CHAPTER III

RESEARCH METHODOLOGY

3.1 Materials

Below are the chemicals and reagents been used in this study.

Table 3.1: List of Chemicals and Reagents.

Materials	Company Brand	Country
n-hexane	R & M Chemical	United Kingdom
Dichloromethane	R & M Chemical	United Kingdom
Acetone	R & M Chemical	United Kingdom
Methanol	R & M Chemical	United Kingdom
DMSO	Sigma-Aldrich	United States of America
Brain Heart Infusion Broth	Himedia	United States of America
Muller Hinton Broth	Himedia	United States of America
Muller Hinton Agar	Himedia	United States of America
Glycerol	Merck	Germany
Penicillin	Sigma-Aldrich	United States of America
Nutrient Agar	Himedia	United States of America

3.2 Plant Material

Acmella paniculata was collected from TKC Herbal Nursery, Seremban (Malaysia). The plant was identified by a botanist, Dr. Mohd Firdaus Ismail from the Institute of Bioscience, University Putra Malaysia, and the voucher specimen (MFI 0164/20) was deposited at the Herbarium of the Institute of Bioscience, University Putra Malaysia (Appendix 1). Start by selecting the plant material, which in this study is the leaves and flower. Figures 3.1 A and B show the drying process of the *A. paniculata* plant. They should be washed and filtered. Then, lay them out on a flat surface for one day. Spread out the plant material evenly on top of the paper and make sure to leave some space between the pieces so that they can dry evenly. After it has dried, place them on newspaper and wrap them well. Then, place it in the microwave oven set to 60 °C for one week to allow the plant material to dry out. This step will aid in the removal of any remaining moisture in the plant material. Once the plants are dry, remove them from the microwave and let them cool completely. After it has cooled, use a blender or food processor to grind the dried plant material into a fine powder. Once you have the powder, you can store it in an airtight container in a cool, dry place for further use.

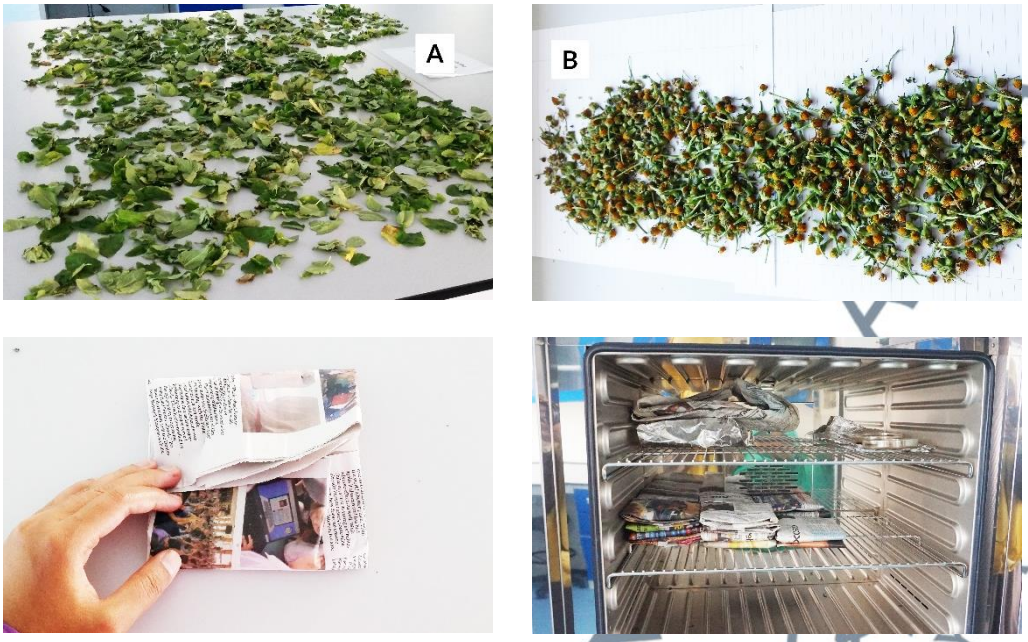

Figure 3.1 (A): *A. paniculata* Leaves (A) and Flower (B) Drying Process using Newspaper and Store in Microwave Oven for 1 Week

Figure 3.1 (B): The Process of Forming *A. paniculata* Powder Using a Grinder

3.2.1 Plant Extraction

A total of 800 g of *A. paniculata* flower and leaves powder were extracted (3 × 1L each) serially with n-hexane (H), dichloromethane (D), acetone (Ac) and methanol (M) in the order mentioned. The extract was then filtered using vacuum filtration and Whatman No. 1 filter paper. The collected filtrate from each solvent were then separately evaporated using a rotary evaporator (Laborota 4000, Germany). Finally, 10% DMSO (Dimethyl sulfoxide, Sigma-Aldrich, USA) was used to dilute each extract.

3.3 Bacterial Growth Conditions, Maintenance and Stock Cultures

The bacterial strain that has been used in this study is *S. mutans* ATCC 25175. The bacteria were cultured at 37 °C under anaerobic conditions (when comparing aerobic and anaerobic incubation, it was discovered that anaerobic conditions performed best) by using Anaerocult® in Brain Heart Infusion (BHI) broth (Himedia, USA). The cells have been harvested by centrifugation and washed twice with phosphate buffer. The pellet was added to fresh BHI broth and glycerol (Merck, Germany) with a ratio of 8:2 and kept at -80 °C as a stock culture.

3.4 Antimicrobial Screening by Disc Diffusion Assay

The disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method (Adzitey, 2020) to assess the presence of antibacterial activities in the plant extracts.

Streptococcus mutans was cultured on Muller Hinton broth (MHB, Himedia, USA) under anaerobic conditions at 37 °C for 24 hours. After that, the bacterial culture was optimised to 2.0 McFarland (Appendix 3) to get an approximately 6 x 10^8 bacterial suspension (McFarland, 1907) by adding 2 mL of the bacterial culture into 8 mL MHB. *S. mutans* were spread evenly onto Muller Hinton agar (MHA, Himedia, USA) using a sterile cotton swab and left to dry for 15 minutes. Six millimetre sterile paper discs were then soaked in both *A. paniculata* leaves (APL) and flower (APF) extracts (n-hexane, dichloromethane, acetone and methanol) with different concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL each. Then, all discs were placed on dried cultured MHA. Penicillin and 10% DMSO have been used as the positive and negative controls, respectively. The plate was then incubated at 37°C under anaerobic conditions. After 24 hours of incubation, the inhibition zones were measured. All tests are done in triplicate.

3.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC were performed using the two-fold serial dilution method described by (Hanafiah et al., 2018). Both MIC and MBC tests were done to determine the lowest concentration at which an antimicrobial agent inhibited and killed a particular organism, respectively.

For the MIC test, the MTT assay was inserted into a 96-well plate to visually differentiate between the live and dead cells after the incubation period. A total of 100 µL of *S. mutans* (108 CFU/mL) was added to various concentrations of APL n-hexane extract (APLHE), APL methanol extract (APLME), APF n-hexane extract (APFHE)

and APF DCM extract (APFDE), ranging from 6.25 to 100 mg/mL, diluted in MHB to a final volume of 200 µL/well. DMSO (10% v/v) was used as a negative control and penicillin (0.04 mg/mL; Sigma-Aldrich, USA) as a positive control. Then, those samples were incubated at 37 °C under anaerobic condition for 24 hours.

After the MIC determination, aliquots of 5 µl from all the tubes that showed no visible bacterial growth were seeded on sterile nutrient agar (Himedia, USA) plates and incubated overnight at 37 °C anaerobically. When 99.9% of the bacterial population is killed at the lowest concentration of an antimicrobial agent, it is termed an MBC endpoint. This was done by observing pre- and post-incubated agar plates for the presence or absence of bacteria.

3.6 Anti-biofilm Assay

Anti-biofilm assay was performed using the method from (Khan et al., 2020) with modifications. The biofilm formation assay was done by crystal-violet assay (Sigma-Aldrich, USA) with a 96-well plate. A total of 1 mL *S. mutans* was transferred to 10 mL BHIB and incubated at 37 °C for 24 hours anaerobically. The culture is then diluted 1:100 in BHIB, and 150 µL of bacteria are added to a 96-well plate, followed by 50 L of APLHE, APLME, APFHE, and APFDE at concentrations ranging from 12.5 to 100 mg/mL in each well. Penicillin (0.04 mg/mL) and 10% DMSO acted as positive and negative controls, respectively. The microplate well was then anaerobically incubated at 37 ˚C for 24 hours. Next, the biofilm formation was quantified by measuring the absorbance of the solutions (biofilm and crystal violet) at 595 nm using a microplate reader (Model 680, Bio-Rad, California, USA).

3.7 Time Kill Assay

A time-kill assay was performed to investigate the rate of killing the *S. mutans* by APLHE, APLME, APFHE and APFDE according to the method by Yadav et al., (2015). *S. mutans* growth was initiated by inoculating MHB for overnight incubation. Then, 2 mL of the *S. mutans* were subculture into 8 mL of fresh MHB to get an approximate density of 2.0 McFarland standards (6 x 10^8 bacterial suspension). All four extracts were prepared at 1 x MIC, 2 x MIC, and 4 x MIC concentrations (1 mL) of APLHE and APLME at 25, 50, and 100 mg/mL, and APFHE and APFDE at 12.5, 25, and 50 mg/mL, respectively. Then, 1 mL of the above-prepared bacterial suspension was added to each extract concentration and incubated anaerobically at 37 ˚C for 28 hours. Positive control includes the bacterial suspension with 0.04 mg/mL of penicillin, while negative control includes the bacterial suspension without *A. paniculata* extracts. A volume of 10 µL of sample was pipetted out of each tube to undergo a serial dilution and each diluted sample was spread on MHA at 0, 4, 8, 24 and 28 hours. The petri dish plates were then incubated anaerobically overnight at 37 ˚C. Colonies on each plate were counted and expressed as number of colony forming units/mL (CFU/mL). The killing rate was determined by plotting logarithm of the viable colony counts against time. The reduction percentage in CFU was calculated in: -

$$\%\ Reduction = \frac{Initial\ count - count\ at\ x\ interval}{initial\ count} \times 100 \quad (1.1)$$

3.8 Statistical Analysis

The results were expressed as mean ± SD (Standard Deviation) in triplicate, and ANOVA was used to determine the differences between treated and untreated cells. All data obtained were analysed using the Statistical Package for the Social Sciences (SPSS). The data from the disc diffusion assays and biofilm assays were determined using two-way ANOVA as presents of two groups of independent variables, which are the four different solvents in four different concentrations. Next, the time kill assay test was then examined using one-way ANOVA. The statistical test was used to find the significant difference of study parameters between the groups, where there was a significant difference if the p value is less than 0.05 ($p < 0.05$).

3.9 Scanning Electron Microscope (SEM) for APFDE and APFHE

S. mutans cultures were grown in MHB for overnight incubation. The bacteria were then subcultured in new MHB to produce approximately 6 x 10^8 bacterial suspensions, which were then treated with 1 x MIC (12.5 mg/mL) and 1 x MBC (50 mg/mL) for both APFDE and APFHE. All the cultures were incubated for 4 hours at 37˚C. Then, the sample suspension underwent a centrifugation process at 13000 rpm for 1 minute using a microcentrifuge (Eppendorf, 5415R, Hamburg, Germany) to get its bacteria pellet. The samples were then fixed in 2.5% glutaraldehyde for 6 hours at 4˚C. Then, wash the pellet with 0.1 M sodium cacodylate buffer for 3 cycles of 10 minutes each. After that, post-fix for 4 ˚C in 1% osmium tetroxide. After 2 hours, repeat the washing process. Then, it underwent a dehydration process that involved immersion in a series of acetone concentrations (35%, 50%, 75%, 95% and 100%). The cell pellet was coated with gold and observation was done via scanning electron microscope

(SEM, Jeol JSM-6700F, Tokyo, Japan). Non-treated *S. mutans* acted as a negative control.

3.10 Transmission Electron Microscope (TEM) for APFDE and APFHE

S. mutans cultures were incubated overnight in MHB using the same initiation process as SEM. Then, the bacteria were subcultured in new MHB (6 x 10^8 of bacterial suspension) and treated with 1 x MIC (12.5 mg/mL) and 1 x MBC (50 mg/mL) for both APFDE and APFHE. All cultures were then incubated for 4 hours at 37˚C. Next, *S. mutans* were centrifuged at 13000 rpm to form pellet. The pellet was incubated with 2.5% glutaraldehyde for 6 hours at 4 ˚C and then continue fix the pellet with 2.5% glutaraldehyde for 6 hours at 4 ˚C and continued with adding an animal serum to clot. Then, the sample clot was diced into 1 mm3 and fix again with 2.5% glutaraldehyde. After 2 hours of fixation, the samples were stabilized with osmium tetroxide. After that, dehydration and infiltration process were done with series of acetone and acetone-resin concentration, respectively. Lastly, the samples were cut with ultra-microtome and *S. mutans* morphology were observed by using transmission electrone microscope (TEM, Joel, Jem-2100, Tokyo, Japan). Non-treated *S. mutans* act as negative control.

3.11 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The volatiles released from APFDE and APFHE were collected using headspace solid phase microextraction (HS-SPME) equipped with a 100 µm polydimethylosiloxane (PDMS) fibre (Supelco) and analysed using an Agilent 7890A gas chromatography coupled to an Agilent 5975C quadrupole mass detector (Agilent

Technologies, Santa Clara, USA). The instrument was equipped with an Agilent HP-5MS capillary column (30 m x 250 µm inner diameter x 0.25 µm film). Helium was used as the carrier gas at a flow rate of 1 mL/min. The SPME fiber was conditioned at the GC injection port for 5 min at 250 ˚C before use. Approximately 1 g of the AP extracts were placed in a 20 mL headspace vial fitted with a silicone septum screw cap. Following 10 min of sample conditioning at room temperature, the SPME fiber was exposed to the headspace for 30 min at 60 ˚C and immediately desorbed in the gas chromatograph injector at 250 ˚C for 15 min using a splitless mode. The oven was kept at 40 ˚C for 2 minutes before increasing to 175 ˚C at a rate of 5 ˚C/min, and then to 250 ˚C at a rate of 90 ˚C/min. The volatile compounds were identified by mass spectra comparison using MSD Chemstation Enhanced Data Analysis Software (E.02.02.1431 version, Agilent Technologies) and the National Institute of Standards and Technology library database (NIST 14). Compound identification within each extract was accomplished by comparing experimental retention indices to theoretical ones obtained from available references. The percentage occurrence of each compound was determined by dividing the peak area of respective with the total peak areas of all identified volatiles.

3.12 RNA Extraction

RNA extraction was carried out using the Innu PREP RNA Mini Kit 2.0. RNA isolation was done on approximately 1 x 109 cells of *S. mutans* treated with APFDE (MBC) and non-treated *S. mutans*. Then, after 4 hours of incubation, the cells are collected by a centrifugation process (10 minutes at 3000 x g), where the supernatant is discarded and pellets are taken. This process is repeated 3 times until the final pellet has

been resuspended in 100 µL of TE-buffer to undergo the pre-lysis steps of the bacterial cell. In the pre-lysis step, 20 µL of lysozyme were added (as it exerts a lysis activity on nucleic acid) and incubated at 37 ˚C for 30 minutes under continuous shaking.

Next, 450 µL of lysis solution was added to the pre-lysed sample and vortexed vigorously. The samples were incubated for another 3 minutes at room temperature. Following that, the lysed sample is transferred to a filter tube and centrifuged at 11000 x g for 2 minutes. Now the filtrate contains the RNA. Then, an equal volume (approximately 600 µL) of 70% ethanol was mixed up with the filtrate. Then again, 650 µL of the sample mixture (RNA and ethanol) was filtered at 11000 x g for 1 minute. Then, the filtered sample was washed a few times with different wash solutions and centrifuged at 11000 x g to remove all traces of ethanol. Lastly, RNase-free water was added to the filtered sample and incubated for 1 minute at room temperature before being centrifuged at 11000 x g for 1 minute. The filtrate was then stored following the purification process.

3.13 Transcriptomic Profile

3.13.1 Transcriptome Sequencing Library Preparation

The RNA sample preparations used a total of 1 µg RNA per sample as input material. Following the manufacturer's recommendations, sequencing libraries were created using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA), and index codes were added to attribute sequences to each sample. Using poly-T oligo-attached magnetic beads, mRNA was purified from total RNA. In the NEBNext First Strand Synthesis Reaction Buffer, fragmentation was carried out using divalent cations at elevated temperatures (5X). The first strand of cDNA was created with a

random hexamer primer and M-MuLV Reverse Transcriptase (RNase H−). Following that, second strand cDNA synthesis was carried out using DNA polymerase I and RNase H. Exonuclease/polymerase activities were used to convert the remaining overhangs into blunt ends. NEBNext adaptor with hairpin loop structure was ligated after adenylation of 32032 ends of DNA fragments to prepare for hybridization. The library fragments were purified using the AMPure XP system to select cDNA fragments of preferentially 150~200 bp length (Beckman Coulter, Beverly, USA). After that, 3 μL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA for 15 minutes at 37°C, followed by 5 minutes at 95°C before PCR. Following that, PCR was carried out using Phusion High-Fidelity DNA Polymerase, Universal PCR Primers, and Index (X) Primer. Finally, PCR products were purified (AMPure XP system), and the quality of the library was evaluated using the Agilent Bioanalyzer 2100 system.

3.13.2 Clustering and Sequencing

The index-coded samples were clustered using an Illumina PE Cluster Kit cBot-HS on a cBot Cluster Generation System in accordance with the instructions provided by the manufacturer. After the clusters had been generated, the library preparations were sequenced on an Illumina platform to generate paired-end reads.

3.13.3 Quality Control of RNA

FASTQ raw data (raw reads) were first processed using fastp. In this step, clean data (clean reads) were obtained by trimming adapter-containing reads and removing poly-N sequences and low-quality reads from raw data. Simultaneously, the Q20, Q30,

and GC content of the clean data were calculated. All subsequent analyses relied on clean, high-quality data.

3.13.4 Mapping to the Reference Genome

Annotation files for gene models and the reference genome were obtained by downloading them directly from the genome website. Bowtie 2 was utilised for both the process of building an index of the reference genome as well as aligning clean reads to the reference genome (Lu & Salzber, 2018).

3.13.5 Quantification of Gene Expression

The featureCounts was used to count the number of reads that were mapped to each gene. The FPKM of each gene was then calculated using the gene's length and the number of reads mapped to it. The expected number of fragments per kilobase of transcript sequence per million base pairs sequenced, or FPKM, takes into account the effect of sequencing depth and gene length on the read count at the same time and is currently the most widely used method for estimating gene expression levels.

3.13.6 RNA-seq Advance QC

Biological replicates are necessary for any biological experiment, including those involving RNA-seq technology (Schurch et al., 2016). In RNA-seq, replicates have a two-fold purpose. First, they demonstrate whether the experiment is repeatable, and secondly, they can reveal differences in gene expression between samples. The

correlation between samples is an important indicator for testing the reliability of the experiment. The closer the correlation coefficient is to 1, the greater the similarity of the samples. ENCODE suggests that the square of the Pearson correlation coefficient should be larger than 0.92, under ideal experimental conditions.

3.13.7 Differential Expression Analysis

Gene expression levels were measured by transcript abundance. The greater the abundance, the higher the gene expression level. The gene expression level is estimated by counting the reads that map to genes or exons. The number of reads is not only proportional to the level of gene expression. However, the read count is also proportional to the gene length and the sequencing depth. In order for the gene expression levels estimated from different genes and experiments to be comparable, the fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) was used. In RNA-sequence, FPKM short for the expected number of fragments per kilobase of transcript sequence per million base pair-sequenced, is the commonest method of estimating gene expression levels, which considers the effects of both sequencing depth and gene length on the counting of fragments (Kukurba & Montgomery, 2015). Feature counts software was used to analyse the gene expression levels in this experiment. The result files present the number of genes with different expression levels and the expression level of single genes. In general, FPKM value of 0.1 or 1 was set as the threshold for determining whether the gene is expressed or not. The comparison of genes treated and non- treated has been done by using duplicate samples.

3.13.8 Gene Ontology (GO) Analysis

Through the enrichment analysis of the differential expressed genes, we can find out which biological functions or pathways are significantly associated with differential expressed genes. GO is the abbreviation of Gene Ontology, which is a major bioinformatics classification system to unify the presentation of gene properties across all species. It includes three main branches: cellular component, molecular function and biological process. GO terms with padj < 0.05 are significant enrichment.

3.13.9 KEGG Pathway

Multiple gene interactions may be involved in certain biological functions. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of manually curated databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances. KEGG is used in bioinformatics research and education, and it is used to analyse data in genomics, metagenomics, metabolomics, and other omics studies. When compared to the whole genome background, pathway enrichment analysis identifies significantly enriched metabolic or signal transduction pathways associated with differentially expressed genes.