CHAPTER 5

DISCUSSION, RECOMMENDATIONS AND CONCLUSIONS

5.1 Introduction

This chapter discussed the results from Chapter 4, including microbial composition in subgingival plaque, antibacterial activities of SPSE against *S. mutans* and *P. gingivalis* and chemical composition in SPSE, which act as a therapeutic agent in oral disease. The recommendation and implications of this study were also detailed in this chapter.

5.2 Summary and Discussion of Results

5.2.1 Microbial Composition in Subgingival Plaques

Carious lesions form in the presence of dental plaque, a thick bacterial formation that adheres to tooth surfaces. Individuals that fail to adequately remove gingival plaque may result in gingival inflammation and deepened periodontal pocket. Thus, subgingival plaque will be developed caused by bacterial accumulation. This study has identified both types of Gram-positive and Gram-negative bacteria from each subgingival plaque sample (Table 4.2) after performing colony forming unit to calculate the viable bacteria (Table 4.1).

Moreover, subgingival plaques have been implicated in periodontitis, a chronic periodontal disease characterised by gingival inflammation and loss of alveolar bone.

Gram-positive bacteria are typically found at the root surfaces by an overlying zone of loosely attached Gram-negative bacteria.

Each bacterial species has its own DNA sequence that encodes the 16S rRNA, which is used in this study method to identify the variety of oral bacteria. In addition, 16S rRNA-targeted probes were used to detect oral bacteria in dental plaque without altering community architecture. Nevertheless, there were different methods used by researchers to identify the composition of oral microflora. For example, the PCR method was applied to detect the presence of the specific target of species in subgingival plaque due to its sensitivity and specificity by using suitable primers. In addition, RT-PCR can quantify a limited number of species because of its advantages of being specific, sensitive, and providing quantification of selected bacteria species (Heller et al., 2012).

From the results, each collected subgingival plaque sample was able to be identified for both Gram-positive and Gram-negative bacteria. A total of 97 (55.11%) and 79 (44.89%) strains of Gram-positive and Gram-negative bacteria were found after being isolated from a few colonies from the media, which was used as a medium for bacterial culture growth and providing nutrients. From 176 of isolated oral bacteria, a total of 17 bacterial species was identified among nine samples, including *S. gordonii, S. mutans, S. sanguinis, S. oralis, S. agalactiae, S. downei, S. luteinsis, S. salivarius* and *S. anginosus* for Gram-positive bacteria and eight types of Gram-negative bacteria, such as *P. gingivalis*, *A. actinomycetemcomitans, K. pneumoniae, E. clocae, E. ludwigii, E. mori, E. tabacii* and *E. raggen kampii*.

The phylogenetic tree of oral bacteria species from 10 subgingival plaque samples was shown in Figure 4.28. To generate phylogenetic tree, alignments of nucleotides from

17 selected strain accession numbers (from NCBI) were compared and analysed using Mega X software. Based on the results, the two species possess a more recent common ancestor (*S. gordonii* and *S. oralis*), while they were a less related when they have less recent common ancestor (*A. actinomycetemcomitans*).

Oral streptococci are predominant and colonise early because they coaggregate with a range of oral pathogens and bind to different host molecules (Kolenbrander et al., 2002). Table 4.3 shows that *S. mutans* (Gram-positive) and *P. gingivalis* (Gram-negative) have high prevalence with 31 and 34 number of respectively isolates from subgingival plaque. Thus, both oral bacteria were selected in this study to determine their growth after being exposed to or treated with SPSE. The amount of *S. mutans* was substantially higher in plaques which initiate carious lesions on tooth surfaces.

Other early colonizers, such as *Actinomyces* spp., *Capnocytophaga* spp., *Prevotella* spp., and *Haemophilus* spp. also interacted with each other. There were researchers performed the restriction fragment length polymorphism (RFLP) method to identify oral bacteria species (Sano et al., 2021). In that study, a total of 848 strains were found with 113 genetic types. Experiments using animal model systems also indicated increased colonization of *S. mutans* in the presence of sucrose. However, *S. salivarious* is usually present in high concentrations in saliva and low concentration in plaques. Besides that, *S. sanguinis* and *S. mitis* were the predominant species found in supragingival plaque (Gibbons, 1980).

The development of biofilm is produced when there is constant bulk fluid at mucosal surfaces with saliva. Mutacins produced by streptococci have been proposed to regulate the formation of dental plaque. In addition, the amount of *S. mutans*-infected tooth

surfaces also correlates with dental caries (Heller et al., 2012). Commonly, Gram-negative bacteria are placed in late colonizers. Moreover, the bacteria species involved in early and late colonizers is *F. nucleatum*, which was found abundant in healthy and diseased individuals (Kolenbrander et al., 2002). The presence of significant numbers of Gram-negative bacterium in subgingival plaque may be due to the existence of crevicular fluid, which includes anaerobic environment and enhance the colonization of anaerobe bacteria, for example, *P. gingivalis* and *A. actinomycetemcomitans*. Furthermore, deeper pockets have a larger epithelial surface area for *P. gingivalis* and *T. denticola* to colonize.

5.2.2 Antibacterial Activity of *Salvadora persica* Stem Extracts (SPSE) Against *Streptococcus mutans* and *Porphyromonas gingivalis*

Biofilms of oral pathogens can be more resistant to antibacterial agents. Previous studies showed the concentration of MIC was not reduced after exposed to amoxicillin and doxycycline. The biofilm of *S. sanguinis* is also resistant to antibiotics and chlorhexidine. However, *S. mutans* has susceptibility to chlorhexidine (Bowden & Hamilton, 1998). *S. mutans* is commonly found in biofilms on tooth surfaces, also known as dental plaque.

Based on the results of antibacterial activities, data showed that SPSE-*n*-hexane exhibited the lowest concentration of MIC against *S. mutans* at 3.13 mg/mL. Whereas the MBC concentration of SPSE-*n*-hexane extract against *S. mutans* was the lowest at 6.25 mg/mL. In contrast, SPSE-*n*-hexane, and SPSE-DCM inhibited *P. gingivalis* at 0.78 mg/mL, but the MBC value for SPSE-*n*-hexane was 0.78 mg/mL and SPSE-DCM was 1.56 mg/mL respectively. Furthermore, this finding is significant because the minimum

concentration of SPSE-*n*-hexane (0.78 mg/mL) to inhibit *P. gingivalis* was lower than CHX (1.56 mg/mL), which acts as a positive control.

S. mutans began to show inhibition when the concentration of *S. persica* methanol extracts at 5 mg/mL. Previous *in vitro* studies reported that, *S. mutans* showed less susceptibility to methanol extract compared to other extracts (Asma & Yasmine, 2021). However, presence study showed that SPSE-acetone from DDA analysis (Table 4.7) is more effective compared to other extracts, whereas SPSE-*n*-hexane from MIC and MBC (Table 4.8) showed better inhibition against *S. mutans* and *P. gingivalis* when compared to other extracts. The results may be contrasted with other studies because of the different bacterial strains tested. Antimicrobial activities of SPSE are related to the release of chemical compounds, such as benzyl isothiocyanate. Benzyl isothiocyanate can inhibit the growth of *S. mutans.* Furthermore, *S. persica* has the tendency to disrupt the QS of the *S. mutans* (Balhaddad et al., 2021). *P. gingivalis* is a well-known oral bacterium for periodontitis. According to Aljarbou et al., (2022), the antimicrobial activity of *S. persica* against *P. gingivalis* was substantial.

5.2.3 Chemical Compositions in *Salvadora persica* Stem Extracts (SPSE)

For centuries, *S. persica* is also known as miswak or siwak, a traditional toothbrush used for maintaining oral hygiene. In this study, SPSE-*n*-hexane, SPSE-DCM, SPSE-acetone, SPSE-ethanol, and SPSE-methanol and the components were identified as *n*-hexadecanoic acid, oleic acid, isopropyl linoleate, benzoic acid, *n*-benzylbenzaide, isopropyl palmitate, tetradecanoic acid, tridecanoic acid and others (Table 4.9).

GCMS analysis was used to detect components in SPSE which act as antibacterial agents for oral health and disease. Analysis by GCMS revealed that SPSE contained 54 compounds, of which *n*-hexadecanoic acid (16.83%), cis-vaccenic acid (25.95%), trans-13-octadecenoic acid (34.53%), oleic acid (49.21%), benzoic acid (47.79%), phenol, 2, 4-bis(1,1-dimethylethyl) (21.60%), tetradecanoic acid (30.99%), tridecanoic acid (30.99%) and cis-13-octadecenoic acid (49.69%) were found to be the major constituents.

In contrast, a study conducted by Khan et al. (2020) identified chemical constituents from *S. persica* which include benzaldehyde (4.7%), benzyl nitrile (26.0%) and benzyl isothiocyanate (68.1%) from SPSE by using GC-FID chromatogram. A high concentration of benzyl isothiocyanate with 73.8% in SPSE has been reported to be effective against *S. mutans*, *P. gingivalis* and *T. forsythia* (Khan et al., 2020). This is due to the antimicrobial, antifungal and anticaries properties of the composition in SPSE. In addition, its activities were possibly due to the presence of several constituents such as flavonoids, saponins, alkaloids and terpenoids (Ahmad & Rajagopal, 2014)

Based on result from Table 4.8 in chapter 4, it showed that SPSE-*n*-hexane is more effective compared to other extracts because presence of hexadecanoic acid and octadecenoic acid which possessed antibacterial properties that contribute to inhibit *S. mutans* and *P. gingivalis* growth.

5.3 Recommendations of the Study

This study can be improved by increasing the number of subgingival plaque samples according to American Dental Association (ADA) guidelines. Furthermore, this study can compare different types of *S. persica* sticks that have been commercialised in the market

and compare them with commercial toothpaste and mouthwash. Besides that, Liquid Chromatography Mass Spectrometry (LCMS) can be used to differentiate compounds that were found with GCMS analysis.

5.4 Conclusions

This study has successfully isolated 176 oral bacteria from 10 human subgingival plaque samples. The majority of the bacteria were Gram-positive such as Streptococcus species with the other were Gram-negative bacteria, such as *K. pneumonia, A. actinomycetemcomitans* and *P. gingivalis*. About 97 Gram-negative bacteria were successfully isolated and identified from all samples. Based on the similarity of bacteria morphology on media and under the microscope, only 17 oral bacteria (nine Gram-positive and eight Gram-negative) were identified from all samples.

To determine antibacterial activity, *S. persica* was extracted using five solvents, which are *n*-hexane, DCM, acetone, ethanol, and methanol. All SPSE exhibited antibacterial activities against *S. mutans* and *P. gingivalis* by using disc diffusion assay, MIC, and MBC. In GCMS analysis, the major phytochemical of SPSE-*n*-hexane, SPSE-DCM, SPSE-acetone, SPSE-ethanol, and SPSE-methanol were benzoic acid, hexadecenoic acid and octadecanoic acid.