CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample Processing

The subgingival plaque samples for this study had been obtained from previous research (PPPI/KHAS_FPG/051007/1521) with ethical approval USIM/JKEP/2019-66. The study had been conducted using a few methods to achieve objectives in this study as shown in the flowchart (Figure 3.1). A total of 10 subgingival plaque samples were collected from Dental Polyclinic, Faculty of Dentistry USIM in the year 2019 with selected inclusion and exclusion criteria as shown in Table 3.1. The plaque samples were mixed properly with thioglycolate broth (1:10) and kept in a -80°C refrigerator until bacteria culture steps.

The supragingival plaque was removed using cotton pellets prior to subgingival plaque collection. The subgingival plaque was collected at the site with probing pocket depth (PPD) ≥ 4mm using gracey curette (Hu-friedy) and pooled in individual tubes containing TB. The method of plaque collection was adapted from the previous study (Al-Otaibi et al., 2004) and laboratory test of colony forming unit (CFU/mL) (Brugger, 2012).

Table 3.1: Selection criteria of the sample.

Inclusion criteria	Exclusion criteria
1. Healthy from periodontitis	1. Individual who wears orthodontic fixed appliances
2. Periodontitis patients (moderate to severe)	2. Individual with severe teeth crowding
3. Age between 18 to 60 years old	3. Electrical toothbrush user
4. New patient: never receive any periodontal treatment/had non-surgical therapy for more than three months	4. At least one year of using *Salvadora persica* stick on a regular basis
5. Physically and mentally healthy	5. Had antibiotics within three months
6. Have at least 18 teeth	6. Pregnant and lactating mother
7. At least have one tooth on maxilla and one tooth on mandible with PPD ≥ 4 mm	7. Consume anti-inflammatory and immunosuppressive drug

3.2 Colony Forming Unit for Each Sample

A volume of 100 µL sample plaque was inoculated in 2.9 mL thioglycolate broth at 37°C in an anaerobic chamber for 24 hours. Serial dilutions were conducted from inoculated thioglycolate broth for bacterial enumeration. The bacteria samples were diluted by a factor of 10 for four times and plated on blood agar. All bacteria samples were cultured at 37°C in an anaerobic chamber for 24 hours and many viable colonies on a dilution plate were calculated using a colony counter machine. If the number of colonies is less than 300, the colony-forming unit per millilitre (CFU/mL) formula was

used to calculate. However, if the number of colonies is more than 300, it cannot be counted and called too-numerous-to-count (TNTC).

3.3 Isolation and Identification of Oral bacteria from Subgingival Plaque

3.3.1 Morphology of Oral Bacteria

3.3.1.1 Gram-staining

Gram-staining was used to verify either Gram-positive or Gram-negative bacteria. A single colony of bacteria sample plaque was placed on a clean glass slide with a small amount of water by using a sterile inoculating loop. There are four main steps for Gram-staining. First, the primary stain of crystal violet was flooded for 60 seconds after heat fixed the bacterial culture from blood, BHI, and WCA agar for a few seconds over the flame of the Bunsen burner and washed gently with tap water. Second, the iodine solution that acts as a secondary stain was flooded for 60 seconds to bind the primary stain in the bacterial cells and washed gently with tap water after removing the excess solution. Third, bacterial cells on the glass slide were decolorized by acetone for five seconds. Fourth, safranin was used as counterstained in the cell for 40 seconds to 60 seconds and washed gently with tap water. The excess water on the glass slide was removed with bibulous paper and air-dried before observing under a microscope.

3.4 Polymerase Chain Reaction (PCR) Analysis

3.4.1 DNA Extraction

DNA extraction of bacteria samples was undergone heat shock and centrifugation to lyse the cell wall. Genomic DNA from the sample of bacteria was extracted by using InstaGene^{TM} Matrix Kit (Bio-Rad, USA). InstaGene matrix was mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. A

single isolated bacterial colony from BHI agar was picked and suspended a volume of 1000 µL of sterile distilled water in a microcentrifuge tube. The solutions were centrifuged for one minute at 10,000 rpm and the supernatant was removed. A volume of 200 µL of InstaGene matrix was added to the pellet and incubated at 56°C for 15 to 30 minutes.

3.4.2 Polymerase Chain Reaction (PCR)

The isolated bacterial samples were further analysed by using the PCR method. Three types of primer were used in this study, including a positive primer for Gram-positive bacteria, a negative primer for Gram-negative bacteria, and a universal primer for both Gram-positive and negative bacteria (Field et al., 2012) as listed in Table 3.2. PCR amplifications were performed in 25 µL in 0.2 mL thin-walled PCR tubes. The amplification cycles in T100 Thermal Cycler (Bio-Rad, USA) were shown in Table 3.3. PCR mixture contained a volume of 5.0 µL of the DNA sample, 2.5 µL of sterile distilled water, forward primer (FP), reverse primer (RP) and 12.5 µL of GoTaq® DNA polymerase (Promega, USA) as tabulated in Table 3.4.

Table 3.2: Details of primer used in PCR.

Primer	Tm (°C)	GC (%)	Sequence 5'-3'	Expected size (bp)
Positive FP	59.5	NA	CCTACGGGAGGCAGCAGTAG	150
Positive RP	59.5	NA	CAACAGAGCTTTACGATCCGAAA	150
Negative FP	84.2	87.7	GGACTAGGGTATCTAATCCTGTT	440
Negative RP	53.6	50.0	TCCTACGGGAGGCAGCAGT	440
Universal FP	60.8	63.2	TCCTACGGGAGGCAGCAG	400
Universal RP	57.2	46.2	GGACTACCAGGGTATCTAATCCTGTT	400

* FP is forward primer; RP is reverse primer Source: Field et al. (2012)

Table 3.3: PCR components used in the amplification of specific DNA target with a total of 25 µL for one reaction.

PCR component	Concentrations (mM)	Volume (µL)
DNA sample	-	5.0
Sterile distilled water	-	2.5
Forward primer	10	2.5
Reverse primer	10	2.5
GoTaq DNA polymerase	1.5	12.5
	Total	25.0

Table 3.4: Details of PCR amplification cycles by using a positive, negative, and universal primer in T100 Thermal Cycler.

PCR process	Temperature (°C)			Time	
	Positive primer	Negative primer	Universal primer		
Initial	95	95	95	2 minutes	
Denaturation	95	95	95	1 minute	30 cycles
Annealing	56	64	54	30 seconds	
Extension	72	72	72	1 minute	
Final extension	72	72	72	5 minutes	
Hold	4	4	4	∞	

3.4.3 DNA Purification

A purification kit was used to purify PCR products from other contaminants. The extracted DNA was purified by using Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA). A volume of 20 µL of membrane binding solution was added into PCR product tubes. The prepared PCR products were transferred into SV mini column with collection tube and incubated at room temperature for one minute. The tube was centrifuged at 14,000 rpm for one minute by using a microcentrifuge machine. The flow-through was discarded and reinserted into a mini-column collection tube. A volume of 700 µL membrane wash solution with ethanol was added into the mini-

column and centrifuged at 14,000 rpm for one minute. The flow-through was discarded and reinserted into a mini-column collection tube again.

A volume of 500 µL of membrane wash solution was added into the mini-column and centrifuged at 14,000 rpm for five minutes. The flow-through was discarded and reinserted into a mini-column collection tube and centrifuged at 14,000 rpm for one minute. The mini-column tubes were transferred into a sterilized 1.5 mL microcentrifuge tube. A volume of 20 µL of nuclease-free water was added to the column without touching the membrane. The sample tubes were incubated at room temperature for one minute and centrifuged at 14,000 rpm for one minute. The mini-column was discarded, and the DNA sample was stored at -20°C.

3.4.4 Gel Electrophoresis

Gel electrophoresis was used to separate the DNA fragments according to molecular size or known as base pair (bp). The PCR products were visualized in 1.5% of agarose gel with a 100 bp DNA ladder. An agarose gel with a 1.5% concentration was prepared by adding 1.5 g of agarose gel powder into 60 mL of 1x TAE (Tris-Acetate-EDTA) buffer solution. The agarose mixture was heated in the microwave for two minutes and swirled until completely dissolved. The melted agarose was poured into the casting tray with a comb. Once the agarose gel solidifies, the gel was transferred to the electrophoresis chamber with a removed comb.

A volume of 1x TAE buffer solution was poured into the chamber until agarose gel is submerged. A volume of 5 µL of DNA samples was mixed with 2 µL of 6x loading dye and transferred into each well. Gel electrophoresis was set at 100 V for 90 minutes. A volume of 5 µL of diamond stain was mixed with 150 mL of 1x TAE buffer before transferring agarose gel into the solution. The agarose gel and buffer were shaken for

about two hours before viewing the result. The band from the gel was visualized using Gel Doc EZ Gel Documentation System (Bio-Rad, USA).

3.5 Sequencing Analysis

3.5.1 16S rRNA Sequencing

A volume of 100 µL of samples was pipetted into a labelled sterile microcentrifuge tube and sealed with parafilm. The samples were stored at -20°C before sending to the sequencing company.

3.5.2 Basic Local Alignment Search Tool (BLAST) Program

Nucleotide sequences were obtained from third-party service and examined for related sequences derived from the GenBank database with the BLAST program which is a registered trademark of the National Centre for Biotechnology Information (NCBI) in the United States National Library of Medicine. The program compares nucleotide sequence to sequence databases and calculates the statistical significance of matches. In addition, BLAST can be used to identify members of gene families. The alignment of nucleotide sequences was generated with nucleotide sequences within the database.

3.6 *Salvadora persica* Stem Extraction

S. persica stem in powder form used was obtained from the Middle East (Biolution Resources Sdn. Bhd.). The extract powder was used from the bark of *S. persica*. A weight of 212 g powdered stems of *S. persica* was extracted with *n*-hexane. The powdered stems were transferred into a 1.5 mL beaker of *n*-hexane filled until 1.8 mL. The extracted stems were soaked for 72 hours at room temperature in a fume hood and mixed for three days. These mixtures were filtered through Whattman no. 1 filter

paper and the filtrate was collected in a 1000 mL flask and continued to be concentrated under low pressure using an RV 8 rotary evaporator (IKA, Malaysia) at 69°C. The supernatants were collected separately in a sterile universal bottle and the remaining solvent was evaporated by drying at 50°C for two to three days.

The steps for the remaining powdered stems were repeated for each solvent by soaking with DCM, acetone, ethanol, and methanol and filling until 1.8 mL. The powdered stems were repeatedly soaked for 72 hours at room temperature in a fume hood and filtered using Whattman no.1 filter paper. The collected filtrate was concentrated under low pressure by a rotary evaporator at different boiling points for each solvent, such as 40°C, 56°C, 78.2°C, and 64.7°C. Next, the solvents were collected separately in a sterile universal bottle and reduced by oven drying at 40°C for three to four days.

3.7 Antibacterial Activities Against *Streptococcus mutans* and *Porphyromonas gingivalis* from Subgingival Plaque

3.7.1 Bacterial Cultures for Selected Oral Bacteria (*Streptococcus mutans* and *Porphyromonas gingivalis*)

S. mutans and *P. gingivalis* identified from previous sequencing analysis were selected to be furthered in this study. Isolated *S. mutans* were cultured on Brain Heart Infusion (BHI) agar. Meanwhile, Wilkins-Chalgren Anaerobe (WCA) agar was for isolated *P. gingivalis*. All bacteria cultures were cultured at 37°C in an anaerobic chamber for 24 hours. The growth colony for *S. mutans* and *P. gingivalis* were inoculated into 5 mL of BHI and WCA broth. The cultured bacteria were maintained in a 10% glycerol stock solution and stored at -80°C after 24 hours.

3.7.2 Disc Diffusion Assay (DDA)

SPSE-*n*-hexane, SPSE-DCM, SPSE-acetone, SPSE-ethanol, and SPSE-methanol were dissolved in dimethylsulfoxide (DMSO) with different concentrations of 25.0 mg/mL, 12.5 mg/mL, 6.25 mg/mL, and 3.125 mg/mL. The solutions were vortexed and sealed with parafilm. Then, the mixture was stored at 4°C for further use.

Disc diffusion assay was done in triplicate for bacteria isolated from samples plaques, for example, *S. mutans* and *P. gingivalis*. A single colony of bacteria were inoculated into 10 mL of BHI broth for *S. mutans* and WCA broth for *P. gingivalis*. The agar plates were incubated at 37°C for 24 hours in an anaerobic chamber. Bacteria cultures were standardized against 0.5 McFarland standard (Mehdipour et al., 2022) after 24 hours. A volume of 100 µL of bacteria suspension was added onto the agar and swabbed evenly with a sterile cotton swab on BHI or WCA agar to get consistent microbial growth.

A sterile cotton swab was dipped into the suspension and spread evenly on BHI and WCA agar to get consistent microbial growth. Sterilized filter paper discs with a size of 6 mm were impregnated with 10 µL of SPSE-*n*-hexane, SPSE-DCM, SPSE-acetone, SPSE-ethanol, and SPSE-methanol at four different concentrations respectively (25.0 mg/mL, 12.5 mg/mL, 6.25 mg/mL, and 3.125 mg/mL) and placed on BHI and WCA agar. 0.2% chlorhexidine (CHX) and DMSO were used as positive and negative controls. All agar plates were incubated in an anaerobic chamber at 37°C for 24 hours to 48 hours. After an incubation period, the inhibition zone was measured.

3.7.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC) of SPSE was determined based on the microdilution method in 96 multi-well microtiter plates (Silva et al., 2014). The dissolved extracts were prepared to the highest concentration to be tested, which is 25.0 mg/mL for each extract. In this study, CHX was used as the positive control, while the negative control was 100 µL of BHI or WCA broth and 100 µL of DMSO and respective broth. The final volume in each well was 200 µL/well. The test bacteria were standardized against 0.5 McFarland standard which is equal to 1.5 x 10^8 CFU/mL and incubated at 37°C for 18 hours to 24 hours anaerobically. A total of 100 µL of *S. mutans* or *P. gingivalis* were added to the wells with 100 µL respective SPSE. The colour changes were assessed visually and recorded. The MIC value was recorded by the lowest concentration of extract that showed no growth after incubation at which the colour change (Al-Bayati & Sulaiman, 2008).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution was prepared by adding 5 mg/mL of sterile phosphate-buffered saline (PBS) solution. The prepared MTT solution was mixed by vortex or sonicate. Then, MTT was added and filtered sterilized solution. The solutions were stored at -20°C.

MBC is a continuous method from MIC. A volume of 5 µL of spot samples from 96-well plates that contain *S. mutans* were taken from microtiter plate wells with visible growth and cultured onto BHI agar and incubated in an anaerobic chamber at 37°C for 24 hours. After incubation, the lowest concentration that inhibits bacterial growth was determined as MBC values. The same MBC steps were repeated for *P. gingivalis*. A volume of 50 µL of MTT solution was added to each well and incubated the plate at 37°C for 3 hours anaerobically to observe the colour of the samples change to black.

3.8 Phytochemistry of *Salvadora persica* Stem Extracts (SPSE)

3.8.1 Gas Chromatography Mass Spectrometry (GCMS)

A total of five extracts (SPSE- *n*-hexane, SPSE-DCM, SPSE-acetone, SPSE-ethanol, and SPSE-methanol) were sent for GCMS analysis. Varian Chrompack CP-3800 ion-trap GCMS 2000 equipped with DB-5.625 GC column (30 m x 0.25 mm) were used. The injector temperature was set at 220°C for five minutes with a split ratio of 10. The gas chromatography (GC) oven was programmed by starting at 50°C and hold for 2 minutes, raise to 150°C at a rate of 10°C/min and hold five minutes, raising to 220°C at a rate of 20°C/min and hold for 10 minutes. The total run time was 30 minutes and five seconds. The mass detector was set to scan between 40 m/z and 40 m/z using fixed mode El. The results were provided in tabulated data from a third-party service provider (Universiti Kebangsaan Malaysia, Malaysia).

3.9 Statistical Analysis

The data was analysed by using Statistical Package for the Social Sciences (SPSS) Statistics. The diameter of the inhibition zone with different types of solvents in SPSE-*n*-hexane, SPSE-DCM, SPSE-acetone, SPSE-ethanol, and SPSE-methanol using MIC and MBC methods against *S. mutans* and *P. gingivalis* were determined using One-Way ANOVA. The statistical test was used to find the significant difference in study parameters between the groups, where there was a significant difference if the *p*-value is less than 0.05 ($p < 0.05$).

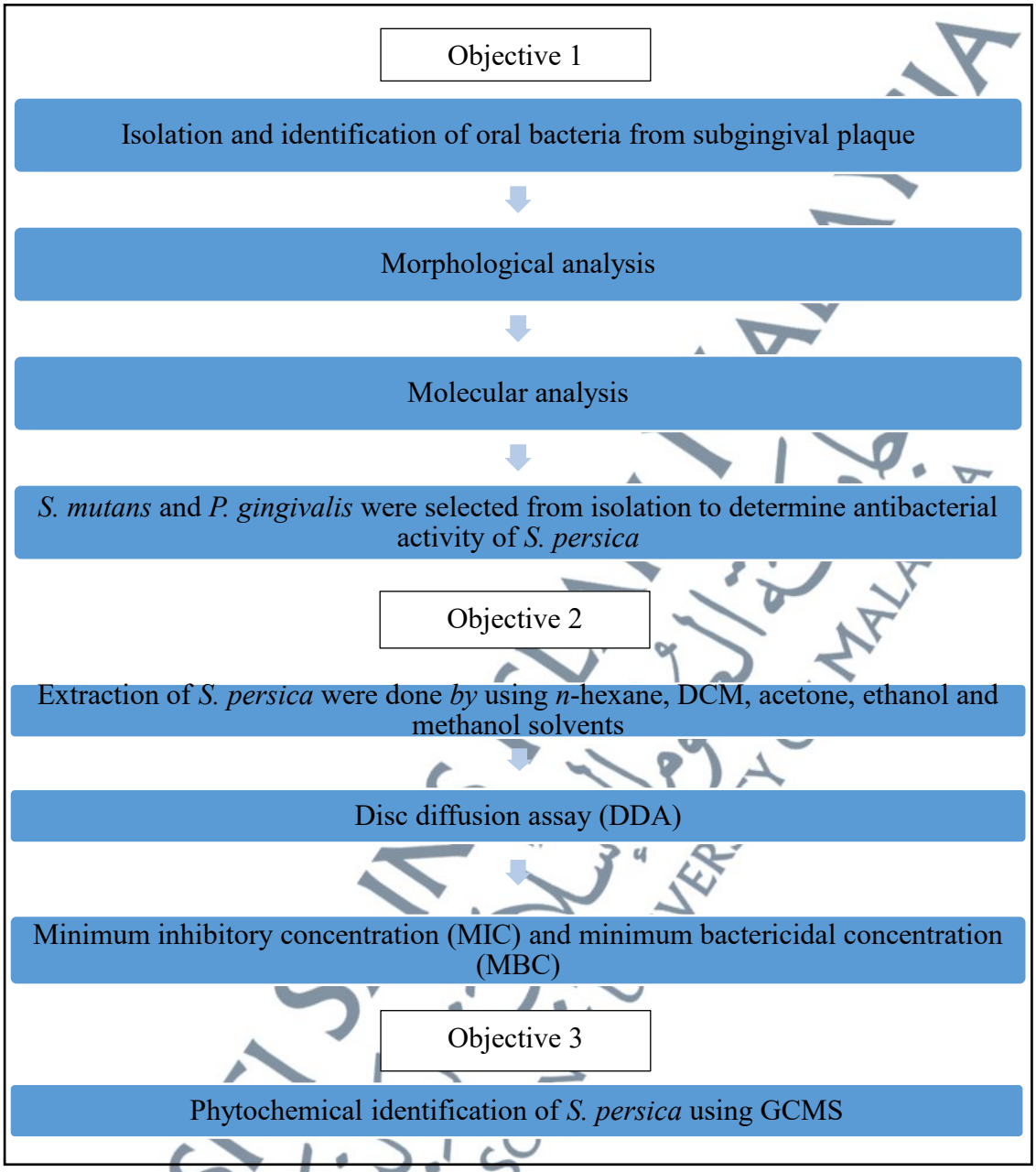

Figure 3.1: Flowchart of research methodology