Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium sp.*, *Mangifera sp.*, and *Mentha sp.*) on single- and dual-species biofilms

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Background: Plant extracts mixture (PEM) and its individual constituent plant extracts (*Psidium* sp., *Mangifera* sp., *Mentha* sp.) are known to have an anti-adhering effect towards oral bacteria in the single-species biofilm. To date, the adhering ability of the early and late plaque colonisers (*Streptococcus sanguinis* and *Streptococcus mutans*) to PEM-treated experimental pellicle have not been investigated in dual-species biofilms. **Methods:** Fresh leaves of these plants were used in the preparation of the respective aqueous extract decoctions. The minimum inhibitory concentration (MIC) of the extracts towards *S. sanguinis* ATCC BAA-1455 and *S. mutans* ATCC 25175 was determined using a two-fold serial microdilution method. The sum of fractional inhibitory concentration (ΣFIC) index of PEM and its constituent plant extracts was calculated using the MIC values of the plants. The minimum bactericidal concentration (MBC) of the plant extracts was also determined. The anti-adherence effect of the plant extracts (individually and mixed) was carried out by developing simulated *S. sanguinis* and *S. mutans* respectively in single- and dual-species of biofilms in the Nordini's Artificial Mouth (NAM) model system in which the experimental pellicle was pretreated with the plant extract before bacterial inoculation. The bacterial population in the respective biofilms was quantified using ten-fold serial dilutions method and expressed as colony forming unit per ml (CFU/ml). The bacterial population was also viewed using Scanning Electron Microscope (SEM). All experiments were done in triplicate. **Results:** The PEM compared with its respective constituent plants showed the lowest MIC towards *S. sanguinis* (3.81 mg/ml) and *S. mutans* (1.91 mg/ml) and exhibited a synergistic effect. The *Psidium* sp. (15.24 mg/ml) and, PEM and *Psidium* sp. (30.48 mg/ml) showed the lowest MBC towards *S. sanguinis* and *S. mutans* respectively. The anti-adherence effect of

the PEM and its respective constituent plants (except *Psidium* sp.) was different for the two bacteria in the single-species biofilm. In the dual-species biofilms, PEM demonstrated similar anti-adherence effect towards *S. sanguinis* and *S. mutans*. The proportions of the bacterial population viewed under SEM appeared to be in agreement with the quantified population. **Discussion:** The combination of the active constituents of the individual plant extracts in PEM may contribute to its low MIC giving rise to the synergistic effect. The different anti-adherence effect towards *S. sanguinis* and *S. mutans* in both single- and dual-species biofilms could be due to the different proportion of the active constituents of the extracts and the interaction between different bacteria. The better adhering ability of *S. sanguinis* towards the PEM-treated pellicle when present together with *S. mutans* in the dual-species biofilms may suggest the potential of PEM in controlling the balance between the early and late colonisers in biofilms.

1   **Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its**

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3   **dual-species biofilms.**

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ABSTRACT

Background: Plant extracts mixture (PEM) and its individual constituent plant extracts (*Psidium* sp., *Mangifera* sp., *Mentha* sp.) are known to have an anti-adhering effect towards oral bacteria in the single-species biofilm. To date, the adhering ability of the early and late plaque colonisers (*Streptococcus sanguinis* and *Streptococcus mutans*) to PEM-treated experimental pellicle have not been investigated in dual-species biofilms.

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Results: The PEM compared with its respective constituent plants showed the lowest MIC towards *S. sanguinis* (3.81 mg/ml) and *S. mutans* (1.91 mg/ml) and exhibited a synergistic effect. The *Psidium* sp. (15.24 mg/ml) and, PEM and *Psidium* sp. (30.48 mg/ml) showed the lowest MBC towards *S. sanguinis* and *S. mutans* respectively. The anti-adherence effect of the PEM and its respective constituent plants (except *Psidium* sp.) was different for the two bacteria in the single-species biofilm. In the dual-species biofilms, PEM demonstrated similar anti-adherence effect towards *S. sanguinis* and *S. mutans*. The proportions of the bacterial population viewed under SEM appeared to be in agreement with the quantified population.

Discussion: The combination of the active constituents of the individual plant extracts in PEM may contribute to its low MIC giving rise to the synergistic effect. The different anti-adherence effect towards *S. sanguinis* and *S. mutans* in both single- and dual-species biofilms could be due to the different proportion of the active constituents of the extracts and the interaction between different bacteria. The better adhering ability of *S. sanguinis* towards the PEM-treated pellicle when present together with *S. mutans* in the dual-species biofilms may suggest the potential of PEM in controlling the balance between the early and late colonisers in biofilms.

87   **INTRODUCTION**

88   Bacterial adherence has a central role in the pathogenesis of oral diseases such as dental caries

89   and periodontal diseases, which are also known as plaque-related diseases. The formation of

90   dental plaque or oral biofilm is as follows. After tooth brushing, the tooth surface is immediately

91   covered with saliva forming an acellular layer known as acquired pellicle. The acquired pellicle

92   provides receptors for the oral bacteria to adhere and colonise. The pioneer bacteria that adhere

93   to and subsequently colonise the acquired pellicle are known as early colonisers which include

94   *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus mitis* (*S. mitis*) and *Actinomyces* sp.

95   (*Fathilah, Othman & Rahim, 1999*). It has been reported that *Streptococcus mutans* (*S. mutans*),

96   the late settlers of the acquired pellicle are the causative agent of dental caries (*Bowen, 2002*).

97   Bacteria in the oral cavity form a balanced population known as homeostasis and if imbalanced

98   can lead to the initiation of oral diseases (*Marsh & Martin, 2009*). Inhibition of bacterial

99   adherence to the acquired pellicle may play a role in the prevention of oral diseases.

100   Fluoride and chlorhexidine are among the chemicals incorporated into oral healthcare

101   products for the prevention of dental caries. They possess bactericidal activities and demonstrate

102   many adverse effects such as vomiting, diarrhoea, taste disturbance and teeth staining (*Chen &*

103   *Wang, 2010; Palombo, 2011*).

104   Past studies have been reporting plants with a wide range of biological activities that could

105   be useful for the development of alternative or adjunctive anti-plaque and anticaries therapies.

106   *Psidium guajava* L. (Myrtaceae) (*P. guajava*) contains phenolic compounds that have multiple

107   biological effects attributing to its antimicrobial and anticariogenic potentials (*Prabu,*

108   *Gnanamani & Sadulla, 2006*). Its leaves decoction have long been used in folklore practices to

109   maintain oral hygiene by exhibiting positive anti-adherence towards the adhesion of the early

110   colonisers onto saliva-coated glass surfaces (*Fathilah & Rahim, 2003*). The extracts have the

111   ability to alter and disturb the surface characteristics of the early plaque settlers thus reducing

112   their adherence (*Fathilah, Othman & Rahim, 2006*). *Mangifera indica* L. (Anacardiaceae)

113   (mango plant) (bark, roots and leaves) have been used in traditional medicine. Its leaves extract

114   can cause significant reduction of *Prevotella intermedia* and *Porphyromonas gingivalis*

115   compared to toothbrushing, a home care hygiene device (*Bairy et al., 2002*). *Mentha piperita* L.

116   (Lamiaceae) leaves extract contains tannin and flavonoids with antibacterial and antifungal

117   activities against selected oral pathogens. Its regular intake can ward off the initial colonisation

118   of pathogenic microbes (*Pramila et al., 2012*).

119   It has been shown *in vitro* that a mixture of aqueous extracts of three plant species (*Psidium*

120   sp., *Mangifera* sp. and *Mentha* sp.) which is referred as Plant Extract Mixture (PEM) in this

121   study has an anti-adherence effect towards early plaque colonisers (*Nordini, Fathilah & Rahim,*

122   *2013*) and towards early and late colonisers (*Rahim et al., 2014*) in single-species biofilm. To

123   date, there is no study on the effect of PEM and its individual extracts on dual-species biofilms in

124   a dynamic environment. Thus, this study investigated the effects of PEM and its individual

125   constituent plant extracts towards the adherence of bacteria in single- and dual-species biofilms.

126

127   **MATERIALS AND METHODS**

128   **Plant collection and extraction**

129   Fresh leaves of *Psidium* sp. (voucher no. 48126) and *Mangifera* sp. (voucher no. 48124) were

130   obtained from Balai Ungku Aziz of the University of Malaya, Kuala Lumpur and the UPM

131   Agriculture Park, Selangor respectively. Leaves of *Mentha* sp. (voucher no. 48127) grown in

132   Cameron Highlands, Pahang, were obtained from a local market in Selangor, Malaysia. The

133   plants were identified at the Rimba Ilmu Herbarium (University of Malaya) and deposited under

134   the stated voucher numbers. The fresh plant leaves were washed with tap water, followed by

135   deionised water, dried using tissue paper, weighed and cut into small pieces before boiling based

136   on the method described by *Nalina & Rahim* (*2006*). Briefly, 1 g of fresh leaves was boiled in 1

137   L of deionised water for several hours until the final volume is one-third of the initial volume.

138   The decoction was then filtered using a muslin cloth to remove any debris and the clear filtrate

139   was further centrifuged at 1500 x g, 4 °C for 15 min to eliminate any sediment. The supernatants

140   were filtered using Whatman No. 1 paper with a diameter of 150 cm and boiled again until the

141   final volume of 100 ml. The supernatants were then frozen overnight at -80 °C followed by

142   freeze-drying for 2 days using freeze dryer (Eyela FDV-1200, China) in a sterile environment.

143   The sterile dried crude aqueous extracts were stored at -20 °C for further use.

144

145   **Preparation of bacterial suspension**

146   *S. sanguinis* ATCC BAA-1455 (strain SK36) and *S. mutans* ATCC 25175 used in this study

147   were obtained from American Type Culture Collection (ATCC, USA). Prior to the experiment,

148   the respective 20% glycerol stocks of each bacterium at -80 °C were thawed at room temperature

149   and 1% of the strains were transferred in 20 ml of sterile fresh Brain Heart Infusion (BHI) broth

150   (Oxoid Ltd, UK), incubated aerobically at 37 °C with shaking at 150 rpm until mid-log phase of

151   growth (6-8 h). The bacterial suspensions were centrifuged at 5800 x g, 4 °C for 10 min and the

152   pellets were washed three times with ice-cold sterile deionised water, suspended in fresh BHI

153   broth and incubated at 37 °C for 15 min to reactivate their growth phase. The turbidity of each

154   strain was standardised by adjusting the absorbance to 0.144 (equivalent to 1.00×10^8 CFU/ml

155   and 1.53×10^7 CFU/ml for *S. sanguinis* and *S. mutans* respectively) at 550 nm using a

156   spectrophotometer (Shimadzu UV-1700, Japan). For dual-species, the standardised suspensions

157   of the two bacteria were mixed (1:1 vol/vol) prior to the experiment. The purity of the bacterial

158   cultures was checked each time prior to every experiment by streaking the culture broth on BHI

159   agar and incubated at 37 °C for 24-48 h.

160

161   **Determination of minimum inhibitory concentrations (MIC), minimum bactericidal**

162   **concentrations (MBC) and the sum of the fractional inhibitory concentration (ΣFIC) index**

163   A broth microdilution method in accordance with standard protocol CLSI (*CLSI, 2012*) and

164   modified method of *Kuete et al.* (*2009*) were used to determine the MIC of PEM and its

165   individual constituent plant extracts (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.). About 1 g of

166   sterile dried extract (*Psidium* sp.) was reconstituted in 7.81 ml sterile deionised water giving a

167   final concentration of extract at 128 mg/ml. After sonication for 30 s and vortexing for 30 min,

168   the reconstituted extract was centrifuged at 1500 x g, 4 °C for 15 min. The supernatant of the

169   reconstituted extract was serially diluted two-fold in BHI broth to obtain a range of concentration

170   (0.25-64 mg/ml) in respective test tubes labelled as T1 to T9. The diluted extracts were

171   transferred into the corresponding wells of a 96-well microtitre plate (NUNC™ Brand products)

172   as follows. To the first three rows of the 96-well microtitre plate labelled as A1 to A9; B1 to B9;

173   C1 to C9 (carried out in triplicate to ensure reproducibility), 100 μl of the respective serially

174   diluted plant extract in BHI broth (T1 to T9) was added. To wells labelled as A11, B11, C11, 100

175   μl of BHI broth was added to ensure bacterial growth. To all of the wells above, 5 μl of *S.*

176   *sanguinis* suspension (standardised to 10^6 CFU/ml) was added. This gave the final concentration

177   of the extract in the respective wells (A1 to A9; B1 to B9; C1 to C9) a range of concentration

178   from 0.24-60.95 mg/ml. In addition to these, wells labelled as A12, B12, C12 was filled with

179   105 μl of BHI broth just to ensure the sterility of the broth. To another row of wells, one row

180   apart from the others (E1 to E9), 105 µl of the two-fold serially diluted plant extract only was

181   added which served as blank control. The microtitre plate was sealed and covered with

182   aluminium foil, incubated aerobically at 37 ºC with shaking at 150 rpm for 24 h. Following

183   incubation, the MIC was determined at 550 nm using ELISA reader based on turbidity

184   determined by comparing the absorbance of the suspension in wells of test extract (A1 to A9; B1

185   to B9; C1 to C9) with that of the corresponding blank control. The MIC of each compound was

186   defined as the lowest concentration that inhibited the bacterial growth. Similar procedure was

187   repeated with *S. mutans*. The similar experiment was repeated for the other plant extracts

188   (*Mangifera* sp., *Mentha* sp. and PEM). For PEM, an equal volume of the stock solution of

189   extracts (*Psidium* sp., *Mangifera* sp. and *Mentha* sp.) each with a concentration of 128 mg/ml

190   was mixed followed by two-fold serial dilution.

191   For the MBC determination, 20 µl of the bacterial suspension from selected wells (A1 to

192   A9; B1 to B9; C1 to C9) which showed almost no turbidity was inoculated on BHI agar (Oxoid

193   Ltd, UK) and incubated for 48 h at 37 °C. The lowest concentration of the extracts that showed

194   no growth corresponded to the MBC value.

195   The sum of the fractional inhibitory concentration (ΣFIC) determines the synergy of the

196   plant extracts, calculated using the following equation:

197    $\Sigma FIC = 1/3(FIC_I + FIC_{II} + FIC_{III})$ and the FICs were calculated as follows:

198   FIC_I (FIC of *Psidium* sp.) $= \dfrac{MIC\ (PEM)}{MIC\ (Psidium\ sp.)}$
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201   FIC_{II} (FIC of *Mangifera* sp.) $= \dfrac{MIC\ (PEM)}{MIC\ (Mangifera\ sp.)}$
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204   FIC_{III} (FIC of *Mentha* sp.) $= \dfrac{MIC\ (PEM)}{MIC\ (Mentha\ sp.)}$
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207 The ΣFIC index determines the interaction between the different plant extracts in the mixture

208 where the interaction is interpreted according to a range of values; value less than 0.5 as

209 synergistic, value greater than 0.5 to 1 as additive, value greater than 1 to 4 as indifferent and

210 value greater than 4 as antagonistic (*Van Vuuren and Viljoen, 2011*).

211

212 **Preparation of Sterilised Saliva**

213 Undiluted sterile saliva was prepared according to the method described by *De Jong & Van der*

214 *Hoeven* (*1987*) and *Nordini et al.* (*2013*). To minimize variation, a single volunteer was used to

215 collect 100 ml of stimulated whole saliva (SWS). The SWS collection was done by expectoration

216 after chewing sugar-free gum (before eating or at least 2 h after eating) in ice-chilled test tubes.

217 The aggregation of the protein in the SWS was minimised by adding 1,4-Dithio-DL-threitol

218 (DTT) (GE Healthcare, Sweden) to a final concentration of 2.5 mM. The mixture was stirred

219 slowly for 10 min followed by centrifugation at 800 x g, 4 °C for 30 min. The supernatant was

220 then filter sterilised through a disposable 0.22 μm low-protein-binding filter (Cellulose acetate

221 syringe filters Sartorius, USA) into sterile centrifuge tubes and stored at -20°C until further use.

222 Prior to the experiment, the sterile SWS was thawed and centrifuged to remove any precipitate. It

223 was later used to coat glass beads (3 mm in diameter) (Merck, Germany) in an artificial mouth

224 (NAM) model forming a layer that mimics the acquired pellicle on the tooth surface and thus

225 referred as the Experimental Pellicle.

226

227 **Ethical approval**

228 The study protocol was reviewed and approved by the Ethics committee of Faculty of Dentistry

229 University of Malaya, Kuala Lumpur, Malaysia (The Ethic committee/IRB reference number:

230   DF OB1506/0070(P). Written informed consent was obtained from one donor for saliva

231   collection.

232

233   **Anti-adherence effect of plant extracts**

234   Prior to use, the dried aqueous extracts were reconstituted in sterile deionised water to a final

235   concentration of 0.5 mg/ml. The reconstituted extracts were further sterilised by filtration using

236   0.22 μm nylon syringe filters (Millipore Billerica, USA). For PEM, the same concentrations of

237   the sterile reconstituted individual extracts were mixed (1:1:1 vol/vol/vol) to give a mixture of

238   extracts of the same concentration (0.5 mg/ml).

239   Nordini's Artificial Mouth (NAM) model developed by *Rahim et al.* (*2008*) was used in the

240   *in vitro* study of the anti-adherence effect of PEM and its individual constituent plant extracts

241   towards the early and late plaque colonisers in single- and dual-species biofilms. The NAM

242   model was used in the study to represent oral cavity. The model consists of a glass chamber (1 x

243   6 cm length), glass beads (3 mm diameter), water bath system, saliva reservoir, bacterial

244   reservoir and a peristaltic pump. In this study, nine glass beads were placed in the glass chamber

245   of the NAM model. The glass beads represent the tooth surface. The glass chamber which was

246   immersed in the water bath system, set at 37 ºC represents the oral cavity. The peristaltic pump

247   (Masterflex L/S, USA) of the NAM model was used to flow the saliva/test extract/bacteria from

248   the reservoir into the system via rubber tubing at a constant flow rate (0.3 ml/min for resting

249   saliva). The experiment was carried out as follows. Saliva was first flowed into the NAM model

250   for 2 min at a constant rate to form an experimental pellicle on the glass beads. Excess saliva was

251   rinsed off by allowing sterile deionised water to flow in for 2 min. This allowed the experimental

252   pellicle to be pretreated with sterile deionised water. Following this, *S. sanguinis* which was

253 standardised to 0.144 absorbance at 550 nm wavelength was allowed to flow into the NAM

254 model for 24 h to form a 24-h biofilm on the experimental pellicle-coated glass beads.

255   Of the nine glass beads, three were taken out randomly whereas the remaining six glass

256 beads were kept for SEM viewing and reserves. The three glass beads were then individually

257 placed in a sterile microcentrifuge tube containing 1 ml Phosphate Buffered Saline (PBS) (pH

258 7.4), sonicated for 10 seconds and vortexed for one minute to dislodge the attached bacteria. The

259 suspension was serially diluted ten-fold (10^{-1} to 10^{-5} dilutions) with PBS. A 100 μl of the

260 homogeneous bacterial suspension from each tube was pipetted out and plated on three separate

261 BHI agar plates using the Lawn method. The plates were incubated for 24 h at 37 °C. The

262 counting of all viable microorganisms formed on the plate done using colony counter. Plates

263 from the dilution which gave a CFU number of between 30-300 colonies were selected and used

264 in the calculation of the growth population. The bacterial suspension used in the plating was 100

265 μl and hence it was expressed as CFU/ml based on the following formula:

266   Total CFU/ml = $\dfrac{\text{Number of colonies}}{\text{Volume plated (ml) x Dilution factor}}$
267
268

269 The deionized water treated experimental pellicle served as negative control for *S. sanguinis* in

270 single-species biofilm and the bacterial population (total CFU/ml) assumed as 100% bacterial

271 adherence. Similar procedure was repeated with *S. mutans* (standardised to 0.144 absorbance at

272 550 nm) for single-species biofilm. For dual-species biofilms, the individual strain was mixed in

273 equal amount (1:1 vol/vol) and a similar procedure was repeated. In the dual-species biofilms,

274 the two bacteria were differentiated according to their colony sizes. *S. mutans* colony is larger

275 than *S. sanguinis* allowing the colonies to be counted separately.

276   The experiment was subsequently repeated where the experimental pellicle was pretreated

277   with respective plant extract (*Psidium* sp./ *Mangifera* sp./ *Mentha* sp./PEM) at sub-MIC (0.5

278   mg/ml) before inoculation with bacterial suspension used for the development of single- and

279   dual-species biofilms. For positive control, the experimental pellicle was pretreated with 0.12%

280   chlorhexidine gluconate (CHX). The individual experiment was carried out in triplicate.

281   Percentage of adherence (X) was calculated using the equation below:

282    $X = \underline{\text{adhered cells (test or positive control)}} \times 100$
283   adhered cells (negative control)

284

285   Assuming the bacterial adherence to negative control was 100%, the percentage of anti-

286   adherence was calculated as:

287   % Anti-adherence = 100 – X

288

289   **Scanning Electron Microscopy (SEM) analysis of cell population on glass beads**

290   The remaining beads from the experiments were processed for SEM viewing according to the

291   method described by *Rahim & Thurairajah* (*2011*) with slight modification. Briefly, three glass

292   beads for each of experiment were fixed with 1 ml of 4% glutaraldehyde solution (prepared in

293   sodium cacodylate buffer, pH 7.4) and kept at 4 ºC until the subsequent analysis. Prior the

294   analysis, 4% glutaraldehyde solution was pipetted out and washed twice with sodium cacodylate

295   buffer for 15 min, followed by postfixing with 2% (vol/vol) osmium tetroxide in 1% (wt/vol)

296   sodium cacodylate buffer solution for 1 hour and kept at 4 ºC. Then, the glass beads were rinsed

297   with deionised water twice for 15 min before dehydration process. A series of 30, 50, 70, 80, and

298   95% ethanol at an interval of 15 min was used for dehydration process. This was followed by

299   dehydration carried out twice for 15 min each using 100% ethanol. Ethanol was gradually

300   displaced with acetone in the following ratios (vol/vol); Ethanol:Acetone 3:1, 1:1, 1:3 each for

301   20 min and finally followed by 100% acetone three times for 20 min. The dehydrated samples

302   were then subjected to Critical Point Drying (CPD) (Balzers CPD 030, Liechtenstein) for 1 h 40

303   min in liquid CO_2 under 95 bar pressure. The glass beads were then kept in a tight container in a

304   desiccator. Prior to SEM viewing, the beads were gold-coated under low pressure with ion

305   sputter coater (Joel JFC1100, Japan). The beads were viewed for cell population at 10,000X

306   magnification using Scanning Electron Microscope (Quante FEG 250, Holland).

307

308   **Statistical analysis**

309   The experiments were repeated and analysed using three triplicates and expressed as a mean ±

310   standard deviation, where the number of determinations, n = 27. Data were analysed using IBM

311   SPSS statistical software, version 23. The Shapiro-Wilk test was used to test assumptions of

312   normality. The results were analysed using Kruskal-Wallis and Mann-Whitney tests for non-

313   parametric statistical analysis to compare the difference between the two independent groups.

314   Results were considered significant at $p < 0.05$.

315

316   **RESULTS**

317   **Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC)**

318   **and the sum of the fractional inhibitory concentration (ΣFIC) index of plant extracts**

319   Table 1 shows the MIC, MBC and ΣFIC index of the PEM and its individual constituent plant

320   extracts against the early (*S. sanguinis*) and late (*S. mutans*) plaque colonisers. The MIC values

321   of PEM (*S. sanguinis* 3.81 mg/ml; *S. mutans* 1.91 mg/ml) were lower than those of the individual

322   extracts for the two bacteria. Among its individual constituent plant extracts, the *Psidium* sp.

323   showed the lowest MIC against *S. mutans* (3.81 mg/ml) whereas *Mentha* sp. and *Psidium* sp.

324 have almost similar MIC against *S. sanguinis* (7.62 mg/ml). It is shown that the MBC were

325 higher than the MIC for all the extracts. *Psidium* sp. (MBC value of 15.24 mg/ml) showed the

326 higher bactericidal effect compared with *Mangifera* sp., *Mentha* sp. and PEM (MBC value of

327 30.48 mg/ml) against *S. sanguinis*. The MBC value exhibited by PEM and *Psidium* sp. against *S.*

328 *mutans* (30.48 mg/ml) was lower compared with *Mangifera* sp. and *Mentha* sp. (60.95 mg/ml). It

329 was found from the calculation of the ΣFIC index, the interactions of the plant extracts are

330 synergistic for the two bacteria where the index is lower for *S. mutans* (ΣFIC = 0.25) compared

331 with *S. sanguinis* (ΣFIC = 0.42).

332

333 **Bacterial population adhering to the experimental pellicle**

334 The bacterial population for *S. sanguinis* and *S. mutans* adhering to experimental pellicle without

335 treatment (negative control) either individually (single-species) or together (dual-species) is

336 shown in Table 2. In a single-species biofilm, the population of *S. mutans* ($(2.48 \pm 0.16) \times 10^7$

337 CFU/ml) adhering to the experimental pellicle was ten times higher compared with *S. sanguinis*

338 ($(2.30 \pm 0.37) \times 10^6$ CFU/ml) and it is statistically significant ($p < 0.05$). However, in dual-

339 species biofilms, the *S. mutans* population ($(1.54 \pm 0.35) \times 10^7$ CFU/ml) adhered in similar

340 proportions with *S. sanguinis* ($(1.49 \pm 0.36) \times 10^7$ CFU/ml).

341

342 **Anti-adherence effect of plant extracts in single- and dual-species biofilms**

343 Figure 1 shows the anti-adherence effects of PEM and its respective constituent plant extracts in

344 single-species biofilm (*S. sanguinis* and *S. mutans*). The anti-adhering effect of all the extracts

345 (except *Psidium* sp.) towards *S. sanguinis* was significantly higher when compared with *S.*

346 *mutans*.

347   Figure 2 shows the anti-adherence activities of the PEM and its individual constituent plant

348   extracts in the dual-species biofilms. It was observed that *Psidium* sp. also demonstrated similar

349   and higher anti-adherence activities towards *S. sanguinis* (88.54 ± 4.47%) and *S. mutans* (88.65

350   ± 3.22%) compared with PEM, *Mangifera* sp. and *Mentha* sp. The *Mentha* sp. exhibited

351   significantly higher anti-adherence activity towards *S. sanguinis* (70.12 ± 6.90%) compared with

352   *S. mutans* (50.54 ± 18.64%). PEM demonstrated comparable anti-adherence effect towards *S.*

353   *sanguinis* and *S. mutans* (47.50 ± 17.93% and 49.08 ± 18.90% respectively).

354

355   **Bacterial adherence to plant extracts treated-experimental pellicle viewed under SEM**

356   Figures 3 and 4 show the SEM photographs of single-species biofilm viewed at 10,000X

357   magnification displaying the cell population adhering to the experimental pellicle (saliva-coated

358   glass beads) treated with plant extracts as well those of the negative and positive controls. The

359   population of *S. mutans* and *S. sanguinis* were almost zero in single-species biofilm adhering to

360   0.12% CHX-treated experimental pellicle (positive control) and very much reduced with plant

361   extract-treated when compared with the negative control.

362   Figure 5 shows the SEM photographs of the dual-species biofilms. It was observed that the

363   population of the two bacteria adhering to *Psidium* sp.-treated experimental pellicle was highly

364   reduced (Fig. 5D). It was different for *Mentha* sp-treated experimental pellicle, the adhered

365   population of *S. sanguinis* was less compared with *S. mutans* (Fig. 5F). The adherence of the two

366   bacteria to *Mangifera* sp.- (Fig. 5E) and PEM-treated experimental pellicle (Fig. 5C) respectively

367   were almost comparable.

368

369   **DISCUSSION**

370 In this study, the MIC value of *Psidium* sp. against *S. sanguinis* was higher (7.62 mg/ml)

371 compared with what has been reported by *Fathilah* (*2011*) which is 4.69 mg/ml. The difference

372 could be due to the bacterial strain used in which used clinical isolate whereas in this study the

373 bacterial strain was purchased from ATCC. *Prabu et al.* (*2006*) have reported the MIC value of

374 guaijaverin (4 mg/ml) towards *S. mutans* MTCC 1943. Guaijaverin is a biologically active

375 flavonoid of *P. guajava* (*Prabu et al., 2006*). The value obtained from our study can be

376 considered within range as we were using the crude aqueous extract.

377 PEM which is a mixture of equal amount of *Psidium* sp., *Mangifera* sp. and *Mentha* sp. has

378 low MIC compared with its individual constituent plant extracts. This suggests that PEM is a

379 better growth inhibitor which may be attributed to the combination of the active constituents of

380 the individual plant extracts, a synergistic interaction as indicated by the ΣFIC index.

381 PEM has the same MBC value towards *S. sanguinis* and *S. mutans* indicating that it exerts

382 similar bactericidal effect against the two bacteria. However, the *Psidium* sp. has lower MBC

383 value compared with PEM towards *S. sanguinis*. The lower MBC value might be due to *S.*

384 *sanguinis* aggregating in the presence of *Psidium* sp. (*Fathilah, 2011*) as the aggregated *S.*

385 *sanguinis* may not be reflected during plating. The similar MBC value of PEM towards the two

386 bacteria indicate that PEM has component(s) that may be bactericidal to both. The synergistic

387 effects of the individual constituent plant extracts in PEM contribute to its bactericidal and

388 growth inhibitory effects. It has been reported that there are synergistic interactions for

389 constituents within a total extract of a single herb, as well as between different herbs in a

390 formulation (*Williamson, 2001; Nahrstedt & Butterweck, 2010*).

391 Bacterial adherence has a central role in the formation of dental plaque (*Jakubovics &*

392 *Kolenbrander, 2010*). It is initiated by the formation of acquired pellicle on the tooth surface

393   which then become the substratum for bacteria colonization (*Jakubovics & Kolenbrander, 2010*).

394   Dental plaque, if not controlled, will subsequently lead to dental caries and periodontitis. It has

395   been a normal practice to brush teeth after meals. The scenario may be different if oral healthcare

396   products are to be used before meals. In this study, we simulate the use of healthcare product

397   before meals by treating the experimental pellicle with plant extracts before inoculating it with

398   the bacteria. This determines the adhering ability of bacteria towards the treated experimental

399   pellicle. Using the plant extract at sub-MIC range (*Hasan, Danishuddin & Khan, 2015*) will

400   allow adherence by viable cells only. The final concentration of PEM and its individual

401   constituent plant extracts (0.5 mg/ml) that were used in this study was well below the MIC which

402   has also been used for PEM in previous studies in single-species biofilm (*Rahim et al., 2014*).

403   In this study, the trend of adhered bacterial population to the experimental pellicle is

404   different in single-species compared with dual-species biofilms. In single-species, the adhered *S.*

405   *sanguinis* population was 10-fold lower compared with that of *S. mutans.* This is agreement with

406   what has been reported by *Rahim et al.* (*2014*). This may suggest that in single-species biofilm,

407   the bacterium (*S. sanguinis* and *S. mutans* respectively) interacts independently with the

408   components of the experimental pellicle. The behaviour of the bacterial population was found to

409   be different when they exist together in dual-species biofilms in which the two bacteria were

410   inoculated at the same time. The adhered bacterial population of the two bacteria was almost in

411   equal proportion. This can be attributed to the reduced competition between the bacteria when

412   they are inoculated at the same time as has been reported by *Kreth et al.* (*2005*).

413   The normal adhering process of the early and late colonisers (*S. sanguinis* and *S. mutans*

414   respectively) is disturbed when the experimental pellicle is pretreated with plant extracts before

415   inoculation (*Rahim et al., 2014*). Their study involved single-species biofilm while our study

416   included single- and dual-species biofilms in a dynamic environment. In a single-species biofilm,

417   the PEM showed a higher anti-adhering activity towards *S. sanguinis* compared with *S. mutans*.

418   The individual constituent plant extracts except *Psidium* sp. also showed the same profile of anti-

419   adherence. The latter is not in agreement with what has been reported by *Rahim et al.* (*2014*) which

420   was carried out in the same dynamic environment. The discrepancy could be due to the different

421   strain of *S. sanguinis* used in our study. In the dual-species biofilms, the pretreatment of experimental

422   pellicle with the plant extracts demonstrated a different profile of anti-adhering activity compared to single-

423   species. The anti-adhering activity exhibited by the *Psidium* sp. towards the two bacteria is highest

424   compared with the other extracts. PEM showed almost similar or about 50% of the anti-adhering activity

425   compared with the negative controls towards the two bacteria. This suggests that the adherence of *S.*

426   *mutans* to PEM-treated experimental pellicle enhanced the adherence of *S. sanguinis* when they

427   are together. The balanced bacterial population is important to maintain oral health against the

428   emergence of pathogenic bacteria following an imbalance in the oral resident microbiota (*Marsh*

429   *& Martin, 2009*). For example, the excess population of *S. mutans* can lead to the initiation of

430   dental caries (*Bowen, 2002*). The increase in the adherence of *S. sanguinis* in the presence of *S.*

431   *mutans* as in the dual-species may be attributed to modification of the complementary binding

432   sites between the adhesins on the bacterial surface and receptors on the treated pellicle (*Rahim et*

433   *al., 2014*). The population of *S. sanguinis,* a normal oral microflora, should be maintained as it

434   has the ability to generate antibacterial substance towards putative periodontal pathogens (*Ma et*

435   *al., 2014*) where the loss of its colonization appears to be associated with aggressive periodontitis

436   (*Stingu et al., 2008*). The colonization of *S. sanguinis* provides suitable substratum for

437   colonization of *S. mutans* following which the population of *S. sanguinis* is reduced (*Caufield et*

438   *al., 2000; Kreth et al., 2005*). The positive control, 0.12% CHX appeared to have no adhering

439   activity as the concentration used is more to the bactericidal (*Rahim & Thurairajah, 2011*).

440   The anti-adhering activity observed in plant extracts treated-experimental pellicle is a reflection of

441   viable cell adherence since the concentration used in the study was in the sub-MIC range. The phenolic

442   content of the plant extracts (*Thimothe et al., 2007*) may interfere the adherence of the bacterial

443   cells to the experimental pellicle. The reduced adherence may subsequently affect the biofilm

444   formation (*Rahim et al., 2014; Barnabé et al., 2014*).

445   The bacterial colonies adhering to the experimental pellicle (or plant extract-treated and

446   untreated) corresponded with the bacterial population viewed under SEM confirming the effect

447   of plant extracts. The *Psidium* sp. appears to aggregate *S. sanguinis* (Fig. 3D) and this is in

448   agreement with what had been reported by *Fathilah* (*2011*). Future studies may include the

449   inoculation of early coloniser prior to the late coloniser for dual-species biofilms. This is to study

450   whether the early coloniser to the plant extracts treated experimental pellicle has any influence

451   on the adherence of the later coloniser.

452

453   **CONCLUSION**

454   PEM appears to demonstrate a balanced population of the early-(*S. sanguinis*) and late-(*S.*

455   *mutans*) plaque colonisers. This may suggest its potential use as an oral healthcare product in

456   controlling the development of periodontitis and caries.

457

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461 of Medicine, UM and Faculty of Dentistry, UM for processing and viewing SEM samples

462 respectively.

463

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583 **Table 1**. The MIC, MBC and FIC value of PEM and its individual constituent plant extracts
584   against tested oral bacteria.

	[a]MIC(MBC) (mg/ml)				Fractional inhibitory concentration index				
	Psidium sp.	*Mangifera* sp.	*Mentha* sp.	[b]PEM	FIC_I	FIC_{II}	FIC_{III}	[c]$\Sigma FIC = 1/3$ ($FIC_I + FIC_{II} + FIC_{III}$)	Interaction
S. sanguinis ATCC BAA-1455	7.62 (15.24)	15.24 (30.48)	7.62 (30.48)	3.81 (30.48)	0.50	0.25	0.50	1/3(0.50 + 0.25 + 0.50) = 0.42	synergistic
S. mutans ATCC 25175	3.81 (30.48)	15.24 (60.95)	15.24 (60.95)	1.91 (30.48)	0.50	0.13	0.13	1/3(0.50 + 0.13 + 0.13) = 0.25	synergistic

585 The results are shown as average values of triplicate.
586 [a]MIC(MBC): minimum inhibitory concentration (minimum bactericidal concentration)
587 [b]PEM: Plant extract mixture of *Psidium* sp., *Mangifera* sp. and *Mentha* sp. in ratio of 1:1:1 (w/v)
588 [c]ΣFIC: the sum of fractional inhibitory concentration and was calculated according to the formula,
589 $\Sigma FIC = 1/3(FIC_I + FIC_{II} + FIC_{III})$
590 The ΣFIC index was interpreted as interactions which are synergistic (if < 0.5), additive (if in the range of > 0.5-1)
591 and indifferent (if in the range > 1-4), or as antagonist (if > 4).
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601   **Table 2**. Bacterial population in CFU/ml for the negative control for *S. sanguinis* and *S. mutans*
602   singly and in the mixture.
603

Bacteria	Bacteria population adhered to the experimental pellicle (negative control)[a] (mean ± SD) (CFU/ml)
S. sanguinis ATCC BAA-1455	$(2.30 \pm 0.37) \times 10^{6}$ *
S. mutans ATCC 25175	$(2.48 \pm 0.16) \times 10^{7}$ *
S. sanguinis ATCC BAA-1455 + *S. mutans* ATCC 25175	$(1.49 \pm 0.36) \times 10^{7} + (1.54 \pm 0.35) \times 10^{7}$

604   The results are shown as average values of triplicate (n = 27) and are expressed in a mean CFU/ml ± standard
605   deviation (SD).
606   [a]Negative control represents bacteria population adhered to the experimental pellicle-coated glass beads and was
607   assumed as 100%.
608   *$p < 0.05$ considered as significant.

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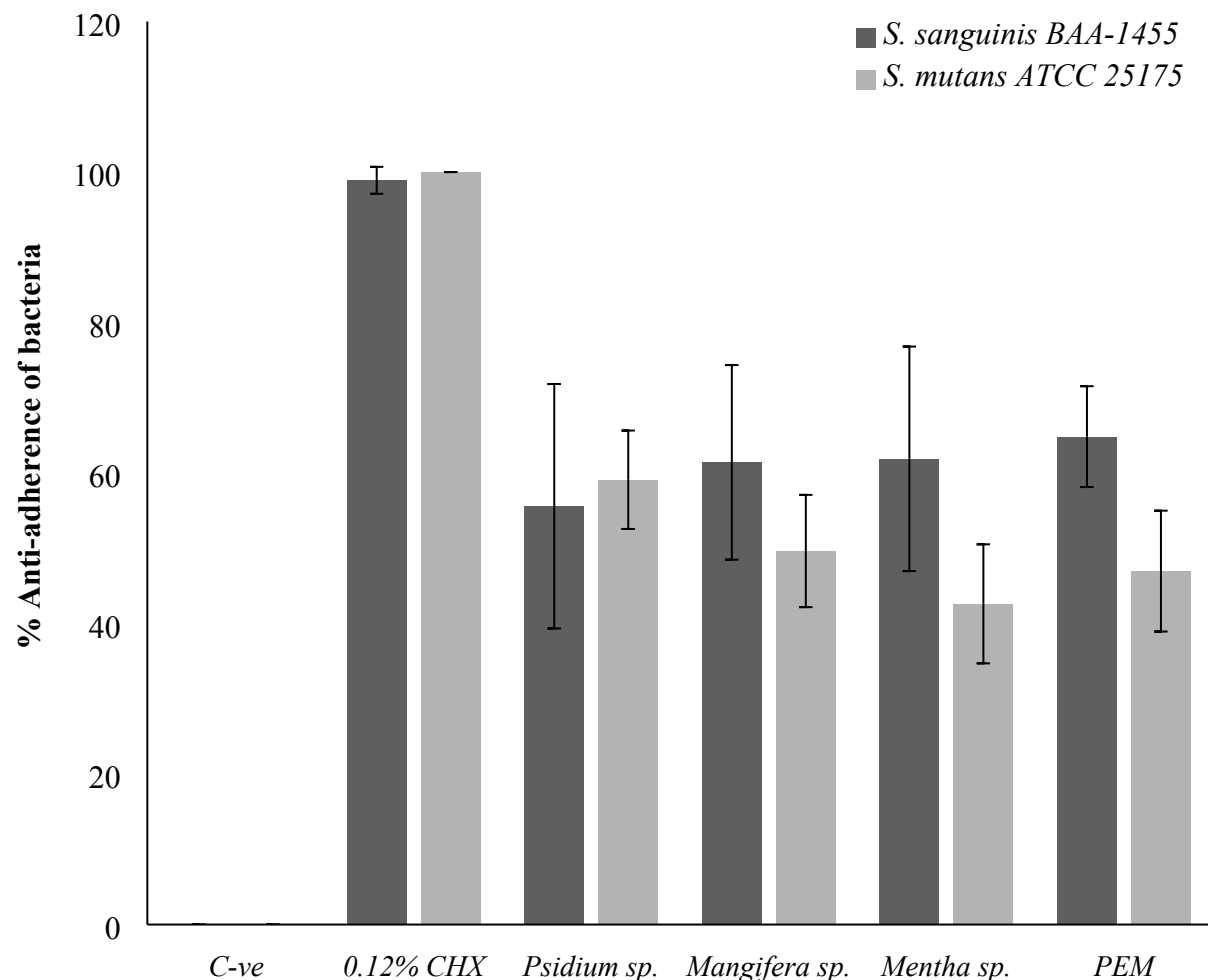
Fig. 1. The anti-adherence effect of PEM and its respective plant extracts in single-species biofilm (*S. sanguinis* and *S. mutans*). The percentage of anti-adhered bacterial cells present in the biofilm was expressed as mean ± standard deviation (SD) where a number of determinations (n) = 27.

a- $p < 0.05$ comparing between PEM, *Psidium* sp., *Mangifera* sp., *Mentha* sp., 0.12% CHX (positive control) and negative control (Kruskal-Wallis test).

b- $p < 0.05$ comparing between PEM and *Psidium* sp., PEM and positive control and PEM and negative control (Mann-Whitney test).

c- $p < 0.05$ comparing between *S. sanguinis* and *S. mutans* (treated with *Mangifera* sp.), *S. sanguinis* and *S. mutans* (treated with *Mentha* sp.) and *S. sanguinis* and *S. mutans* (treated with PEM) (Mann-Whitney test).

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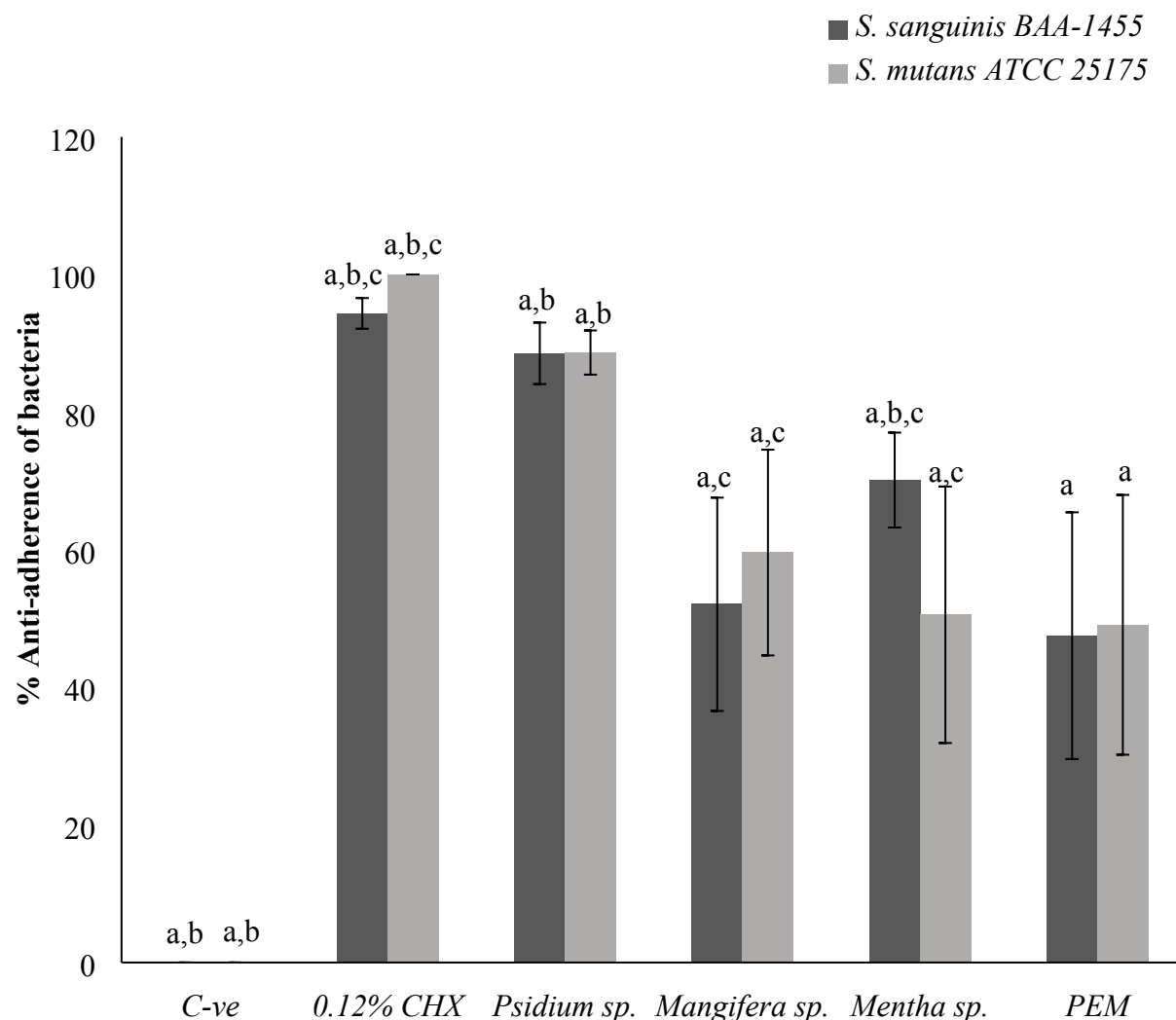
Fig. 2. The anti-adherence effect of PEM and its respective plant extract on dual-species (*S. sanguinis* + *S.mutans*) biofilms. The percentage of anti-adhered bacterial cells present in the biofilm was expressed as mean ± standard deviation (SD) where a number of determinations (n) = 27.

a- $p < 0.05$ comparing between the respective PEM, *Psidium* sp., *Mangifera* sp., *Mentha* sp., 0.12% CHX (positive control) and the negative control (Kruskal-Wallis test).

b- $p < 0.05$ comparing between PEM and *Psidium* sp., PEM and *Mentha* sp. (for *S. sanguinis*), PEM and positive control and, PEM and negative control (Mann-Whitney test).

c- $p < 0.05$ comparing between *S. mutans* and *S. sanguinis* (treated with *Mangifera* sp.), *S. mutans* and *S. sanguinis* (treated with *Mentha* sp.), *S. mutans* and *S. sanguinis* (treated with positive control) and, *S. mutans* and *S. sanguinis* (treated with negative control) (Mann-Whitney test).

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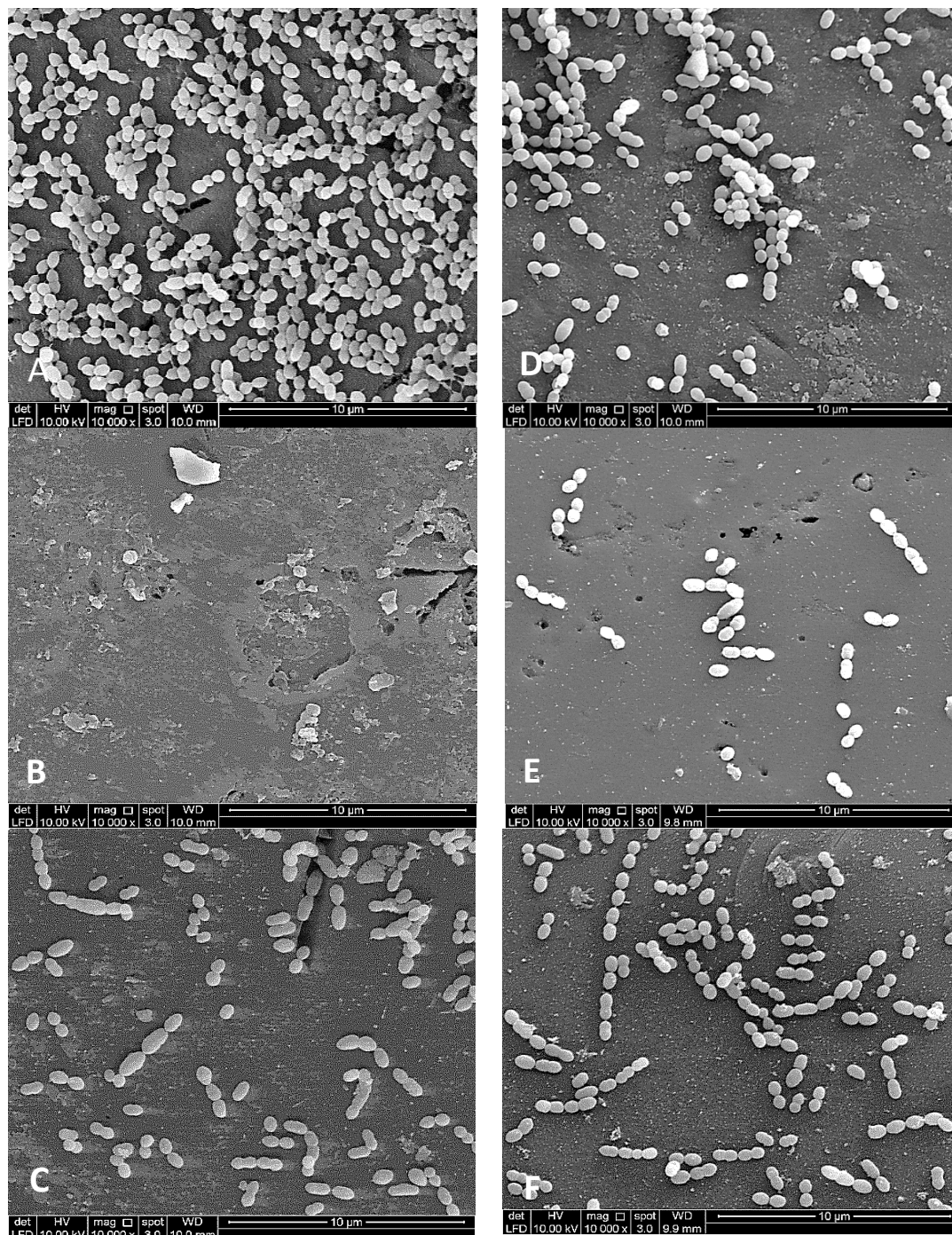
Fig. 3. The cell population of *S. sanguinis* viewed by Scanning Electron Microscopy (SEM) where the pellicle was treated with PEM {C} and its respective plant extracts (*Psidium* sp {D}; *Mangifera* sp. {E} and *Mentha* sp.{F}). The 0.12% chlorhexidine gluconate {B} and deionised water {A} served as positive and negative controls respectively. Magnification: 10,000X.

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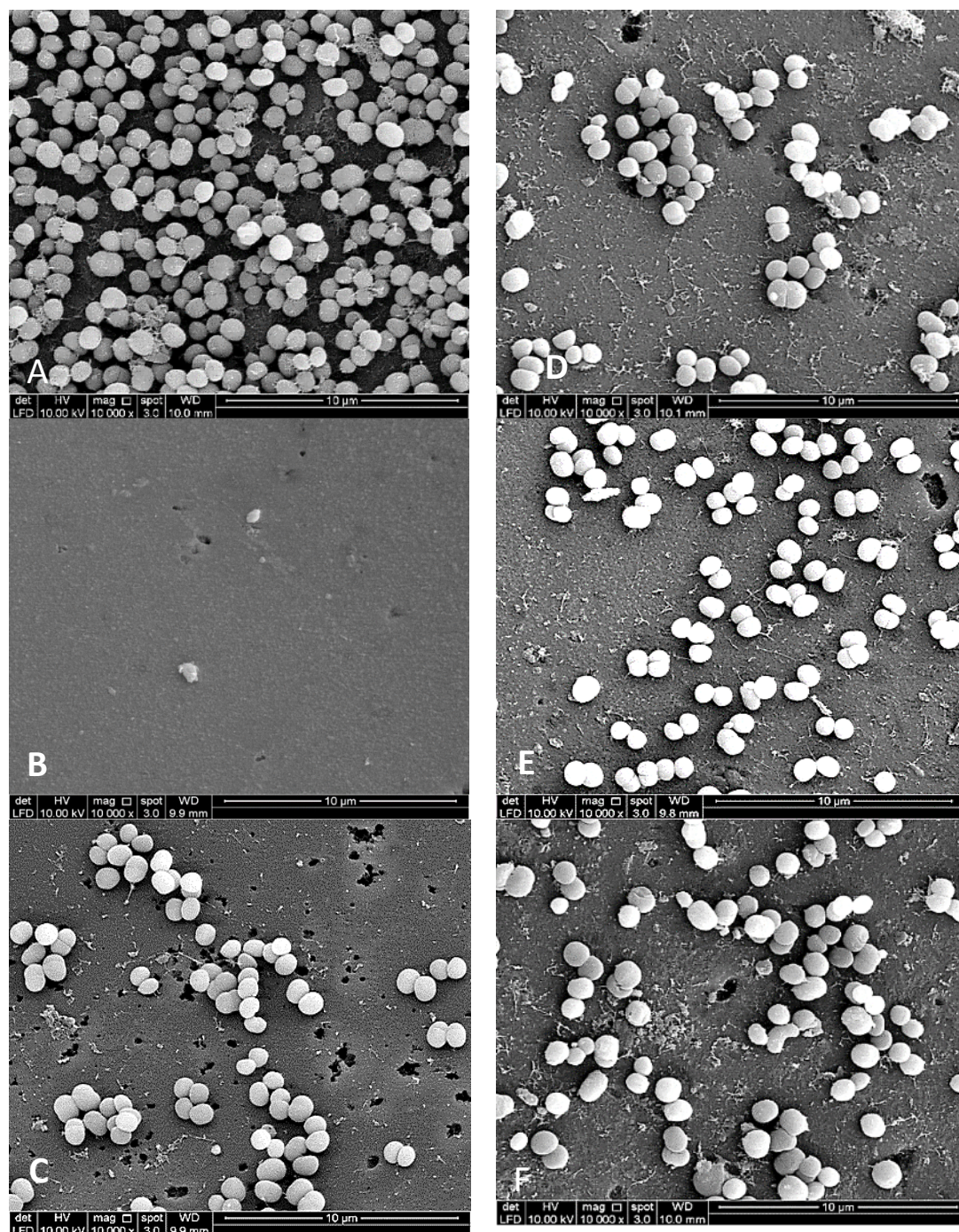
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672   **Fig. 4.** The cell population of *S. mutans* viewed by Scanning Electron Microscopy (SEM) where
673   the pellicle was treated with PEM {C} and its respective plant extracts (*Psidium* sp {D};
674   *Mangifera* sp. {E} and *Mentha* sp. {F}). The 0.12% chlorhexidine gluconate {B} and deionised
675   water {A} were served as positive and negative controls respectively. Magnification: 10,000X.

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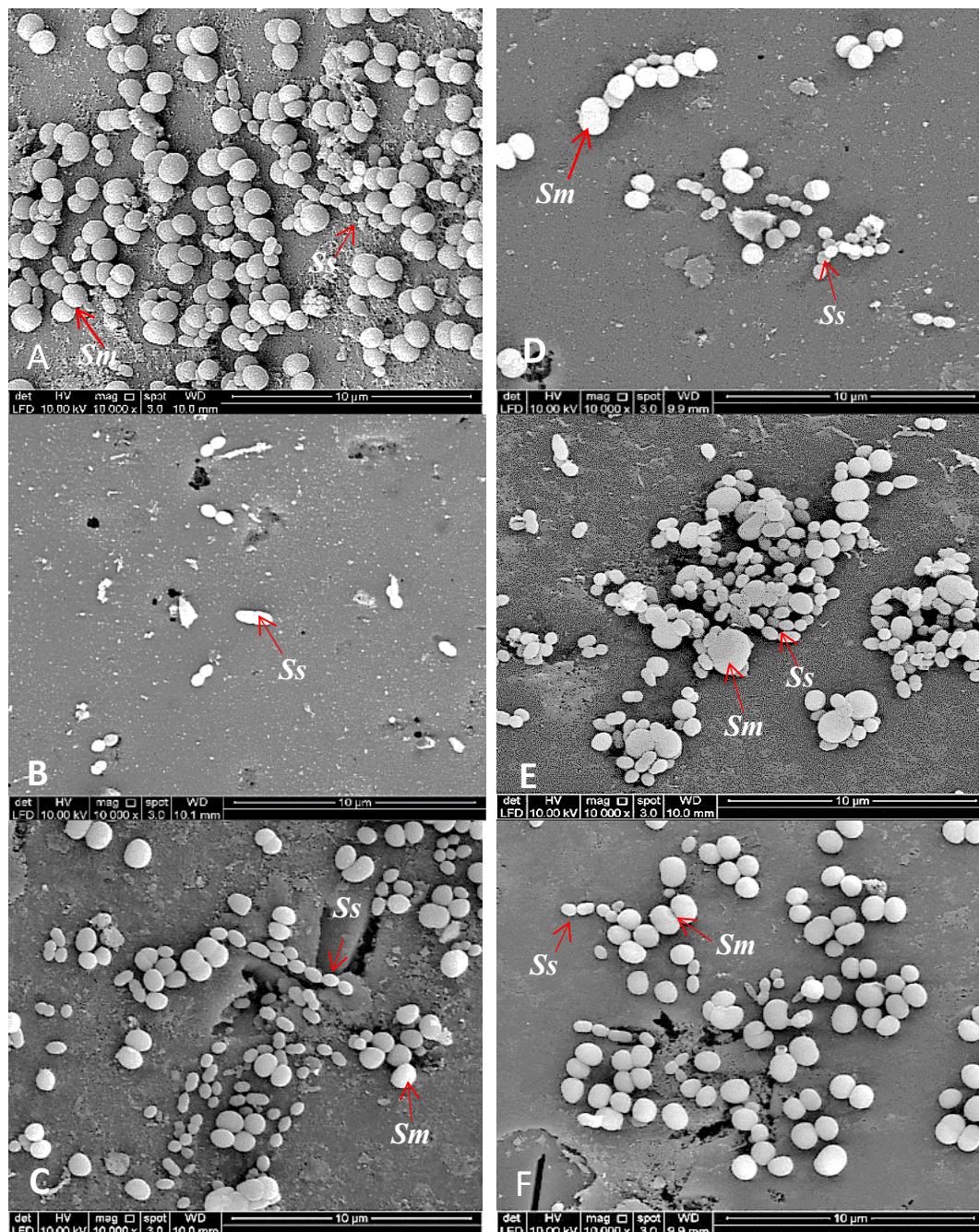
Fig. 5. The cell population of dual-species biofilms (*S. sanguinis* (*Ss*) + *S. mutans* (*Sm*)) viewed by Scanning Electron Microscopy (SEM) where the pellicle was treated with PEM {C} and its respective plant extracts (*Psidium* sp. {D}; *Mangifera* sp. {E} and *Mentha* sp.{F}) on cell adherence. The 0.12% chlorhexidine gluconate {B} and deionised water {A} were served as positive and negative controls respectively. Magnification: 10,000X.

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