CHAPTER 3 : MATERIAL AND METHODS

3.1 Study Design

This study consists of two phases, i.e. Phase 1: Determination of antibacterial activities of date fruits (**Figure 3.1**) and Phase 2: Understanding the mechanisms underlying the antibacterial activities of date fruits (**Figure 3.2**). Phase 1 started by extraction of date fruits by aqueous and methanol extracts followed by testing for their antimicrobial and anti-adhesion activities. In addition, the date fruits were also soaked in water at room temperature to mimic the practice of the Prophet Muhammad SAW, and the infused water was tested. Antimicrobial activities were determined using well-diffusion assay agar, Minimum Inhibitory concentration (MIC) assay and Minimum Bactericidal Concentration (MBC) assay. On the other hand, anti-adhesion activities were determined using bacterial adhesion assay with Caco-2 cells model.

In phase 2, the phytochemicals content in date fruit extracts were screened using UHPLC-ESI_QTOF-MS/MS and later quantified using colourimetric assay, which was Total Phenolic Content (TPC) assay, Total Tannin Content (TTC) assay, and Total Flavonoid Content (TFC) assay. To further explore the mechanism underlying the antibacterial activity, active compounds responsible for antimicrobial activity were isolated and tested for their antimicrobial and anti-adhesion activity.

To evaluate the possible mechanisms of action and cellular targets of the date extract and the isolated active fraction against selected bacteria, a morphological analysis of

the bacteria was carried out using electron microscopy. The summary of the workflow for Phase 1 and Phase 2 are as shown **in Figure 3.**1 and **Figure 3.2**.

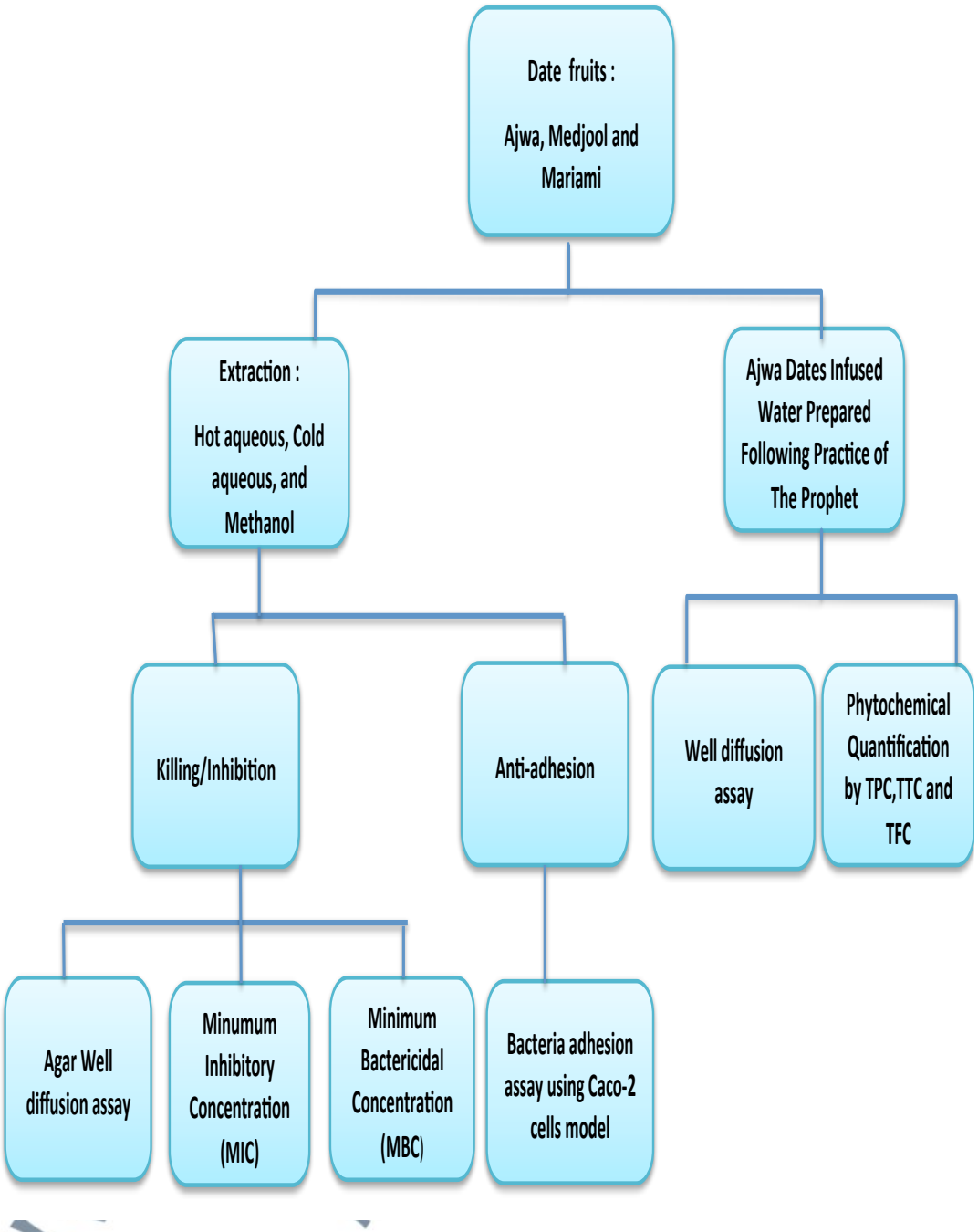

Figure 3.1: Flowchart of phase 1: determination of the antibacterial activity of date fruits.

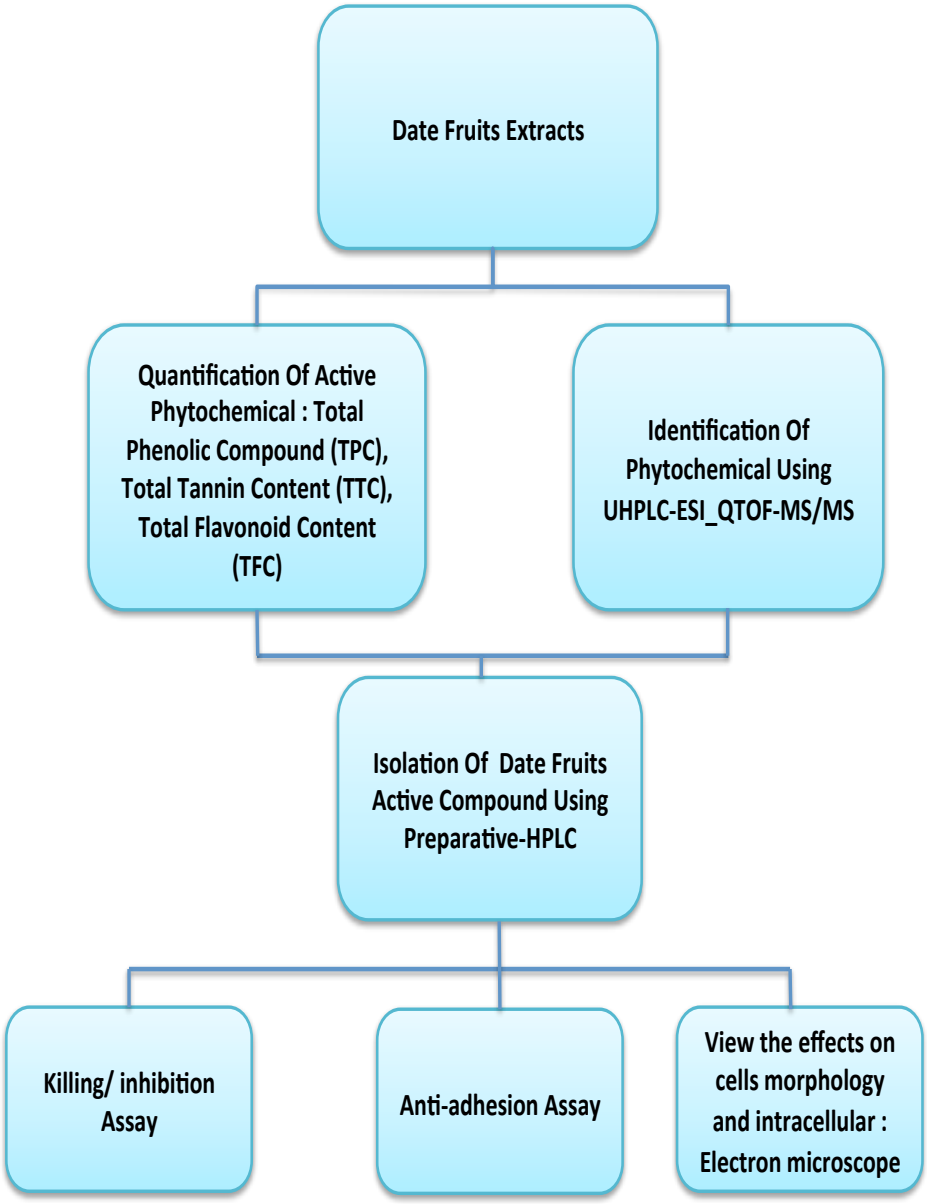

Figure 3.2: Flowchart of Phase 2: understanding the mechanism underlying the antibacterial activity of dates fruits.

3.2 Microorganism

3.2.1 Test Bacterial Strain

Six strains of bacteria were used in this study; gram-positive bacteria: *Staphylococcus aureus (*ATCC 6538) and gram-negative bacteria: *Escherichia coli, Salmonella enterica serotype Typhi, , Salmonella enterica* serovar *Typhimurium* (ATCC 14028*), Shigella flexneri* (ATCC 12022), and *Vibrio cholerae*. Three references strain of American Type Culture Collection (ATCC) were obtained from Thermo Fisher Scientific USA and three clinicals isolated of *Vibrio cholerae, Salmonella Typhi* and *Escherichia coli* were obtained from Universiti Kebangsaan Malaysia Medical Centre (UKMMC). All bacteria were maintained in a cryopreservation medium (1:1 ration of 30% glycero1 and bacterial culture in nutrient broth). They were stored and maintained in -80^{o}C freezer prior to any screening.

3.2.2 Preparation of Inoculum

Each bacterium was subculture on a fresh nutrient agar plate and incubated at 37^{o}C for 24 hours for optimum growth. For broth culture, 2-3 isolated bacterial colonies were taken from the agar plate using a sterile cotton swab and transferred into fresh nutrient broth or Mueller-Hinton broth and incubated at 37^{o}C for 20-24 hours in a shaker incubator [N-Biotek, Korea].

To ensure the purity, each bacterium was culture in selective medium. *S. aureus* was cultured in blood agar and colonies appear with beta-hemolysis and golden coloured colonies. *S. Typhi* and *S. Typhimurium*, were cultured in McConkey agar and appear pale or colourless colonies (non-lactose fermenter). While, *E. coli* culture in MacConkey agar appear dry, dark pink colonies with surrounded with dark pink area of precipitated bile salts (lactose fermenter). *S. flexneri* culture on McConkey agar appear

non-lactose fermenting and colourless colonies. *V. cholerae* cultured in TCBS agar, appear yellow colonies with opaque centre and translucent surrounding.

3.3 Chemicals and Reagent

Mueller-Hinton agar [OXOID™, USA], Mueller-Hinton Broth [OXOID™, USA], Nutrient Agar [OXOID™, USA], Nutrient Broth [OXOID™, USA], Ampicillin Sodium Salt [AMERCO, Life Science, USA], Ciprofloxacin [Fluka analytical, Germany], Gentamicin Sulfate [AMERCO, Life Science, USA], Alamar blue [Invitrogen, USA], Dulbecco's Modified Eagle medium [Gibco, Life technologies Corp, NY, USA], Phosphate Buffer saline [Gibco, Life technologies Corp, NY, USA], Fetal Bovine Serum [Gibco, Life technologies Corp, NY, USA], 0.25% Trypsin [Gibco, Life technologies Corp, NY, USA], Penicillin-streptomycin (10,000 U/mL] [Gibco, Life technologies Corp, NY, USA], Trypan blue [Gibco, Life technologies Corp, NY, USA], Dimethyl Sulfoxide [Vivantis, USA], Folin & Ciocalteu's Phenol Reagent [Sigma Aldrich, USA], Sodium Carbonate [Sigma Aldrich, USA], Gallic acid [Sigma Aldrich, USA], Aluminum Chloride [Sigma Aldrich, USA], Rutin [Sigma Aldrich, USA], Formic acid [Emsure, USA], Ammonium formate, Acetonitrile (ACN) for UHPLC-QTOF [Sigma Aldrich, USA], Acetonitrile and methanol for Prep-HPLC [Merck, Germany], Glutaraldehyde [Sigma Aldrich, USA], Sodium Cacodylate Buffer [Sigma Aldrich, USA], Osmium tetroxide [Sigma Aldrich, USA], 100 Resin [Agar Scientific Ltd, UK], Toluidine blue [Sigma Aldrich, USA], Uranyl Acetate [Agar Scientific Ltd, UK].

3.4 Culture Media and Reagent Preparation

3.4.1 Nutrient Agar

Nutrient agar was made by dissolving 28g of dehydrated nutrient agar in 1L of distilled water. Media were boiled until completely dissolved and then autoclaved [Hirayama, Japan] at 121°C for 15 minutes. The media then were poured into sterile culture plate in aseptic condition under class II biosafety cabinet [Esco, Singapore] until hardening. The agar plates were stored at 4^0C.

3.4.2 Nutrient Broth

The nutrient broth was made by dissolving 13g of dehydrated nutrient broth in 1L of distilled water. Then, the media was stirred well and aliquot in an appropriate container. The media were then sterilized by autoclave at 121°C for 15 minutes.

3.4.3 Mueller-Hinton Agar

Mueller-Hinton (MH) was made by dissolving 38g of dehydrated MH agar in 1L of distilled water. The media was boiled until completely dissolved and then autoclaved at 121°C for 15 minutes. The media then were poured into sterile culture plate in aseptic condition under class II biosafety cabinet until hardening. The agar plates were stored at 4^0C.

3.4.4 Mueller Hinton Broth

MH broth was made by dissolving 21g of dehydrated nutrient broth in 1L of distilled water. Then, the media was stirred well and aliquot in an appropriate container. The media were then sterilized by autoclave at 121°C for 15 minutes.

3.4.5 Cryopreservation Medium for Bacteria

30% of glycerol was mixed with 70% distilled water and sterile the medium by autoclaved at 121^0C for 30 minutes. Cryopreservation made by adding 1:1 ratio of bacterial culture and 30% glycerol.

3.4.6 Antibiotic Stock Preparation

Few types of antibiotic were selected in this study, since six different strains of bacteria might have different sensitivity. Two wide spectrum antibiotics i.e.; Ampicillin and Gentamicin and one antibiotic widely used for bacterial gastroenteritis such as ciprofloxacin were used in this study.

3.4.6.1 Ampicillin Stock Solution

Ampicillin stock (100mg/mL) solution was made by dissolved 1g of Ampicillin sodium salt in 100mL of distilled water and mixed well the solution using vortex. The stock solution then filtered using a sterile 0.2µm syringe filter. Aliquot the solution and stored the stock solution in -80°C.

3.4.6.2 Gentamicin Stock Solution

Gentamicin stock solution (100mg/mL) solution was made by dissolved 1 g of Gentamicin Sulphate into 100mL of distilled water and mixed well the solution by the vortex. The stock solution then filtered using a sterile 0.2um syringe filter. Aliquot the solution and stored the stock solution in -80°C.

3.4.6.3 Ciprofloxacin Stock Solution

Ciprofloxacin stock solution (20mg/mL) was made by dissolved 400mg of Ciprofloxacin powder in 20 mL of acidic aqueous (pH 4.5). Mixed well the solution by the vortex. The stock solution then filtered using a sterile 0.2um syringe filter. Aliquot the solution and stored the stock solution in -80^{o}C

3.4.7 Complete medium Dulbecco's Modified Eagle medium (DMEM)

The basal medium of (DMEM) was supplemented with 10% of Fetal Bovine Serum (FBS) and 1 % of Penicillin-Streptomycin (10,000 U/mL) to make 500mL of complete medium DMEM. Complete DMEM medium was stored in 4^{o}C until used. This medium was used to culture Caco-2 cells and for cryopreservation medium for Caco-2 cells.

3.4.8 Cryopreservation Medium for Caco-2 Cells

10% of Dimethyl Sulfoxide (DMSO) was mixed with complete DMEM to make cryopreservation medium. This medium was used to freeze cells for long-term storage.

3.4.9 Antibiotic-Free Complete Medium DMEM

The basal medium of Dulbecco's Modified Eagle medium (DMEM) was supplemented with 10% of Fetal Bovine Serum (FBS) to make 500ml of complete medium DMEM. Complete DMEM medium was stored in 4^{o}C until used. This medium was used in bacterial adhesion assay.

3.4.10 7.5% of Aqueous Sodium Carbonate

A 7.5g of Sodium carbonate powder was dissolved in 100mL of distilled water. Then, the solution was stirred well with a magnetic stirrer.

3.4.11 10% of Folin-Ciocalteau's Reagent

100% of Folin-Ciocalteau's Reagent was diluted with distilled water to get 10% of Folin-Ciocalteau's Reagent.

3.4.12 2% of Aluminum Chloride

This solution was done under fume cupboard because Aluminum chloride was hazarded. Briefly, 4g of Aluminum chloride was dissolved in 200mL methanol. Mixed well the solution in a Scoot bottle.

3.4.13 Gallic Acid Standard Stock Solution

Gallic acid standard stock solution was made by adding 10mg of Gallic acid powder in 10mL of methanol to make a stock solution of 1mg/mL and mixed well. The Further dilution was made by diluted stock solution with distilled water by serial dilution technique.

3.4.14 Rutin Standard Stock Solution

Rutin standard stock solution was made by adding 10mg of Rutin powder in 10mL of methanol to make a stock solution of 1mg/mL and mixed well. Further dilution was made by diluted stock solution with methanol by serial dilution technique.

3.5 Date fruits

Date fruits were purchased from a local supplier, Syarikat Abdul Ghaffar Trading Sdn. Bhd. (Saudagar Kurma) Penang, Malaysia. Ajwa dates were originated from Madinah, Saudi Arabia. Medjool dates originated from Palestine, and Mariami dates originated from Iran.

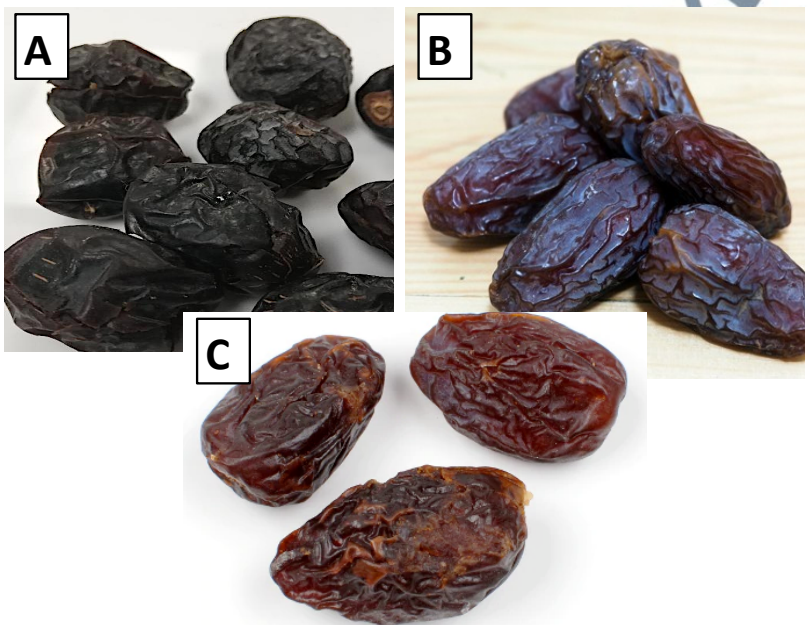

Figure 3.3: Dates fruits (*Phoenix dactylifera*) used in this research. **A**- Ajwa Dates, **B**- Mariami Dates, **C**- Medjool Dates

Figure 3.4: Packaging of date fruits sourced from Syarikat Abdul Ghaffar Sdn. Bhd. **A**- Ajwa Dates, **B**-Medjool Dates, **C**-Mariami Dates

3.5.1 Date fruits preparation

Date fruits were deseeded and rinsed with distilled water. Date fruits were dried in 40 ºC oven and then kept at 4 ºC until further used/analysis.

3.5.2 Preparation of Date Fruit Extract

3.5.2.1 Aqueous extraction

3.5.2.1.1 Hot aqueous extraction

Hot aqueous extraction was performed as described previously with modification (Al-Daihan & Bhat, 2012). Briefly, 100 g of date fruits were crushed using laboratory-grade blender [Waring, USA] and extracted in 1000 mL of distilled water. Then, the mixture was heated at 60 ºC for 1 hour using a hot plate [Daihan Labtech, Korea]. Subsequently, filtered using cotton gauze followed by Whatman® No. 1 filter paper [Whatman® Int. Ltd., Maidstone, UK]. The filtrates were later dried in an oven at 60 ºC [Memmert, Schwabach, Germany]. The extracts yields were stored at -20ºC until further analysis

3.5.2.1.2 Cold Aqueous Extraction

Cold aqueous extraction was performed as described as previously with slight modification (Al-Daihan & Bhat, 2012). Briefly, 100 g of date fruits were crushed using a laboratory-grade blender and extracted in 1000mL of cold distilled water and soaked for 24 hrs at 4 ºC. Subsequently, filtered using cotton gauze followed by Whatman® No. 1 filter paper. The filtrates were then dried using freeze dryer machine [SCANVAC, Coolfsafe™, Labogene ApS, Lillerod, Denmark] and stored at -20 ºC until further analysis.

3.5.2.2 Methanol extraction

Methanol extraction was performed as described previously with modification (Bouhlali et al., 2016). Briefly, 100 g of date fruit was crushed using l laboratory-grade

blender and mixed in 1000 mL of methanol, and the mixture was seated in room temperature (24 ^{o}C) for 24 hours. The mixture then filtered using cotton gauzed followed by Whatman® No. 1 filter paper. The filtrates volume was reduced under pressure at 40^{o}C using Digital Distilling Rotary Evaporator [IKA-Werke GmbH, Germany] and t further dried in an oven at 60 ^{o}C. The extract yields were stored at -20 ^{o}C until further analysis.

3.5.2.3 Prophetic Methods Extraction

Samples were prepared by immersing an odd number of Ajwa dates (one, three, five, and seven dates) individually in a glass of water (240 ml). Each glass was covered using aluminum foil and seated in room temperature (24 ^{o}C). The infused water was taken at a different time interval (0 h, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, and 24 hrs).

3.6 Antibacterial Assay

3.6.1 Screening of Antimicrobial Activity using Agar Well Diffusion Assay

The antibacterial activities of date extract were evaluated using the agar well diffusion assay. Each of bacterial strain suspension (adjusted at 1.5 x 10^{8} CFU @ 0.5 McFarland standard) was spread over Mueller-Hinton agar media using a sterile cotton swab. Four wells (7.00mm in diameter) were cut from the inoculated medium using flame-sterilized metal cork borer. For each agar plate, three wells were filled with 100uu µL of samples and one well filled with 100 µL of antibiotic (Ampicillin, gentamicin and ciprofloxacin) as a positive control. All plates were then incubated at 37^{o}C for 16-20h. The diameter of the inhibition zone was then measured using a digital caliper and recorded. The test was performed in triplicate.

3.6.2 Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial activity of various date fruit extract was tested using a broth micro-dilution method of Wiegand et al. (2008) with modification. The MIC for 96-wells plate template was followed by **Figure 3.5**. Date fruit extracts were prepared by dissolving each dried date fruit extracts with nutrient broth (1000mg of dried extract dissolved in 1ml of nutrient broth). Then, all extracts were diluted with nutrient broth to prepared at four serials of two-fold dilution, forming a concentration of 1000 mg/ml to 62.5 mg/ml. All extracts were filtered using a sterile 0.20µm membrane filter [Sartorius, Germany] before use. The bacterial suspension was then adjusted at 0.5 McFarland standard and measured spectrophotometrically at (0.09-0.12) OD 625nm [GE healthcare, England] and then dilute to 1:100. 50µL of each extract dilution were added in sterile 96-well plate [Corning Costar Ltd, USA] and followed by 50µL of the adjusted bacterial suspension. Appropriate positive control (antibiotic and inoculum), negative control (medium and inoculum), and sterility control (medium only) were included to aid interpretation. The plate then incubated at 37^{o}C for 16-20 hours. The well with visible no growth (turbidity) of the microbes was taken as a MIC.

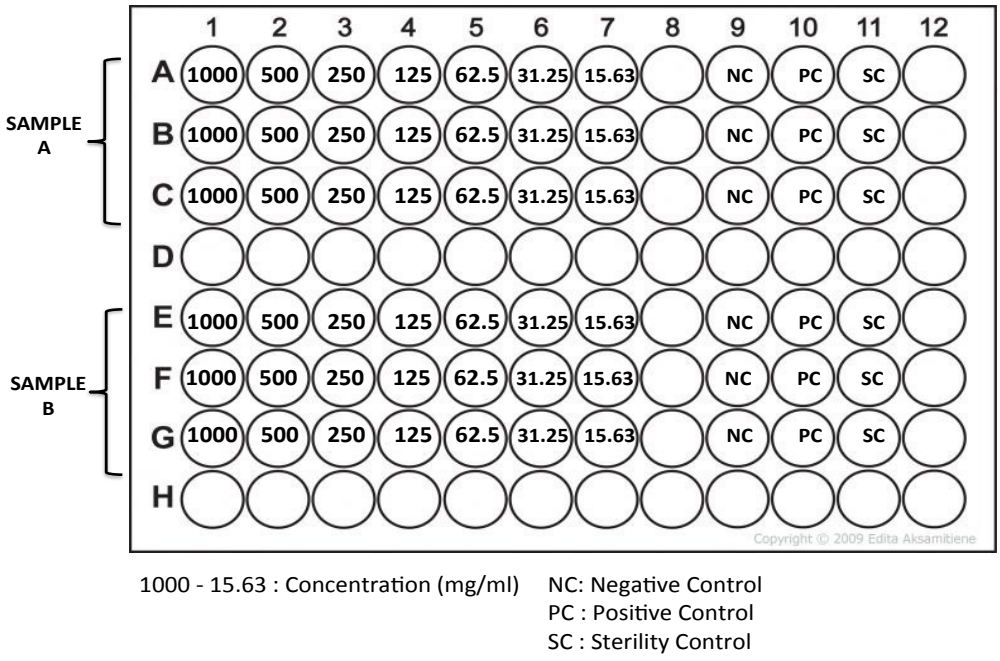

Figure 3.5: Minimum Inhibitory Concentration (MIC) layout in 96-wells plates.

3.6.3 Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) of date fruit extract was assessed in accordance to Clinical and Laboratory Standard Institute [CLSI] (2012) standard whereby 10µL of the sample from MIC well plate which showed no visible growth of microbes were streak on nutrient agar plates (OXOID, USA). The plates were incubated for about 24 hours at 37ºC. The concentration of the extract that showed no colony growth on the plates was taken as the MBC.

3.7 Bacterial Adhesion Assay

3.7.1 Caco-2 cells

Caco-2 cells were purchased from the American Type Culture Collection (ATCC), and the culture was maintained in T25 or T75 flasks [SPL Life Science, Korea] with complete medium of DMEM. Caco-2 cells from passage 2 (P2) to 15 (P8) were used in this study. The cells were incubated in humidified incubator [Galaxy, Eppendorf, Germany] at 37°C in 5% CO_2 and 95% air.

3.7.2 Cells Passaging

Passaging is a processed of sub-culture the cells when it reached 80% to 90% of confluences. The cells were washed twice with 1x PBS before cells were trypsinized with 0.25% trypsin-EDTA for cell detachment. A 2mL of trypsin-EDTA was used for T25 culture flask and 5 mL for T75 culture flask. Then the cells were incubated for a maximum of 10 minutes until cell detached from the culture flask surface. After that, a complete medium of DMEM was added to inactivate the trypsin-EDTA. The cell suspensions were transferred into a 15mL tube and centrifuge at 1500 rpm for 10 minutes. The supernatant was discarded, and fresh complete medium DMEM was added to re-suspend the cells. The cell suspension was re-plated with the desired expansion ratio or cell number. Desired cell number was counted using hematocytometer. 10µl of Trypan blue stain and 90 µL of cells suspension were mixed and unstained cells (lived) cells were counted by light microscopy. The calculation formula for cell suspensions was based on the following;

$$\frac{\text{Number of cells under microscopy} \times 10^4 \times \text{cell suspension (mL)} \times \text{dilution factor}}{\text{Number of fields counted}}$$

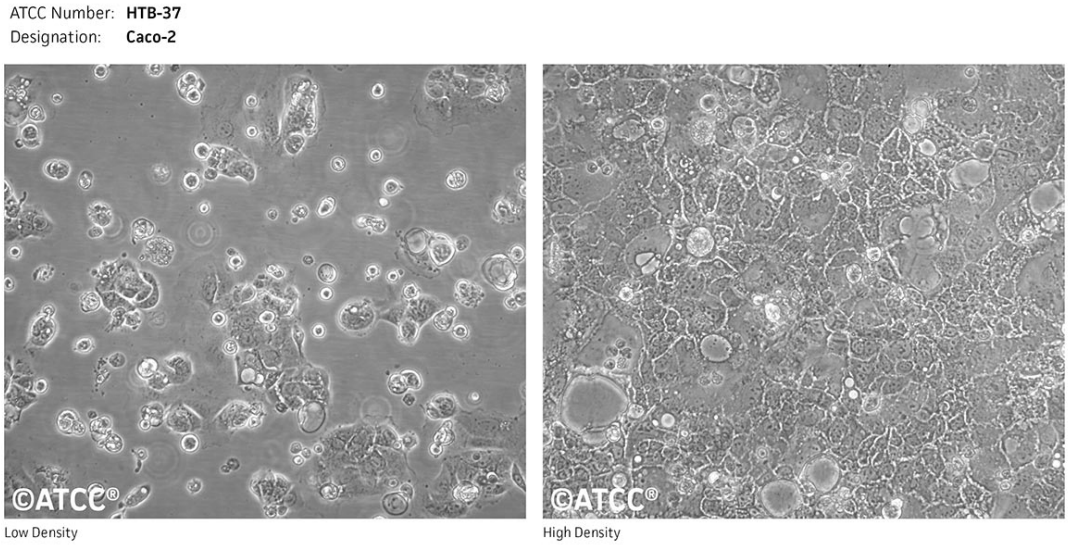

Figure 3.6: Caco-2 cells used in the anti-adhesion assay. Left picture caco-2 cells at low density and right picture caco-2 cells at high density.

Source: ATCC: The Global Bioresource Centre http:/www.atcc.org

3.7.3 Bacterial adhesion assay

Bacterial adhesion assay was performed as Parkar, Stevenson, and Skinner (2008) method with modifications (Parkar et al., 2008). The cells used for this study were between passage 2 and 15. Caco-2 cells were seeded at $1x10^4$ cells/well in sterile cell culture grade 96-well plates [Corning Costar Ltd, USA] and incubated at 37 ^oC in 5% CO_2 and 95% air to obtain confluence prior to the adhesion assays. Overnight bacterial cultures in a broth of all tested bacteria were harvested by centrifugation (3500xg, 10 min), then washed twice with 1x PBS buffer. The bacterial cells were re-suspended in antibiotic-free DMEM at a cell density of approximately $5x10^8$ CFU/ml. Date fruits extract were dissolved in antibiotic-free DMEM at a concentration of 100mg/ml. Cell line monolayers were washed twice with 1x PBS to remove antibiotics before a 50 µL of extract solution was added and incubated for 1 hour at 37^{o}C in a CO_2 incubator. Followed by adding bacterial suspension (10µL) to obtain a multiplicity of infection ratio at 100:1 (bacteria to Caco-2 cells) and a 2 h-incubation. For control wells,

bacterial suspensions were incubated in the same medium without addition of sample extracts. After the incubation period, supernatants were removed, and wells were gently washed twice with 1x PBS to remove non-adhered bacteria. Finally, Caco-2 monolayers were trypsinized by adding 100µL 0.25% trypsin–EDTA solution. The viable bacterial cells were counted using serial dilution methods and plated on appropriate agar plates. Percentage of adhesion was calculated compared with the control as follows:

$$\text{Relative percentage of adhesion (\%)} = CFU_{sample}/CFU_{control} \times 100;$$

Where CFU_{sample} is the number of bacteria adhered in wells containing sample extracts, and $CFU_{control}$ is the number of bacteria adhered in the control wells.

3.8 Isolation of Active Compound

3.8.1 Phytochemical Screening Using UHPLC-ESI_QTOF-MS/MS analysis

This analysis was performed using an LCMS/MS system with Sciex 3200QTRAP hybrid trap mass spectrometer coupled with Perkin Elmer FX-15 UHPLC. The separation was performed on 100mm x 3uM x 2.0mm Phenomex® Synergy Reverse-phase C18 at flow-rate 0.25mL/min and an injection volume at 20µL. The detection was by MS–ESI (-) spectroscopy at a probe temperature of 500°C. All samples were filtered with 0.45µm nylon syringe filter [Merck Millex®] before injecting into a column. The mobile phase used was (A) deionize water with 0.1% formic acid and 5nM ammonium formate, (B) Acetonitrile with 0.1% formic acid and 5nM ammonium formate. The Sciex Triple TOF® 5600+ high-resolution accurate mass spectrometer [AB Sciex Pte. Ltd., Biopolis, Singapore] was used to obtain the mass spectrum. The ionization source

used was Sciex patented Duo Spray source with electrospray ionization (ESI) in negative mode. Ionization voltage used was 5500kV with 500°C source temperature. For the identification of phytochemical compound, the mass scan range was set from m/z 100 to 1200 with the information-dependent acquisition (IDA) of mass fragment spectrum. Identification of the phytochemical was done via chemical formula finding, Sciex internal natural product database and also cross-referencing with existing publication to obtain more data.

3.8.2 Quantification of Phytochemical Using Calorimetric Assay

3.8.2.1 Total Phenolic Content (TPC)

The total phenolic content assay was conducted based on method describes in previous studies (Singleton et al., 1999; Khorasani Esmaeili et al., 2015) with minor modification. Briefly, 2.5 ml of 7.5 % aqueous sodium carbonate (7.5g in 100ml distilled water) [Sigma-Aldrich, Germany] into test tube followed by adding 2.5 ml of 10% Folin-Ciocalteau's reagent and 0.5mL of the date fruit extract (20mg/ml). The mixture was incubating in the dark for 2 hours. Blank was prepared by adding of 99.8% methanol instead of date fruits extract. After incubated, the absorbance was determined at 760nm by using plate reader [Tecan infinite M200, Switzerland]. The TPC was then determined from the standard calibration curve (R2 = 0.9242) using Gallic acid as standard (0.01-0.2 mg/ml). The TPC in the extracts was expressed as mg Gallic acid equivalent per 100g of dry weight (mg GAE/100 g DW).

3.8.2.2 Total Tannin Content (TTC)

The total tannin content was determined by Follin-Ciocalteu method after removal of tannin by their absorption to the insoluble matrix Polyvinylpolypyrrolidone (PVPP). This method was based on (Kchaou et al., 2013); 1mL of date fruit extract (20mg/ml) was added to 100mg of PVPP and incubated for 15 min at 4ºC. The mixture was vigorously shaken and centrifuged for 15min at 13,000g, where the supernatant was collected, and non-absorbed phenolic were subjected to the Follin-Ciocalteu assay for total phenolic content. Results were subtracted from total phenolic content, and total tannins were expressed as mg GAE/100g dry weight.

3.8.2.3 Total Flavonoid Content (TFC)

The flavonoids content was determined by the aluminum chloride method using rutin as a reference compound. This method based on the formation of a complex flavonoid-aluminum having the absorptivity maximum at 430nm. Rutin was used to make the calibration curve. About 500 µL of date fruit extract in methanol was mixed with 500µL of 2% aluminum chloride in methanol. The mixture was incubated for 15 min, and the absorbance was determined at 430nm by using plate reader . The content of flavonoids is expressed in mg rutin equivalent (RE)/100g samples.

3.8.3 Isolation of Active Compound from Ajwa Dates Extract

3.8.4 Sample preparation

Ajwa hot aqueous dried extract was dissolved in methanol at concentration 500mg/ml. The extracts were filtered using 0.45µl syringe filter and aliquots in 1.5ml HPLC vials.

3.8.5 Isolation using Preparative High-Performance Liquid Chromatograph (Prep-HPLC)

The isolation of active compound, a preparative scale reverse-phase chromatography was performed using a C-18 column (Zorbax 300sb), 250mm in length and 2.6mm internal diameter. The HPLC elution used was a gradient mobile phase consisting of 0.0125% Formic Acid in water: acetonitrile (100%), at a flow rate of 3ml/min for the period of 70 minutes. The detection was at 206nm and 320nm. The gradient was as followed: 0-5% B for 0-4 min, 2-25% B 4-5min, 25-25%B 4-40 min, 25-80% B 40-55 min, 80-80% B 55-60 min, 80-5% B 60-65 min, and 5-5% B 65-70 min. Each compound in the fraction then identified using UHPLC-ESI_QTOF-MS /MS approach.

3.8.6 Characterization and Identification of Isolated Compound Using UHPLC-ESI_QTOF-MS/MS

Characterization and identification were followed method **3.8.1**

3.9 Investigation of Bacterial Morphological Changes via Electron Microscope

3.9.1 Scanning Electron Microscopy (SEM)

3.9.1.1 Sample Preparation

Bacteria were cultured in nutrient broth and incubated using shaking incubator at 37ºC for 16-24 hours. After incubation, the bacterial suspensions were divided into two portions, one portion for control and one portion for treatment. For control, bacterial harvested by centrifugation and the bacterial palette immediately fixed using 2.5% glutaraldehyde. For treatment, bacterial cells were harvested using centrifugation (5000rpm x 5mins) and discarded the supernatant. Then extracts (500 mg/ml) then was added into bacteria palette and further incubated for 16 hours.

3.9.1.2 Fixation

The samples were fixed by harvested the cells by centrifugation at 5000rpm for 5 minutes. The supernatant was removed, and the bacterial palette was fixed using 2.5% glutaraldehyde for 4-6 hours at 4ºC.

3.9.1.3 Washing

After fixation for 4-6 hours, samples were washed three times with 0.1M Sodium Cacodylate buffer and sample were centrifuged at 5000rpm for 5 minutes each.

3.9.1.4 Post-Fixation

The samples then were post-fix in 1% Osmium tetroxide for 2hours at 4ºC.

3.9.1.5 Washing

After post-fixation with 1% osmium tetroxide, cells were washed three times with 0.1M Sodium Cacodylate with 10 minutes interval.

3.9.1.6 Dehydration

The cells then dehydrated with a series of acetone at 10 minutes interval (**Table 3.1 & Figure 3.7**)

Table 3.1: A series of sample dehydration in acetone

Concentration	Time
35%	10 minutes
50%	10 minutes
75%	10 minutes
95%	10 minutes
100%	15 minutes

Figure 3.7: Sample dehydration using a series of acetone.

3.9.1.7 Mounting and Critical Point Drying (CPD)

The cells mounted and fixed on a sheet of aluminum foil, which pre-coated with egg white to help the cells to sticks on the surface. The cells were then transferred into the chamber of Critical Point Machine (CPD) [Leica EM CPD030, Wetzlar, Germany] and the cells let to dry for 90 minutes (**Figure 3.8**). In the chamber of CPD, the acetone was replaced by liquid carbon dioxide. The liquid carbon dioxide then converted into gas whereas leaving the cells in a super dry state.

Figure 3.8: Critical point drying machine in IBS, UPM

3.9.1.8 Coating

Prior viewing, cells were coated with gold using sputter coater Bal-tec Scd 005 Leica (**Figure 3.9**) [Wetzlar, Germany]. Firstly, the chamber was evacuated until it reached a pressure of approximately 1x $10^{-1.063}$m bar. The chamber than was flushed with argon gas several times. To activate the sputter coating, the start button was pressed, and the process was stopped when the sputtering time has elapsed.

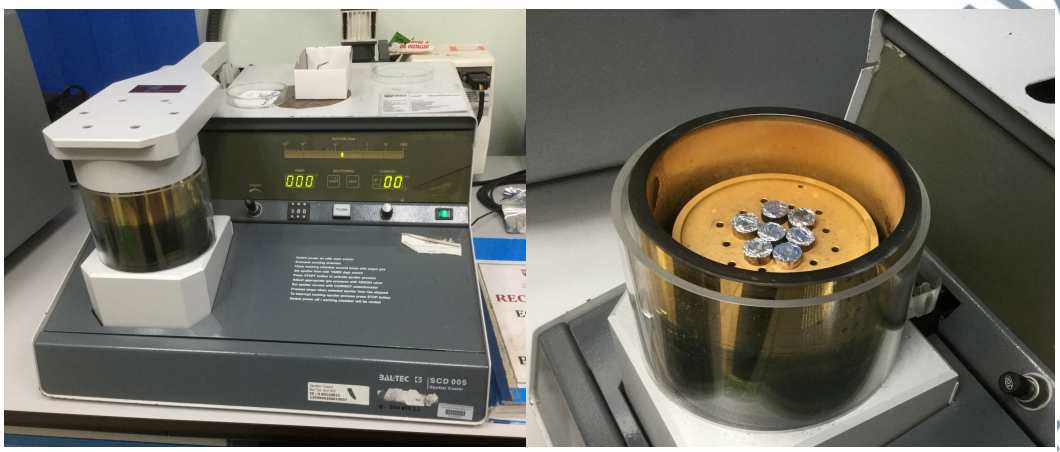

Figure 3.9: Gold coating using sputters coater in IBS, UPM

3.9.1.9 Viewing

All samples were view and examined using a JOEL JSM-IT InTouchScope Scanning electron microscope (**Figure 3.10**) (JOEL, Japan).

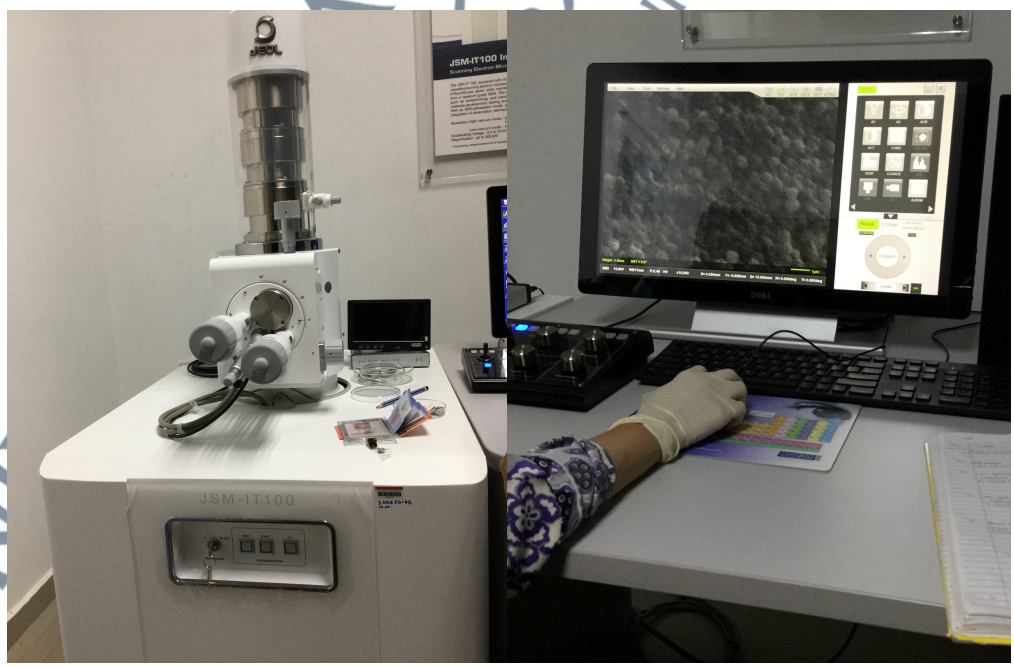

Figure 3.10: Viewing sample using Scanning electron microscope in IBS, UPM.

3.9.2 Transmission Electron Microscopy (TEM)

3.9.2.1 Sample Preparation

Bacteria were cultured in nutrient broth and incubated using shaking incubator at 37ºC for 16-24 hours. After incubation, the bacterial suspensions were divided into two portions, one portion for control and one portion for treatment. For control, bacterial harvested by centrifugation (5000rpm x 5mins) and the bacterial palette immediately fixed using 2.5% glutaraldehyde. For treatment, bacterial cells were harvested using centrifugation (5000rpm x 5mins) and discarded the supernatant. Then extracts (500mg/ml) then was added into bacteria palette and further incubated for 16 hours.

3.9.2.2 Fixation

The cells were harvested by centrifugation, and an appropriate quantity of horse blood serum was added to submerge the samples. The sample was allowed to clot, and the clotted samples were diced into $1mm^3$. Then fixed with 2.5% glutaraldehyde for 4-6 hours at 4ºC.

3.9.2.3 Washing

After fixation for 4-6 hours, cells were washed three times with 0.1M Sodium Cacodylate buffer with 10 minutes time interval.

3.9.2.4 Post-Fixation

The cells then postfix in 1% Osmium tetroxide for 2hours at 4ºC.

3.9.2.5 Washing

After post-fixation with 1% osmium tetroxide, cells were washed three times with 0.1M Sodium Cacodylate with 10 minutes interval.

3.9.2.6 Dehydration

The cells then dehydrated with a series of acetone at 10 minutes interval (**Table 3.2)**

Table 3.2: A series of sample dehydration in acetone

Concentration	Time
35%	10 minutes
50%	10 minutes
75%	10 minutes
95%	10 minutes
100%	15 minutes

3.9.2.7 Infiltration

The samples were infiltration with 100% acetone and resin mixture accordingly (**Table 3.3)**

Table 3.3: Infiltration samples with acetone and resin mixture

Acetone: Resin	Time
1: 1	1 hour
1 : 3	2 hours
100% Resin	Overnight
100% Resin	2 hours

3.9.2.8 Embedding

After infiltration, samples were placed into a beam capsule and filled up with resin.

3.9.2.9 Polymerization

Samples in beam capsule were polymerized in an oven at 60ºC for 24-48 hours.

3.9.2.10 Thick Sectioning

Thick sectioning was performed by cut the samples into 1µm thick sections. This was performed using a glass knife maker and ultramicrotome [JOEL, Japan]. The sections then were placed onto a glass slide and stained with toluidine blue. The sections were dried on a hot plate and stain was washed. These sections were examined under a light microscope to select an interesting area.

3.9.2.11 Ultrathin sectioning

Ultrathin sections were done by a selected area of interest and cut for ultrathin sections. The thickness of sections was determined by the colour of sections floating in the boat. Thick section appeared blue and gold while the ultrathin section appeared silver. The silver colours sections were pick up onto the bright surface of the copper grid.

3.9.2.12 Staining

Sections on the copper grid were stained in Uranyl acetate for 15 minutes and washed with double distilled water. The copper grid then immersed in the lead stain for

10 minutes following washing with double distilled water and let dry with a piece of filter paper.

3.9.2.13 TEM Viewing

The samples were viewed using a JOEL JEM-210F transmission electron microscope (**Figure 3.11**)[JOEL Japan]

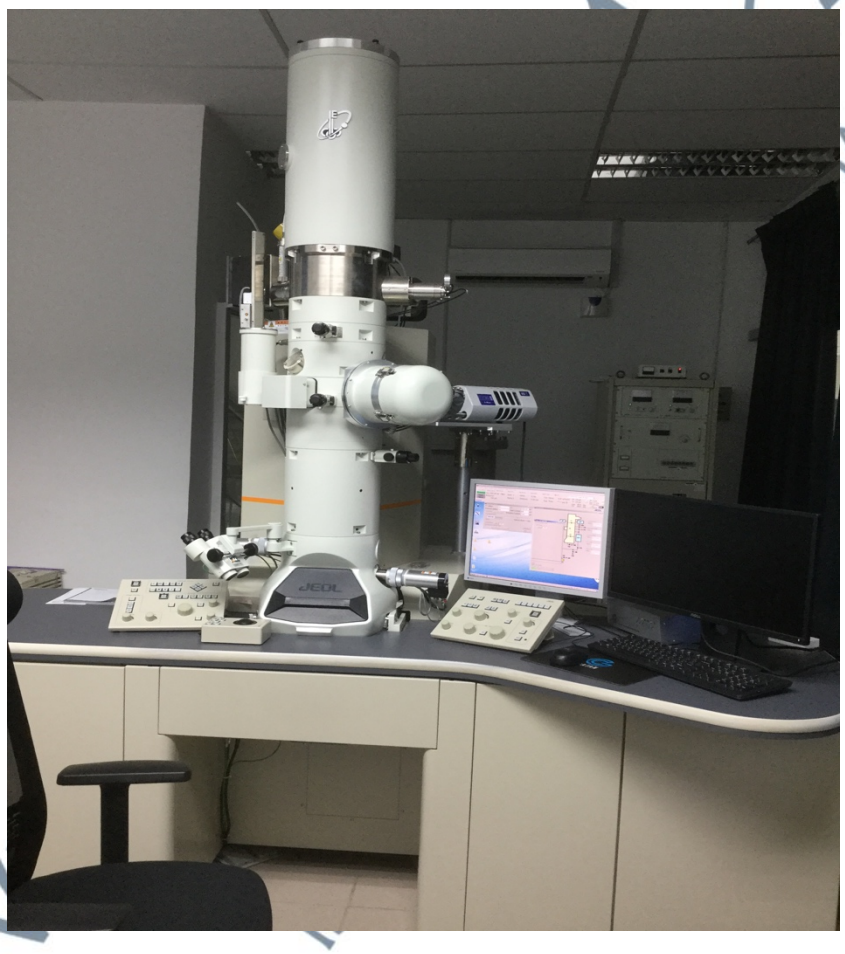

Figure 3.11: Viewing Transmission Electron Microscope (TEM) in IBS, UPM.

3.10 Statistical Analysis

Samples were tested in triplicate. Data were expressed as mean (± standard deviation). Error bars represent Standard deviation (SD). Statistical analysis was done using Graph Pad Prism version 6.0 for Mac, Graph Pad Software, San Diego California USA (www.graphpad.com). Data were subjected to Kruskal-Wallis one-way analysis of variance, and significant results were subjected to post-hoc analysis (Dunn test) for multiple comparisons. The correlation between antibacterial activity and phytochemical contents (TPC, TFC, and TTC) were evaluated by Spearman correlation coefficient test. All data with p-value of < 0.05 were considered statistically significant.