by allowing sterile deionized distilled water to flow into the model before the respective bacterial inoculums were introduced. Biofilms were allowed to form over a period of 24 hours as in the above procedure.

Similar procedure was repeated using Thymol (0.064%) instead of the PEM. Thymol (0.064%) was used as a positive control as it contains thymol which is also of a plant source (Thymus sp). The effect of PEM and Thymol (positive control) on adhering ability of the respective bacterial species was expressed as a percentage of adhering bacteria. Based on the assumption that the bacterial population adhered in the negative control biofilm is maximum and equal to 100%, the postulated formula to calculate percentage values for bacterial adherence was:

\[
\text{Percentage of adhering bacteria} = \frac{Y}{X} \times 100
\]

where, \(X\) = Bacterial population in the negative control biofilm and \(Y\) = Bacterial population in the treated experimental pellicle (PEM or positive control biofilms) determined by estimating the sum of 30 determinations. The percentages of adherence for the respective bacterial species are outlined in Table I.

The above experiment was repeated with a slight modification. In this experiment, biofilms were allowed to form first by inoculating the experimental pellicle with the individual bacterial inoculum (S. mitis, S. sanguinis and S. mutans) and then treating with deionized distilled water (negative control). The bacterial population (CFU) retained in the respective single-species biofilms formed on the experimental pellicle was referred to as 100%.

The procedure was repeated using PEM and mouthrinse containing thymol (positive control) respectively instead of the deionized water. The effect of the PEM was determined from the percentage of bacteria retained in the respective biofilms using the equation below and compared with those of the positive (Thymol) and negative controls.

Based on the assumption that the bacterial population retained in the negative control biofilm is maximum and equal to 100%, the postulated formula to calculate percentage values for bacterial retention was:

\[
\text{Percentage of bacteria retained in the treated biofilms} = \frac{B}{A} \times 100
\]

Where \(A\) = Bacterial population in the negative control biofilm, and \(B\) = Bacterial population in the treated biofilms (PEM or Thymol) determined by estimating the sum of 30 determinations. The percentages of retention for the respective bacterial species are outlined in Table II.

Each glass bead with the biofilm was carefully taken out from the artificial mouth model and placed into a microcentrifuge tube (1.5 mL) containing 1.0 mL phosphate-buffered saline (PBS). The tube was sonicated for 10 seconds and vortexed for 1 minute to dislodge the bacteria that adhered to the glass beads. Following this, serial dilutions were carried out and 100 µL of the diluted bacterial suspensions from the 10³ dilution was pipetted out and streaked onto Brain Heart Infusion (BHI) agar. This procedure was carried out in triplicates. The plates were then incubated aerobically at 37°C for 18 - 24 hours followed by the determination of viable count of bacterial colonies the next day. This procedure was repeated with all the glass beads. The Colony Forming Units (CFU) in the 24 hours single-species biofilms were enumerated and presented as CFU mL⁻¹ x 10⁶. The plates with the CFU between 30 and 300 were used in the determination of adhered bacteria.¹⁶

All the data obtained (with one decimal point) was analyzed using Predictive Analysis Software (PASW) Statistics 18.0. The data in each group was normally distributed and hence one-way analysis of variance (ANOVA) was used to compare the respective PEM-treated and Thymol-treated with negative control. The values were expressed as mean ± SD. P-value of less than 0.05 is considered as statistically significant.

**RESULTS**

The adhering ability of the respective bacteria to the deionized distilled water-treated experimental pellicle (negative control) in this study was referred as 100%. It was found that the population of the early colonizers, S. mitis (605.7 ± 125.7 CFU mL⁻¹ x 10⁴) (p < 0.001) is significantly higher (2.4-folds) than that of the S. sanguinis (249.7 ± 20.1 CFU mL⁻¹ x 10⁴). S. mutans which is a secondary colonizer of dental biofilm...
Table I: The bacterial population (percentage of adherence) within single-species biofilm developed on PEM-treated saliva-coated glass beads compared to controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial population (CFU/mL x 10^4) expressed as mean ± SD</th>
<th>S. mitis</th>
<th>S. sanguinis</th>
<th>S. mutans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized distilled water</td>
<td>605.7 ± 125.7 (100%)</td>
<td>249.7 ± 20.1 (100%)</td>
<td>1165.7 ± 98.7 (100%)</td>
<td></td>
</tr>
<tr>
<td>PEM</td>
<td>(88.8 ± 0.6) (14.4 %)</td>
<td>(165.4 ± 10.3) (66.2 %)</td>
<td>(479.3 ± 144.2) (41.0 %)</td>
<td></td>
</tr>
<tr>
<td>Thymol (positive control)</td>
<td>(3.8 ± 1.1)h (0.6 %)</td>
<td>(139.0 ± 8.5) (56 %)</td>
<td>(4.9 ± 0.9)h (0.4 %)</td>
<td></td>
</tr>
</tbody>
</table>

Table II: The effect of Plant Extracts Mixture (PEM) on the bacterial population (percentage retained) within the single-species biofilms.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial population (CFU/mL x 10^4) expressed as mean ± SD</th>
<th>S. mitis</th>
<th>S. sanguinis</th>
<th>S. mutans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized distilled water</td>
<td>728 ± 80.3 (100%)</td>
<td>276.0 ± 8.0 (100%)</td>
<td>1787.7 ± 120.0 (100%)</td>
<td></td>
</tr>
<tr>
<td>PEM</td>
<td>(38.8 ± 5.6h) (5.3 %)</td>
<td>(212.7 ± 11.1) (77.1 %)</td>
<td>(100.4 ± 10.9) (5.6 %)</td>
<td></td>
</tr>
<tr>
<td>Thymol (positive control)</td>
<td>(14.6 ± 5.1h) (2%)</td>
<td>(113.5 ± 11.4) (41.1 %)</td>
<td>(13.9 ± 0.8)h (0.8 %)</td>
<td></td>
</tr>
</tbody>
</table>

For calculation of percentage values, the bacterial population (CFU/mL x 10^4) in biofilms was determined by estimating the sum of 30 determinations and the bacterial population adhered in the negative control biofilm is assumed to be maximum and equal to 100%; (mean ± SD) implies statistical significance (p < 0.001) when the PEM-treated beads were compared with the negative control treated beads, (mean ± SD)h implies statistical significance (p < 0.001) when the Thymol treated beads was compared with the negative control treated beads and (mean ± SD)h implies statistical significance (p < 0.001) when the PEM-treated beads was compared with Thymol treated beads; PEM = Plant Extracts Mixture.

There was a statistically significant reduction (p < 0.001) in the bacterial population in biofilms forming on the PEM-treated experimental pellicle when compared with that in the negative control treated experimental pellicle, indicating a reduction in the adherence. The corresponding (mean ± SD) values for bacterial population within the PEM-treated experimental pellicle was found to be (68.8 ± 18.4) for S. mitis, (165.4 ± 10.3) for S. sanguinis and (479.3 ± 144.2) for S. mutans.

It was shown that the bacterial population is also reduced in biofilms forming on the positive control treated experimental pellicle. The decrease in the adhering ability of the respective bacteria was significantly greater (p < 0.001), on comparison with that in the negative control. For S. mitis, the mean value representing its adherence in Thymol treated experimental pellicle was 3.8 ± 1.1 and for S. mutans it was, 4.9 ± 0.9. For S. sanguinis, the mean value of adherence was 139.0 ± 8.5 compared to the PEM-treated experimental pellicle (165.4 ± 10.3). This is statistically insignificant.

Table I shows the adhering ability of the early and secondary colonizers towards deionized distilled water (negative control), Thymol (positive control) and PEM-treated experimental pellicles.

The bacterial population retained in biofilm of the negative control represented maximum (100%) population. It was shown that the population of bacterial cells retained in the PEM-treated S. mitis biofilm (38.8 ± 5.6) and PEM-treated S. mutans biofilm (100.4 ± 10.9) was significantly reduced (p < 0.001) as compared to the population of bacterial cells in respective negative control biofilms for S. mitis (728 ± 80.3) and for S. mutans (1787.7 ± 120.0). In S. mitis (14.6 ± 5.1), S. sanguinis
(113.5 ± 11.4) and S. mutans (13.9 ± 0.8) biofilms, respectively treated with Thymol, the retention of bacterial population was significantly lowered with a p-value < 0.001 when compared with those in the negative control treated biofilms. For S. mitis, the mean value of retention in Thymol treated biofilm was (14.6 ± 5.1) which was almost statistically similar to that observed for PEM-treated S. mitis biofilm (38.8 ± 5.6). The bacterial population retained in the positive control treated S. sanguinis biofilm (113.5 ± 11.4) on statistical analysis was found out to be slightly lower (p < 0.001) than that in the PEM-treated S. sanguinis biofilm (212.7 ± 11.1).

Table II illustrates the bacterial population (in terms of percentage of retention) of the respective single-species dental biofilms treated with the PEM compared with negative and positive controls.

**DISCUSSION**

In the oral cavity, saliva is continuously flowing providing constituents for the formation of the acquired pellicle on tooth surfaces. The saliva-coated glass surface in this study was assumed to mimic the acquired pellicle on the tooth surface in the oral cavity and thus subsequently referred as an experimental pellicle. S. mitis and S. sanguinis are the early colonizers and S. mutans, the secondary colonizer of dental plaque. The adherence and colonization of the early colonizers to the acquired pellicle will provide an environment suitable for the adherence of secondary colonizers.2

It was shown in this study that the adhering abilities (in terms of bacterial population) of the respective S. mitis and S. mutans to experimental pellicle are higher than that of S. sanguinis. The different adhering ability of the early colonizers (S. mitis and S. sanguinis) towards the saliva-coated glass experimental pellicle is in agreement with what has been reported in the study by Wan Nordini Hasnor et al.,17 in which the adhering ability of S. mitis was found to be higher than that of S. sanguinis. However, this contradicts the report by another researcher,18 who have suggested that the high hydrophobicity of S. sanguinis contributes to its higher adhering ability than S. mitis. The discrepancy could be attributed to the difference in the environment used in the study. In the present study, the researchers used a dynamic environment whereas in previous studies,6,7 a planktonic situation was employed. Interactions other than hydrophobic interaction may be involved in the binding of the bacteria to the pellicle. It has also been reported that S. mutans, S. sanguinis and S. mitis interact with different pellicle receptor sites.19 S. mutans cells react with high molecular weight salivary glycoproteins which may be responsible for the direct attachment of the bacteria to the pellicle.19 Thus, the adhering ability demonstrated by the bacteria in this study may be attributed to the salivary components which possess surfaces complementary to those of the adhesions on the bacterial cell walls. In this study, the nutrient broth used did not contain sucrose and therefore, any adherence observed should not reflect sucrose involvement.

In this study, the effect of PEM on the adhering ability of bacteria was investigated. Thymol (0.064%) a commercially available mouthrinse containing thymol was considered appropriate for use as a positive control. Unlike chlorhexidine which is usually used as a positive control in previous studies, thymol is plant-based from the Thymus sp. and has been reported to inhibit the growth of oral bacteria including mutans streptococci.20 Jeon et al.21 indicated in their review paper of the great potential for the discovery of therapeutically relevant compounds from nature that can be used in antacaries chemotherapy. However, studies may need to include the implementation of standardized and stepwise cross-disciplinary approaches.

The PEM contains the extracts of Psidium sp., Mentha sp. and Mangifera sp. The phytochemical analysis of Psidium sp. showed that it possesses active ingredients like tannins, saponins, flavanoids and terpenes which have been reported to exhibit antibacterial and antifungal activity.22 It has also been reported to reduce bacterial aggregation of the early plaque colonizers.13 The Mentha sp. is known to contain active ingredients like carvone, menthone, menthol and methyl acetate all of which have the potential antibacterial and antifungal activity.23 In this study, the addition of the extract of Mentha sp. was more for its natural preservative and fungicidal potentials. The extract of Mangifera sp. contains tannins in addition to bitter gum and resins. The concentration of each of the plant extracts, made into a mixture (PEM) that was used in this study was below the sub-minimum inhibitory concentration (MIC) values, allowing for minimal if any bactericidal effect to be exhibited. The antimicrobial activities exhibited by Psidium guajava has a MIC value ranging from 1.25 to 10 mg/ml,22 and 4.69 mg/ml.13 For the Mentha sp. and Mangifera sp., the MIC values were above 5 mg/ml (unpublished data).

There is a decreasing trend in the adhering abilities (in terms of percentage values) of the three bacterial species to the PEM-treated experimental pellicle. S. mitis demonstrated the least adhering ability (11.4%), followed by the S. mutans (41.0%) and then S. sanguinis (66.2%).

The PEM contains a mixture of components that have antibacterial antioxidant properties which could modify the receptors on the experimental pellicle, reducing their abilities to interact with the adhesins on the bacterial cell walls.24 PEM has antiplaque and antacaries activities which are bacteria-specific, the results obtained in this study showed that the PEM appears to be more effective towards S. mitis and S. mutans than S. sanguinis. This trend was also observed with that of Thymol treated experimental pellicle.

In dental plaque formation, the acquired pellicle provides substratum for the early colonizers (S. mitis and S. sanguinis) to adhere. Both of the colonizers belong to the Streptococcus mitis-group which is considered to be important for the formation of dental biofilm.2,24 The reduced adhering abilities of S. mitis and S. sanguinis to PEM-treated experimental pellicle may provide a control
to the development of dental plaque. The PEM may exert its anti-adherence effect by modifying the receptors on the experimental pellicle. The modified receptors may be less efficient in its ability to interact with the incoming bacteria, resulting in reduced number of bacteria with the ability to adhere to the treated experimental pellicle. The PEM which is a crude preparation have ions that may have a role in the modification of the receptors on experimental pellicle. *S. mutans* is said to be responsible for the initiation of dental caries. Controlling its adhering ability and colonization can provide a mechanism which will be inhibitory to the development of cariogenic plaque.

Inoculating the experimental pellicle first with the respective inoculums and thereafter treating it with the PEM or thymol containing mouthrinse was used to determine the effect of them on the retention of bacterial population within the respective biofilms (*S. mitis*, *S. sanguinis* and *S. mutans*-biofilms). It is known that some bacteria can actively detach themselves within the biofilm and these detached cells then colonize some other intra oral surfaces. The bacterial populations (*S. mitis* and *S. mutans*) of the PEM-treated biofilms which were observed to decrease in this study could be attributed to cell detachment. The PEM may have influence on the secondary attachment forces like van der Waals forces. These forces may not involve specific interaction between the receptors on the acquired pellicle and the adhesins on the bacteria. The presence of PEM could have weakened these forces, resulting in cell detachment. The potential effect of PEM on the adherence of bacteria to the experimental pellicle and the population of bacteria within the treated biofilms is shown diagrammatically in Figures 1a and 1b.

Moreover, through toxicity studies PEM extracts showed no toxic effect on *Artemia salina* (unpublished observation), thereby ensuring the safe use of the extracts that can be developed into commercial oral products without any potential contraindications.

**CONCLUSION**

The results obtained in this study suggest that the PEM may have an influence on the development of dental biofilms by affecting the adhering ability to the pellicle and retention of bacteria in biofilm. Thus, the incorporation of PEM in a mouthrinse formulation may be considered for use in dental plaque control.

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