CHARPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Preparation of Coated Gauze

3.1.1.1 Gauze Dressing

3.1.1.2 Sodium Alginate (Na-AG)

3.1.1.3 Calcium Chloride

3.1.1.4 Mangosteen Extracts

3.1.1.5 Deionized Water (pH 6.5)

3.1.1.6 Ethanol (C_2H_5OH)

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 MRSA (Methicillin-resistant Staphylococcus Aureus)

3.1.2.4 S. epidermidis (Staphylococcus Epidermidis)

3.1.2.5 E. faecalis (Enterococcus Faecalis)

3.1.2.6 P. aeruginosa (Pseudomonas Aeruginosa)

3.1.2.7 VRE (Vancomycin-resistant Enterococci)

3.1.2.8 MDR A. Baumannii (MDR Acinetobacter Baumannii)

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 Human Fibroblast Cells

3.1.3.2 Human Keratinocyte Cells (HaCat)

3.1.3.3 Mouse Fibroblast Cells (L929)

3.1.3.4 DMEM (Dulbecco's MEM) or MEM (Minimum Essential Medium)

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 22

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

3.2.2 Shaking bath for in vitro drug release study

3.2.3 UV-Vis spectrophotometer (UV-2500)

UV-Vis spectrophotometer (UV-vis; Shimadzu UV-1800) was used to confirm the mangosteen extracts drug releasing.

3.2.4 Micropalted Reader

Microplate reader model Infinities 200 PRO NanoQuant Multimode Microplate Reader from Tecan AG was used to determine the optical density of the samples in MTT assay.

3.3 Methodologies

3.3.1 Characterization and Testing of coated gauze with Mangosteen Extract

3.3.1.1 Oxygen Permeability

The oxygen permeability followed by B. Singh *et al.*, 2012. The samples were cut to circle shape. Then, all of the samples were covered on the top of the flask that contained 20 ml of boiling de-ionized water. The negative control was the closed flask with blocking oxygen. On the other hand, the positive control was an open flash. All of them were kept at open environment for 24 hr. and then used Winkler's method (Winkler, 1888) to determine the dissolved oxygen.

3.3.2.2 Winkler's Method

First, added 0.08 ml of 48% $MnSO_4$ solution to 20 ml the test sample, and added 0.16 ml of alkaline KI (15% KI in 70% KOH) solution. Then a brown precipitate appeared. Second, the sulphuric acid (50% v/v) was added in the solution from first step. And brown precipitate was dissolved completely. Next, 5 ml of clear solution from second step was titrated with sodium thiosulfate ($Na_2O_3S_2$) and added 0.1% starch solution used as an indicator.

3.3.2.3 In Vitro Blood Clotting

The in vitro blood clotting follows by M. shih *et al.*, 2006. All of sample were cut and placed into flat-bottom bottles. The bottles were pre-warmed at 37 °C for 5 minutes. Then 0.3 ml of mixing blood and ACD (anticoagulant citrate dextrose) ratio 9:1 was slowly dropped on each sample, and followed by 0.03 ml of 0.2 M $CaCl_2$. The bottles and samples were kept in 37 °C for 10 minutes. Then 10 ml of DI water was carefully added into each bottle without disturbing the blood clot. Sampling 5 ml of solution and diluting 5 times. All of sampling was kept at 37 C for 60 minutes. The blood clotting test was determined by UV-Vis spectrophotometer at 542 nm wave number. The blood clotting index (BCI) was calculated by following equation:

$$\text{BCI Index} = \frac{100 \times (abs\ of\ blood\ solution\ thant\ sampling\ from\ sample\ at\ 542\ nm)}{abs\ of\ ACD\ whold\ blood\ in\ DI\ water\ at\ 542\ nm)}$$

3.3.2.4 Swelling Ratio

Swelling ratio followed by Straccia *et al.*, 2014 method. Before testing, all samples were dried in an oven at 60 °C 24 hours and measured the average thickness and weight. Then dried films were washed one time in deionized water for 5, 10, 30, 60, 120, 400 minutes and 24 hrs, withdrawn by briefly blotting between two filters papers and then weighed. The swelling ratio that is function of time were determined by

$$\%\Delta W_{st} = \frac{Wt - Wd}{Wd} \times 100$$

Where $\%\Delta W_{st}$ was the swelling ratio, W_t and W_d were the weights at time t and the weight of dried films, respectively.

3.3.2 Bacterial Culture Evaluations

3.3.2.1 Evaluating MIC and MBC Value by Dilution Test and Agar Test

All bacteria were activated in different flasks. In each flask, there was 3 g/ml of tryptic soy broth (TSB) in DI water as the bacterial growing supporter and kept them in an incubator at 37 °C for 18 hours. Then the activated bacteria solution was added in 0.85%w/v NaCl and adjusted the turbidity of bacterial suspensions or McFarland Standard No. into 0.5 (that means cell density was approximately 1.5×10^8 CFU/mL).

Mangosteen extracts were dissolved in DI water and used DMSO as a surfactant. Then the mangosteen extracts solution was diluted to 10 times. In each dilution, activated bacteria in NaCl solution (McFarland Standard No. as 0.5) was added and kept it in an incubator at 37 °C for 24 hours. The MIC (Minimum Inhibiting Concentration) is the lowest concentration of an antimicrobial that inhibit the visible growth of a microorganism after overnight incubation. The lowest concentration that noticed clear solution is MIC value.

After 24 hours, each dilution was placed on agar plates in order to evaluate MBC value. The MBC (Minimum Bactericidal Concentration) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. Both values can be the same number. In this study, the concentration of

MBC was decided by lowest concentration that each bacteria was not appear on the agar plates.

3.3.2.2 Zone Inhibition

An inhibitory test of initial gauze, coated gauze by calcium alginate with and without mansosteen extracts were investigated by disc diffusion method (Bauer et al., 1966). All samples were cut in circle shape (diameter as 0.5 cm), purified them by ultraviolet radiation (UV).

All bacteria were prepared for activated bacteria in NaCl solution (McFarland Standard No. as 0.5). The bacteria solution was smeared onto different prepared agar plate. Them placed the prepared sample on the agar's surface and kept in an incubator at 37 °C for 18 hours. The diameters of the inhibition zones were measured in diameter by transparent ruler.

3.3.2.3 Bacteria Reduction

Bacteria reduction method use to confirm antibacterial efficacy of all simples. All of samples were cut into circular shape (a diameter was 1.2 cm). First, plastic film was placed into each bottle, and placed each samples in different bottles and then drop bacterial solution (McFarland Standard No. as 0.5) on the samples and covered them with plastic film. All of the bottles were kept at 37 °C for 0, 1, 12 and 24 hours. At any time required, 2 ml PBS was added in each bottle. Sampling 0.1 ml of each solution and diluted by PBS 10 times. In each dilution, the solution was drop into prepared agar plate and kept in an incubator again at 37°C for 18 hours. Finally, count colony of bacteria in each plate.

3.3.3 Biological Characterization

Human fibroblast cells, human keratinocyte cells (HaCaT) and mouse fibroblast cells (L929) were used to determine the biological response of coated gauze by calcium alginate with and without mangosteen extract. They were cultured in α-MEM medium which was changed every 2 days. The cultured cells were kept at 37 °C under 5% CO_2.

3.3.3.1 Indirect Cytotoxicity Evaluation

All of the gauzes, initial gauze and coated gauze with and without mangosteen extracts, were cut into circular sharp (a diameter was 1.2 cm.) and purified them by X-ray. Placed all sample in each well in 24-well tissue-culture

polystyrene plate (TCSP) and added SFM (SFM: containing MEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic) overnight.

Cell preparation, first added α-MEM medium in each well in TCSP and cultures cells in prepared well overnight. The density of cells about 40,000 cells/cm^2 for L929 and 10,000 cells/cm^2 for human fibroblast cells and HaCat cell. Then α-MEM medium was remove and changed to SFM to starve cell and leaved overnight again. After 1 day, SFM was removed and added prepared SFM (SFM was immersed with sample). They were placed under 5% CO_2 at 37 °C in 24-well plate for 1 day and 3 days. After time required, MTT assay was used to determine the number of viable cells.

3.3.3.2 MTT Assay

MTT assay used to determine the number of viable cells. It noticed concentration of purple formazan crystals by using UV-Vis Spectrophotometer. The amount of purple formazan crystal formed is proportional to the number of viable cells. First of all, the cultured medium was moved out in each well and added in 400 μL MTT solution at 37°C for 30 minutes. Then, MTT was replaced by a buffer solution containing 900 μL/well of DMSO and 125 μL/well glycine buffers (pH 10) in each well to dissolve the formal crystal. The solution was shaken for 15 minutes and then transferred into a spectrophotometer to measure the number of viable cells at absorbance 540 nm.