# CHARPTER III
# EXPERIMENTAL

## 3.1 Materials

3.1.1 Spray Solution

*3.1.1.1 Poly(vinyl acetate)*

*3.1.1.2 Mangosteen Extract*

*3.1.1.3 Ethanol Solvent*

*3.1.1.4 Propanol Solvent*

*3.1.1.5 Ethyl Acetate Solvent*

3.1.2 For Bacterial Culture

*3.1.2.1 E. coli (Escherichia coli)*

*3.1.2.2 S. aureus (Staphylococcus aureus)*

*3.1.2.3 Methicillin-resistant Staphylococcus Aureus (MRSA)*

*3.1.2.4 Staphylococcus Epidermidis*

*3.1.2.5 Enterococcus Faecalis*

*3.1.2.6 Acinetobacter Baumannii*

*3.1.2.7 Pseudomonas Aeruginosa*

*3.1.2.8 Vancomycin-resistant Enterococcus*

*3.1.2.9 Deionized Water (pH 6.5)*

*3.1.2.10 NaCl 0.8%w/v*

*3.1.2.11 Peptone Broth*

*3.1.2.12 Agar Powder*

*3.1.2.13 Tryptic Soy Broth (TSB)*

3.1.3 For Cytotoxicity Test

*3.1.3.1 HaCaT Cells*

*3.1.3.2 Mouse Fibroblast Cells (L929)*

*3.1.3.3 Normal Human Fibroblast Cells (NHF)*

*3.1.3.4 DMEM (Dulbecco's MEM)*

*3.1.3.5 Simulated Body Fluid (SBF) Buffer Solution (pH 7.4)*

*3.1.3.6 L-glutamine 100x*

*3.1.3.7 Lactabumin*

*3.1.3.8 Ab/Am 100x*

*3.1.3.9 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl Tetrazoliun Bromide (MTT)*

*3.1.3.10 Phosphate Buffer Saline or PBS (pH 7.4)*

*3.1.3.11 Dimethyl Sulfoxide (DMSO)*

*3.1.3.12 0.1 M Glycine Buffer Solution*

3.1.4 For *In vitro* Drug Release Study

*3.1.4.1 Acetate Buffer Solution (pH 5.5)*

*3.1.4.2 Phosphate Buffer Saline (pH 7.4)*

## 3.2 Equipments

3.2.1 Petri Dish Plastic 15 x 90 mm and a 24-well Plate from Corning for Culturing and Investigating Bacteria and Cells Viability

3.2.2 Microscope was Chosen to Capture Characteristic of Cells after Cultured in Extraction Media on 1 and 3 Days

3.2.3 Shaking Bath for *in vitro* Drug Release Study at 37 °C by varying time

3.2.4 Incubator was chosen to culture bacteria at 37 °C

3.2.5 UV-Vis Spectrophotometer (UV-2500) was Used to Characterize The Formation of Drug Releasing

3.2.6 Contact Angle was Used to Measure Waterproof Function

3.2.7 Ubbelohde Viscometer was Used to Measure Kinetic Viscosity of Spray Solution at Room Temperature

## 3.3 Methodology

3.3.1 Turbidity

Any solution were investigated complete solubility by using turbidity measurement (JIS K0101). Turbidity of solution was performed from UV-absorbance at 660 nm.

3.3.2 Evaporation Rate

All spray's solution were investigated rating of evaporation by observing weight change depened on times. 10 ml of spray's solution drop on plate by fix temperature at 25 °C. Next, weight of spray's solution were recorded every 10 secounds.

3.3.3 Solution Viscosity

Ubbelohde viscometer (υ) was chosen to detect kinetic viscosity of all solutions. In an ubbelohde viscometer, the time that liquid take between two ring marks is proportional to the viscosity of liquid. The kinetic viscosity is measured following procedure in the ASTM 445 (Berger *et al.*, 2004). First the viscometer is cleaned with acetone solvent .Then dry air is passed through the instrument to remove final traces of solvent. After cleaning the instrument, charge the viscometer by introducing sample through tube into lower reservoir. The kinematic viscosity (υ) at room temperature is estimated using the equation 1.

$$\upsilon = t \times C \qquad (1)$$

Where t is the measured flow time to cross the two ring marks and C is the calibration constant.

3.3.4 Contact Angle

Advancing and receding air-water contact angles were measured at room temperature by an image analyzing system, using the air bubble technique. The advancing angle was obtained by placing a needle in deionized water and creating a bubble on the sample surface (1–1.5 mL) and carefully expanding the bubble volume until the part of carefully expanding the bubble volume until the part of the bubble adjacent to the sample surface stopped changing. Similarly, the receding angle was obtained when the air was sucked out of the bubble.

3.3.5 Drug Release

Drug release study is investigating in vitro for a human blood's and skin's pH at 7.4 and 5.5, respectively. Released drug from film was measured by placing a known mass and approximate dimensions (1.2 × 1.2 cm) of material into 25 mL of PBS (pH 7.4) and acetate buffer (pH 5.5) under constant stirring at 37°C. At certain time points, 20 μL the buffer was taken out of the buffer and kept into a micro tube for measuring the absorbance further. The amount of drug released was determined using a UV-vis spectrophotometer at λmax = 380 nm for acetate buffer (pH 5.5) and λmax = 380 nm for PBS (pH 7.4). The release experiments of each sample were performed in triplicate, and average values are reported.

3.3.6 MIC and MBC

The antibacterial activity of the sprayed film was tested against gram positive such as *S. aureus* (*Staphylococcus aureus*), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* and gram negative bacteria for instant *E. coli* (*Escherichia coli*), *Enterococcus faecalis, Acinetobacter baumannii* (MDR), *Pseudomonas aeruginosa* and Vancomycin-resistant *Enterococcus* (VRE). For the minimum inhibitory concentration (MIC) test, every bacteria were cultivated in Tryptone soy broth (TSB) in a shaking incubator at 37°C and 100 rpm for 24 h. The bacterial suspension was diluted until the bacterial concentration was approximately $1 \times 10^6$ CFU/ml, and it was then pipetted into a 24 well plate at a concentration of 1 ml/well. The different ratio of the mangosteen extract (0.25–5 mg) were placed into the wells that contained the bacterial suspension and incubated at 37°C for 24 h. The MIC was defined as the mini-mum concentration of mats where no growth was observed after 24 h of incubation. The optical density (OD) at 550 nm was measured using a microplate reader. For determining the minimum bactericidal concentration (MBC), the mixtures from the wells with no growth (100 _l) were spread onto agar plates for the MBC determination. The MBC was defined as the minimum concentration of mats where no colony growth was observed on the agar plates after 24 h of incubation at 37◦C. The MIC and MBC determinations were conducted in triplicates, and 1 mg/ml penicillin was used as a positive control.

3.3.7 Disk Diffusion Method ATCC 147

The stock solutions of each extract were prepared in dimethyl sulfoxide (DMSO). Bacteria were incubated in tryptic soy broth (TSB) for 24 h at 37 °C in shaker and were adjusted to yield approximately $10^8$ CFU/ml. The inoculum was spread on Mueller Hinton agar (MHA) and air-dried at room temperature. A 6-mm sterile paper disc was impregnated with test materials and the disc was placed on the agar. The plates were left to dry, then were incubated at 37 °C for 24 hr under aerobic condition. All disc diffusion tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced by the medicinal extracts.

3.3.8 Time-kill Kinetic

The inoculum was prepared by making a direct suspension of isolated colonies selected from the 18–20 h Tryptic Soy Agar (TSA) plate in PBS solution. The concentration of the inoculum suspension was adjusted with the buffer. After inoculation, each tube with the desired concentration of sprayed film and commercial products also contained bacteria at $10^5$ to $10^6$ CFU/ml. The tubes were then incubated at 35 °C and samples were withdrawn at the specified time intervals for viable plate counts. Serial 10-fold dilutions of the sample were made in the diluent, D/E Neutralisation Broth. A 20 µL aliquot of each dilution was plated out on TSA plates using the surface-spread plate method. The plates were incubated at 35 °C for 0, 1, 12 and 24 h. The possibility of carry-over of the antimicrobial agents onto the plates was examined using the test organism as control in the serial dilutions in the presence and absence of the antimicrobial agents.

3.3.9 Cytotoxicity

HaCaT cells, Mouse fibroblast cells (L929) and Normal human fibroblast cells (NHF) were from the American Type Culture Collection. Cells were cultured in DMEM (for SK-MEL-28) or IMDM (for CCD-1064Sk) (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corporation, Australia), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Scientific, Melbourne, Australia) and maintained in an incubator with 5% CO2 at 37 °C.

The indirect cytotoxicity of the nanofiber mats was evaluated based on a procedure adapted from the ISO10993-5 standard test method (indirect contact).

The sprayed film and commercial products were sterilized with UV radiation for 1 h. The mats were then immersed in a serum free medium (SFM; containing Dulbecco's modified Eagle's medium (DMEM), 1% (v/v) l-glutamine, 1% (v/v) lactalbumin and 1% (v/v) antibiotic and antimycotic formulation) in an incubator for 24 h to produce different concentrations of extraction media (1, 2.5, 5, 7.5and 10 mg/ml). Each cells were plated in 500 µl of DMEM, which was supplemented with 10% FBS,at a density of 10000 cells/well in 24-well plates. When the cultures reached confluence (typically 48 h after plating), the varying concentrations of the tested extraction media were replaced, and the cells were re-incubated for 24 h. After treatment, the tested extraction solutions were removed. Finally, the cells were incubated with 100 µl of a MTT-containing medium (1 mg/ml) for 30 minutes.The medium was removed, the cells were rinsed with PBS (pH7.4), and the formazan crystals that formed in living cells were dissolved in 100 µl of dimethyl sulfoxide and glycerine per well. The relative cell viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, ModelAOPUS01 and AI53601, Packard BioScience, CT, USA). The cell viability of the non-treated control (TCPS) was defined as 100%.