Full Length Research Paper

Piper betle L. and *Psidium guajava L.* in oral health maintenance

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The objective of the study was to seek for new agents from plant extracts for use in dental plaque control. The emphasis was directed at the initial stage of dental plaque formation, specifically the adherence of bacteria referred to as the early plaque colonisers, to the acquired pellicle on the supragingival tooth surface. *Streptococcus sanguinis, Streptococcus mitis* and *Actinomyces* sp., the predominant bacteria involved at this initial stage of plaque development were used as test organisms. The aqueous extracts of *Piper betle* and *Psidium guajava* exhibited antimicrobial activities with MIC values in the range of 2.61 to 4.69 mg/mL and toxicity values (LC$_{50}$ and EC$_{50}$) well above their toxic concentrations. *P. betle* and *P. guajava* extracts contained 9.25 and 11.5 ppm fluoride, respectively. Some aggregating activity was also displayed by the extract of the latter. Both extracts showed positive antiadherence activity and reduced the cell-surface hydrophobicity of the bacteria which might have rendered them less adherent and hence, minimising their adhesion to the tooth surface during the early stage of plaque development. Both extracts also suppressed the growth of these bacteria. Such an activity was reaffirmed and supported by SEM micrographs whereby the bacterial cells were unable to divide or grow successfully and hence suggesting bacteriostatic effect of the extracts. Extracts of *P. betle* and *P. guajava* may work by first preventing and reducing the adhesion of primary bacterial colonisers to the tooth surface and second, to prevent or inhibit the growth and proliferation of microorganisms adhering on to the tooth surface and may potentiate their antiplaque activities.

Key words: Dental plaque, experimental pellicle, antiadherence, cell-surface hydrophobicity.

INTRODUCTION

Dental plaque continuously forms on the tooth surface. It is a localised concentration of bacteria embedded in a soft matrix consisting of extracellular polysaccharides, degraded mammalian cells and components of the saliva. In the oral cavity the flow of saliva over the surfaces of teeth plays a crucial role in plaque formation. Deposition of components from the saliva on to the tooth surface form a layer known as the acquired pellicle which through its components, provide binding receptors for the early plaque bacteria to colonise the tooth surface. The hydrophobic property of a bacterium cell-surface is an important factor and assists in the adherence of the bacterium to the host's hard tissues. As bacteria cells approach the acquired pellicle which also has hydrophobic residues, the chances of hydrophobic interactions between them are high.

Subsequent to the adhesion of these early colonisers, the cells may grow and multiply to form the ecosystem of the dental plaque. If allowed to accumulate as seen in subjects not practising good oral hygiene, dental plaque can lead to the development of oral diseases such as dental caries and periodontal diseases. Hexadecane has been used by researchers to mimic the hydrophobic nature of the acquired pellicle (McBride et al., 1984; Jenkinson, 1986; Koga et al., 1990). Effective plaque control strategies have become the basic principle in the prevention of plaque-related diseases like periodontitis.

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By preventing or limiting bacterial adhesion and their subsequent growth on the tooth surface, the severity of these diseases can be reduced. Chemical-based antimicrobial agents such as chlorhexidine gluconate (Cummins, 1992; Jones, 1997) are used extensively in mouthrinses to help maintain dental plaque at a level compatible with oral health. However, due to several reports on some local side effects of this compound (Matsumoto, 1999; Koo et al., 2000), finding alternative agents to be used as adjuncts in oral healthcare regimens became a prime interest.

The tropical rainforest of Southeast Asia has been estimated to house some 6,500 medicinal plant species. Malaysia, with her rainforest of about 19.12 million hectares is estimated to have about 8,100 plant species and 10% of these have been reported to have some medicinal value (Samy et al., 2009). Many of the plants which form the basis of many local folklore medicines have been known to have antimicrobial properties as they have been proven effective in treating microbe-related diseases such as vaginal thrush, psoriasis, chicken pox, shingles and the like. Betelvine or scientifically known as *Piper betle* L. is in the family *Piperaceae* and is native to the Indo-Malayan region. In India, it is one of the highly regarded herbs that are used in religious rituals and traditional ceremonies such as weddings. In Malaysia and also Indonesia, the plant is known as sireh. Like India, the use of sireh in many cultural functions is significant and explains the widespread cultivation to almost every backyard of Malay and Indian houses. The plant is propagated through cuttings taken from the top of mature vine.

Guava is the common name given to the fruits of *Psidium guajava* within the family of *Myrtaceae*. Originally found in the region between Peru and Mexico, this plant has spread to various parts of the tropical and sub-tropical areas. Various parts of the guava tree have been used traditionally to maintain oral hygiene (Prabu et al., 2006) and have displayed various biological activities such as antibacterial and antiviral (Ponglux et al., 1987; Hobert and Tietze, 2001; Obinna et al., 2008; Palombo, 2009). The leaf extracts of *P. guajava* and *P. betle* have been reported to exhibit therapeutic properties and are popularly used in the formulations of traditional medicines (Indu and Ng, 2000; Samy et al., 2009).

The objective of the study was to investigate the biological activities of *P. betle* and *P. guajava* extracts that may contribute to their application in plaque control. Data gathered will be used to elucidate the mode of action and efficiency of these extracts as antiplaque agents.

MATERIALS AND METHODS

Preparation of Plant Extracts

Aqueous crude extracts (CE) of *P. betle* and *P. guajava* were prepared by concentrating decoctions of the fresh leaves of the plants using a speed-vacuum concentrator (HETO/HS-1-110, Denmark). The dried extracts were weighed into sterile microfuge vials and prepared into stocks of 20 mg/mL using sterile distilledwater as the diluents.

Preparation of bacterial suspensions

Pure cultures of *S. sanguinis, S. mitis* and *Actinomyces* sp., representatives of the early dental plaque bacteria, were isolated from plaque specimens and stored at -80°C in glycerol stocks. The bacteria cells were revived in Brain Heart Infusion broth (BHI, Oxoid) and harvested by centrifugation at 10,000 rpm for 10 min. The cells were resuspended in BHI broth and the concentration was standardised to 10^6 cells/mL by using a spectrophotometer read at 550 nm (optical density of 0.014).

Determination of biological activities of plant extracts

Minimal Inhibition Concentration (MIC)

MIC was determined using broth dilution method (Smith et al., 1985). The CE was serially diluted in 10 tubes with the highest concentration in tube-1 and the least in tube-9. A volume of bacterial suspension at a turbidity of #0.5 Mc Farland standards was added to tube-2 through tube-10. After incubation at 37°C for 24 h, the least concentration in tubes with no visual growth or cloudiness represented the MIC of the extracts. Tube-1 with no bacteria cells represented the positive control while tube-10 without the extracts represented the negative control for the test.

Toxicity level

The toxicity of the extracts was determined using the brine shrimp bioassay (Meyer et al, 1982). The doses causing a 50% death (LD_{50}) was analysed using dead counts of shrimps at five different CE concentrations; 200, 400, 600, 800 and 1000 ppm (1 to 5 mg/mL). Dead shrimps were counted after 24 h. ED_{50} values were determined using tissue culture technique (Fathilah et al, 2010).

Determination of free water-extractable fluoride

The ion selective electrode, ISE (Istek, UK) was used to measure the level of fluoride ions in the extract preparation. In its ionized form, free F^- ions are easily detected by the electrode and measured in mV (Light and Cappucino, 1975).

Anti-adhesion study

Stimulated whole saliva was collected in ice-chilled tubes from a single donor by expectoration. The saliva was clarified by centrifugation at 17,000 g for 30 min and the pelleted debris was discarded. This effort was to render the saliva samples free of microorganisms from the donor. The inner surface of 9 triplicate sets of borosilicate glass culture tubes 75 x 12 mm were coated with the clarified saliva for 2 min and then lightly rinsed with sterile distilled water. Each set represented the experimental pellicle-coated binding surface for *S. mitis, S. sanguinis* and *Actinomyces* sp., respectively. The saliva-coating was to simulate the saliva-coated tooth surfaces in the oral cavity known as the acquired pellicle.

Bacterial suspension was then introduced and the tubes were incubated for 24 h to allow for the adhesion of bacteria to the experimental pellicle on the glass surface. Similar procedure was

repeated but this time the clarified saliva was replaced by sterile distilled water. The difference in the readings between the number of cells adhering to the saliva-coated to those adhering to the water-coated surfaces was determined. In another 12 triplicate sets of culture tubes, the experimental pellicle was first pre-treated with 2 ml of the plant extracts, CHX (positive control) and sterile distilled water (negative control), respectively before each of the respective bacterial suspensions was introduced. The reduction in the amount of bacteria cells adhering to the treated experimental pellicle as compared to those adhering to the untreated pellicle, was determined to indicate the antiadherence effect of the extracts. Throughout the study, chlorhexidine-containing (CHX) mouthrinse was used as a reference based on its widely accepted antimicrobial activities against oral microorganisms (Jones, 1997).

Bacterial cell-surface hydrophobicity study

The intensity of the hydrophobic interaction between surfaces of the bacteria cells and the experimental pellicle was measured using the method described by Gibbons and Etherden (1983). The hydrocarbon hexadecane was used to provide the hydrophobic binding surface in this experiment. To test for the effect of the plants extracts on the cell-surface hydrophobicity, the bacterial cells were first exposed to various concentrations of the extracts prior to the addition of hexadecane. Following rigorous agitation of the suspensions, the optical densities (OD) of the unbound cells which corresponded directly to the opacity of the bottom layer were determined. The percentage of adsorption of the treated cells to hexadecane was calculated and compared to that of the untreated. A detailed procedure of the experiment is available in Fathilah et al. (2006).

Growth profile and ultrastructural study

The method of Matsumoto et al. (1999) was closely followed. Sterile metal capped test tubes (13 x 75) mm containing 5 mL of growth medium were used as culture tubes. The tubes were divided into four sets of triplicates to represent growth of *S. sanguinis, S. mitis* and *Actinomyces* sp. under four different growth conditions; (1) normal growth in BHI broth, (2) growth in the presence of *P. betle* extract (4 mg/mL), (3) growth in the presence of *P. guajava* extract (4 mg/mL), and (4) growth in the presence of CHX-containing mouthrinse (0.12 µg/mL).

Following the inoculation of bacteria cells, the growth in each of the sets was monitored periodically every 15 min intervals over a period of 9 h based on changes in their optical absorbance at 550 nm. The growth profiles of the bacteria were then plotted and analysed against the controls. The detail of the procedure is available in Fathilah et al. (2009). At the mid of the logarithmic phase of growth, an inoculum of bacteria cells was pipetted out and processed for scanning electron microscope (SEM) examinations. Alterations to the morphology involving cell sizes and surface characteristics as compared to the controls were recorded.

RESULTS AND DISCUSSION

Amongst the biological activities of the aqueous extracts of *P. betle* and *P. guajava* identified in this study include positive antimicrobial activities towards the three bacteria with MIC values ranging from 2.61 to 4.69 mg/mL. Some aggregating activity on the bacteria cells was displayed by *P. guajava* extract. As indicated in the toxicity studies, both extracts were not toxic. The LC_{50} of *P. betle* was

3.6 mg/mL and not determinable for *P. guajava.* The EC_{50} of *P. guajava* was at 51.5 mg/mL but not determinable for *P. betle.* Both extracts contained fluoride at 9.25 and 11.5 ppm for *P. betle* and *P. guajava*, respectively.

Fluoride is an effective cariostatic agent and has been known to affect the adhesion, aggregation and metabolism of bacteria (Maltz and Emilson, 1982; Lagerlof and Oliveby, 1994). It has been reported to be bactericidal as it inhibits the enzyme enolase which is crucial in the functioning of the glycolytic pathway that supplies the bacteria cell with energy for the maintenance of cells viability (Hamilton, 1990). The presence of fluoride on the surfaces of a substratum has been shown to inhibit the growth of *S. mutans* and *Actinomyces naeslundii* biofilms significantly.

A simple effective assay was designed to screen the plant extracts for anti-adhesion activities. In this assay the activity of the plant extracts was targeted at the experimental pellicle. The number of cells attached to the untreated experimental pellicle is a representative of the maximum adhesion capacities of the experimental pellicle for the bacteria. Based on the results obtained from the adherence studies both *P. betle* and *P. guajava* extracts showed positive anti-adherence activity (Figure 1) and exhibited the ability to reduce the cell-surface hydrophobicity (Figure 2) of the bacteria cells (Fathilah and Rahim, 2003; Fathilah et al., 2006).

In this *in vitro* experiment, it was observed that bacteria cells with hydrophobic cell-surface bind to the non-aqueous hydrophobic hexadecane layer on top of the assay mixture. The unbound non-hydropbobic bacteria cells stayed in the aqueous portion at the bottom of the test tube. In the oral cavity, both these effects may render the bacteria cells to become less adherent which may restrict and minimise their ability to adhere to the acquired pellicle during the early stage of plaque formation.

The growth patterns displayed in Figure 3 showed that *P. betle* and *P. guajava* extracts exerted bacteriostatic effects on the growing cells. This was indicated by the extension of the generation times and reduction of the specific growth rates (Fathilah et al., 2009a), characteristics often observed when cells are grown under a suppressed growth condition. Figure 4, 5 and 6 were micrographs of the bacteria cells examined under the normal and suppressed growth conditions following treatment with the extracts. The micrographs showed that under the untreated condition, optimum cell sizes and volumes were attained that enable them to reproduce actively. Under the influence of the extracts however, the bacteria cells did not attain the predetermined sizes as they were observed to be slightly smaller. Since the basic requirement for reproduction was not met, many of the treated cells were observed to be at the non-dividing state (Figure 4c and d, 5c and d, and 6c and d).

The many biological activities possess by the extracts of *P. betle* and *P. guajava* collectively contribute to their positive antimicrobial effects on the early plaque bacteria;
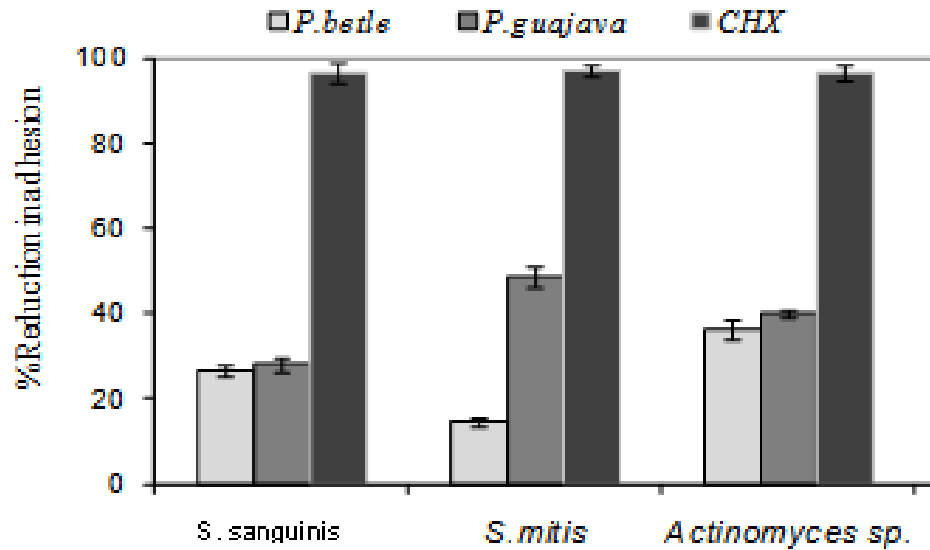
Figure 1. The antiadherence effect of *P. betle, P. guajava* and CHX-containing mouthrinse which act as a positive control in the study. Though not comparable to the antiadherence activity of CHX, the extracts of both plants exerted considerable reduction in bacterial adhesion to the saliva-coated glass surface. The percentages of reduced adherence showed in the chart were expressed as means ± standard deviation of nine determinations.
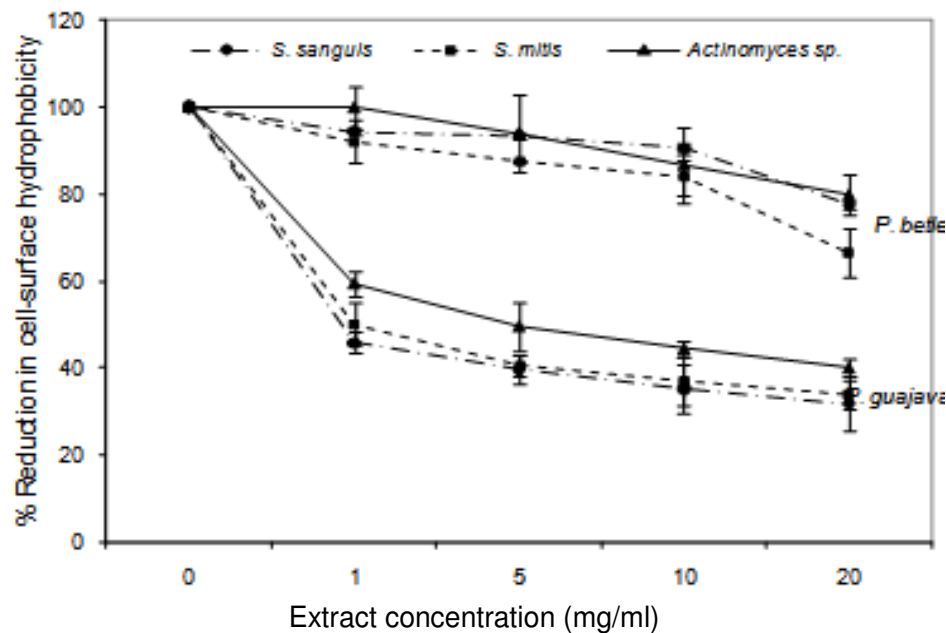

Figure 2. The effect of *P. betle* and *P. guajava* on the cell-surface hydrophobicity of *S. sanguinis, S. mitis* and Actinomyces sp. The percentages were means ± standard deviations of nine determinations.
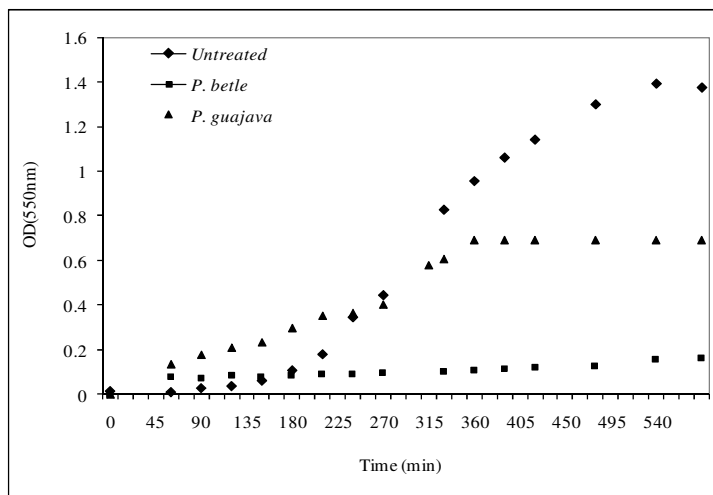
S. sanguinis, S. mitis and *Actinomyces* sp. As an antiplaque agent, it can be suggested that the extracts work by first, modifying the properties of the acquired pellicle and second, reducing the hydrophobicity of the bacterial cell surface rendering them less compatible for adherence to the acquired pellicle on the tooth surface. For the attached bacteria, the growth suppressive effect of the extracts may control the development of plaque by
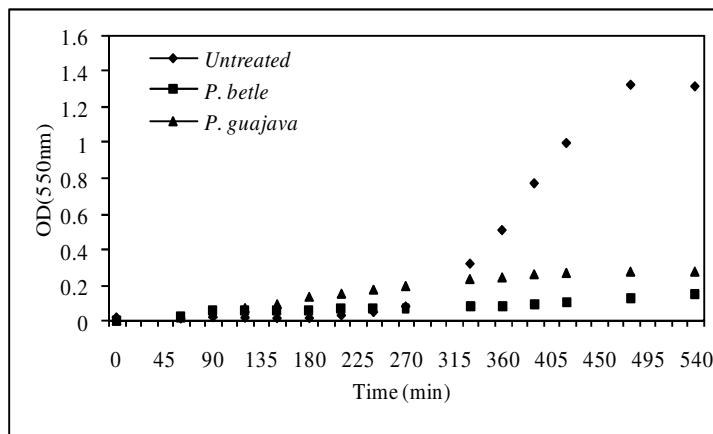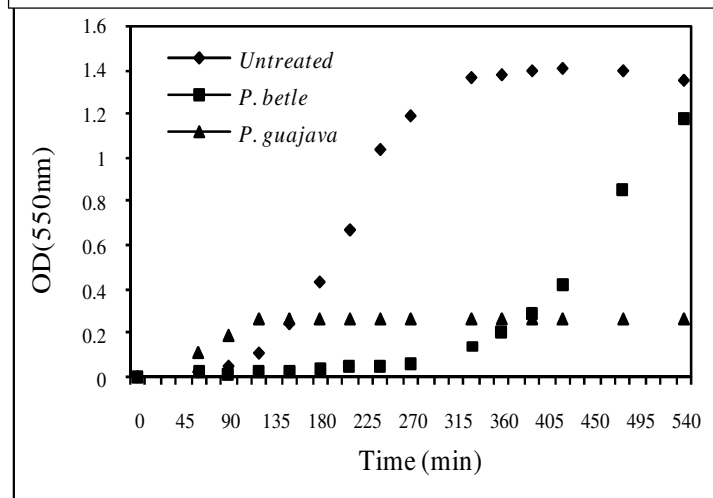
Figure 3. The growth profiles of (a) *S. sanguinis,* (b) *S. mitis* and (c) *Actinomyces* sp. plotted under the untreated, *P. betle-* and *P. guajava*-treated growth conditions. Deviation of curves from the untreated profile indicated the effect of the extracts on the growth cycles.

minimising the growth and proliferation of the bacteria cells. All the properties exhibited by the extracts towards the early plaque bacteria potentiate the antiplaque activities of *P. betle* and *P. guajava* extracts. An antiplaque is an agent or compound that exerts effect on the plaque, which will later result in the reduction of
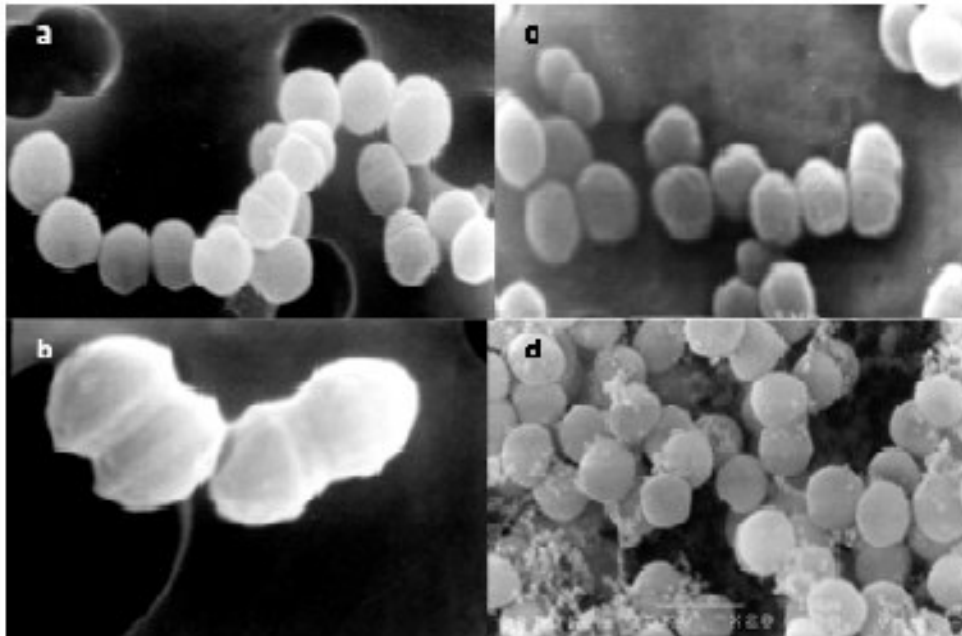
Figure 4. SEM micrograph of *S. sanguinis.* (a) Majority of the cells cultured under the untreated condition were at the dividing state (90,000x). (b) Higher magnification of dividing cells showing the groove where the cell would split into two identical cells (200,000x). (c) Cells cultured following exposure to *P. betle* extract were of variable sizes and majority existed as single, non-dividing cells (90,000x). (d) Under the influence of *P. guajava*, the size of the cells were more uniform and were clumped together in extracellular matrices (90,000x).
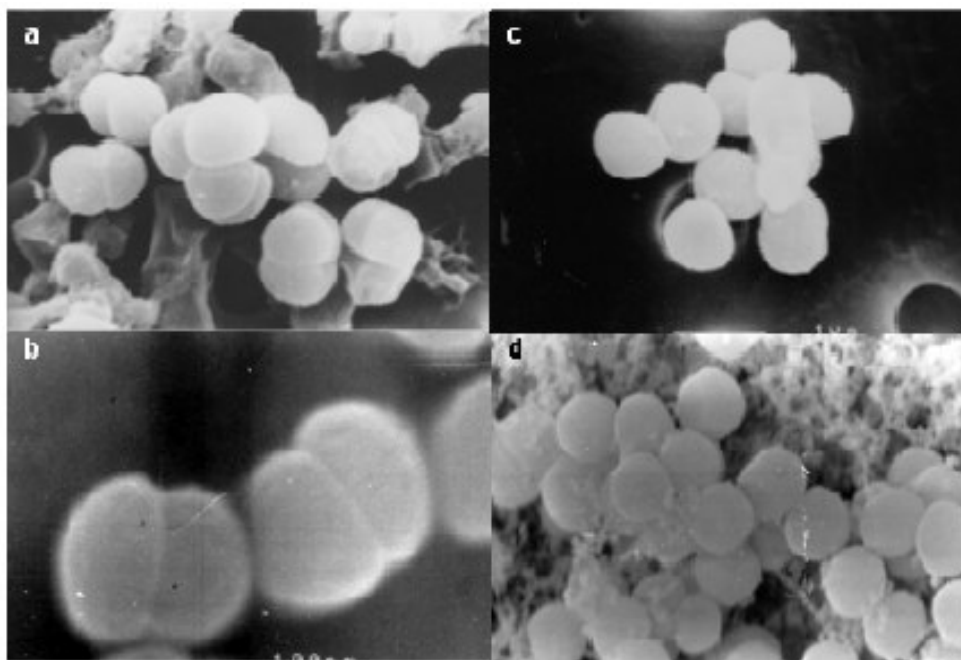
Figure 5. SEM micrographs of *S. mitis.* (a) Majority of the cells cultured under the untreated growth condition were in the dividing state (90,000x). (b) The dividing cells at higher magnification (200,000x). (c) Under the influence of *P. betle*, cell were observed to be of uniform sizes and existed as single non-dividing cells (90,000x). (d) Cells cultured in the presence of *P. guajava* were also of uniform sizes and aggregated together in heavy extracellular matrices (90,000x).
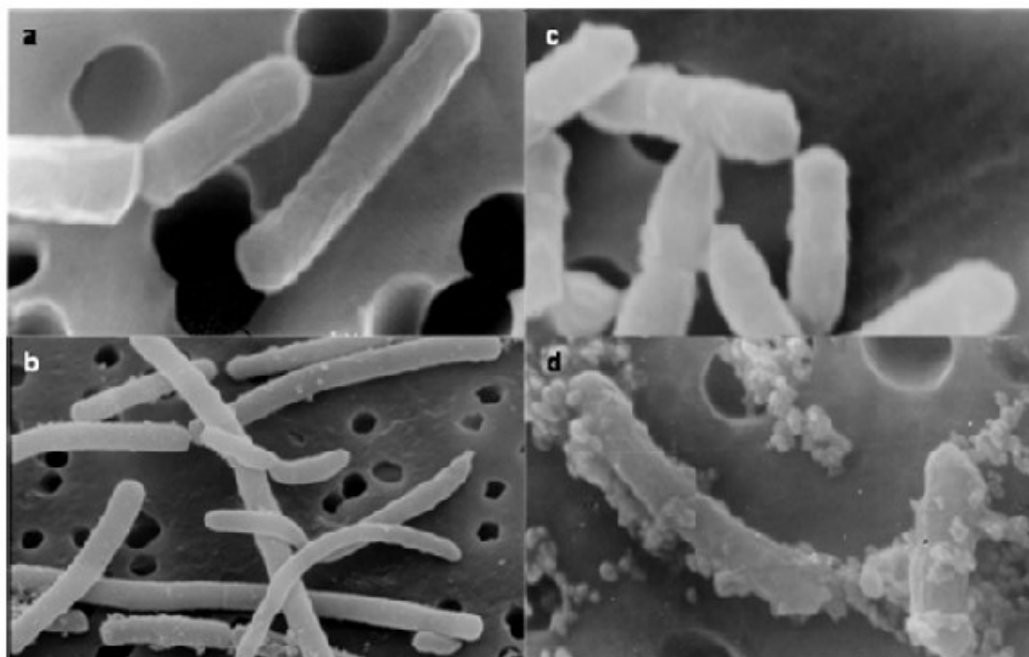
Figure 6. SEM micrographs of *Actinomyces* sp. (a) Cells resumed rod shape with smooth surface under the untreated growth condition (90,000x). (b) The varying length resumed by the cells under the untreated condition was obvious at lower magnification (30,000x). (c) Cells cultured under the influence of *P. betle* display tapering ends and irregular surfaces (90,000x). (d) In the presence of *P. guajava* extracts, the cells were observed to be covered in extracellular matrices (90,000x).

caries and gingivitis (Addy et al.*,* 1992). The basic scientific data on the properties possessed by *P. betle* and *P. guajava* gathered in this study may assist in the formulation of these extracts for use as antiplaque agent in mouthrinses.

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