CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Larvae

The fly colonies of *L. cuprina* were maintained at the insectarium of Medical Entomology Unit, IMR, Kuala Lumpur under 12:12 hour of light dark cycle at 22 ± 2˚C and 77.0 ± 2.53% humidity with continuous supply of tab water and granular sugar (Figure 3.1.1). Female flies were provided with raw cow liver for oviposition. The resultant eggs were transferred onto fresh pieces of raw cow liver and mouse pellet in clean containers and the hatched larvae were constantly supplied with fresh, raw cow liver and tap water for development into late second-instar larvae.

Figure 3.1.1: Fly colonies of *L. cuprina* at the insectarium of Medical Entomology Unit, IMR

3.1.2 Bacteria

Staphylococcus aureus ATCC25923, Methicillin-resistant *Staphylococcus aureus (*MRSA S914, a clinical isolate), *Staphylococcus epidermidis* ATCC14990, *Streptococcus pyogenes* (S630, a clinical isolate), *Klebsiella pneumoniae* (ATCC700603), *Pseudomonas aeruginosa* (ATCC27853) and *Escherichia coli* (ATCC25922) were kind gift from the Bacteriology Unit, IMR, Kuala Lumpur. These bacterial cultures were maintained on blood agar (BA).

3.1.3 Chemicals

All chemicals were purchased from Bio-Basic, Canada and Oxoid Ltd (BioFocus Saintifik Sdn.Bhd).

3.2 METHODS

3.2.1 Production of Larval Extract

Approximately 500 unsterile, two to three days-old *L. cuprina* larvae were collected from raw cow livers and transferred into a clean, disinfected 50 ml washing tube. The unsterile larvae were washed with 40 ml of 5% formaldehyde for five minutes and rinsed three times with sterile distilled water. Washed larvae were blot-dried with sterile paper towels and transferred into sterile universal bottles (200 larvae per bottle). The universal bottles were covered with sterile nylon netting and secured with liner; then 500 µl of sterile normal saline was added into the bottles. The larvae were incubated at 37 ℃ overnight.

Following an overnight incubation, the overnight-starved larvae were washed three times with 70% ethyl alcohol and rinsed three times with sterile distilled water. Again, larvae were blot-dried with sterile paper towels and transferred into another

clean, disinfected 50 ml washing tube. Then, the larvae-containing washing tubes were placed onto ice to deactivate the larvae.

Deactivated larvae were homogenized manually with 15-ml glass Dounce homogenizer (Figure 3.2.1 a) and adequate amount of absolute methanol was added from time to time during homogenization. The homogenate was then transferred into clean, disinfected centrifuge tubes and centrifuged at 8,000 x g for five minutes (Figure 3.2.1 b). The resultant supernatant was collected and transferred into clean, disinfected eppendorf tubes. Lastly, the supernatant was concentrated using a vacuum concentrator (Eppendorf Concentrator 5301 – Figure 3.2.1 c) to remove methanol. The vacuum-concentrated product, i.e., the larval extract was weighed before being kept at -70 ℃. Prior to use, 200 mg larval extract was re-suspended in 1 ml sterile distilled water. For each batch of larval extract, 10% of the product was tested for sterility on blood agar (BA) and brain-heart infusion agar (BHIA).

3.2.2 Preparation of the 0.5 McFarland Standard

The preparation of the 0.5 McFarland Turbidity Standard was carefully performed according to the Clinical and Laboratory Standards Institute (CLSI) protocols. A volume of 0.5 ml of 0.048 M barium chloride (1.17% w/v $BaCl_2.2H_2O$) was added to 99.5 ml of 0.18 M sulphuric acid (1.0% v/v H_2SO_4) with constant stirring. The prepared 0.5 McFarland Standard was transferred into universal bottle, sealed tightly with parafilm to prevent loss by evaporation and finally wrapped with aluminium foil to avoid exposure to light and stored at room temperature.

Figure 3.2.1a: 15-ml glass Dounce homogenizer used to homogenize *L. cuprina* late second-instar larvae

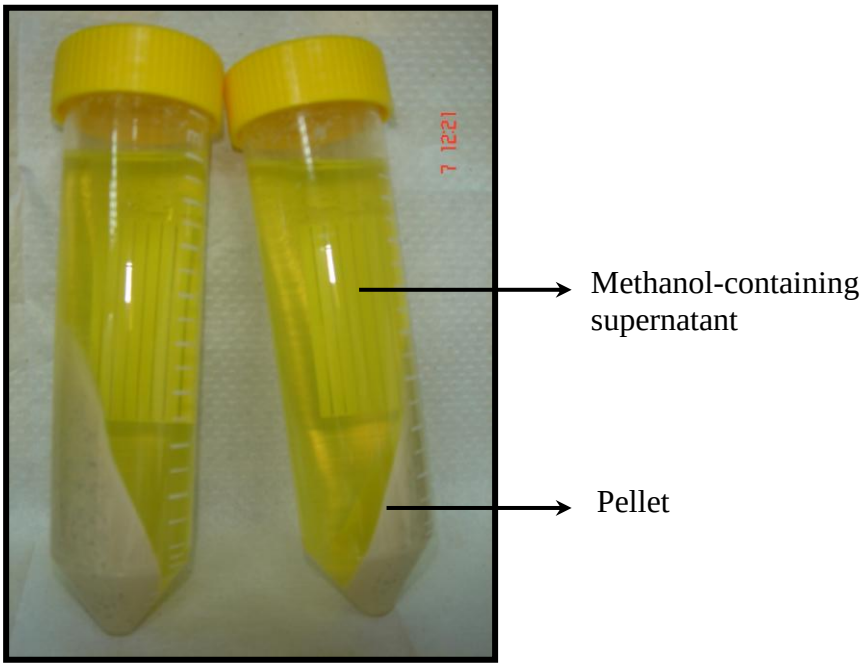

Figure 3.2.1b: Centrifuged *L. cuprina* larval homogenate

Figure 3.2.1c: Vacuum-concentrator used for concentrating the larval extract and removing methanol

3.2.3 Preparation of Bacterial Suspension

Bacteria stock cultures (*S. aureus,* MRSA*, S. epidermidis, S. pyogenes, K. pneumoniae, P. aeruginosa* and *E. coli*) were sub-cultured onto BA plates and incubated overnight at 37℃. The next day, three to four discrete bacterial colonies with similar morphology were inoculated into 25 ml sterile BHI broth and incubated overnight at 37℃ and 150 rpm. The overnight bacterial suspensions were adjusted to 0.5 McFarland Standard with sterile normal saline for Agar Well Diffusion and Minimum Inhibitory Concentrations Assays or sterile BHI broth for Turbidometric and Colony-Forming Unit Assays on ice. To aid comparison, the adjustment of bacterial suspensions to the density of the 0.5 McFarland Standard was done against a white background with contrasting black lines.

3.2.4 Turbidometric (TB) Assay

This assay was adapted from Jaklic *et al.* (2008). In a 96-well flat-bottom microtiter plate, 100 µl bacterial suspensions after diluted to 1.0 x 10^2 cell/ml with sterile BHI broth were pipetted into the corresponding Growth Control Wells and Test Sample Wells. Another 100 µl of sterile distilled water was added into the Growth Control Wells, making a final bacterial concentration of 0.5 x 10^2 cell/ml; whilst 100 µl of larval extract (200 mg/ml) was added into the Test Sample Wells, making a final concentration of 100 mg/ml larval extract.

For each set of Growth Control and Test Sample Well, there was a corresponding pair of Broth Sterility Control and Larval Extracts Sterility Control Wells. The Broth Sterility Control Well contained 100 µl sterile distilled water and 100 µl BHI broth; whilst the Larval Extract Sterility Control Well contained 100 µl larval extract and 100 µl BHI broth. All bacteria samples and controls were run in triplicates and repeated ten times. Before and after an overnight incubation, microtiter plates were

agitated and the optical density (OD) of each well at 625 nm wavelength was determined using spectrophotometer.

3.2.5 Colony-Forming Unit (CFU) Assay

After an overnight incubation and OD_{625} measurement, 100 µl of aliquots from each well was diluted to the designated concentration and spread onto BHIA plates and incubated overnight at 37℃. Plates were run in triplicates and repeated ten times. The CFU count of each sample was determined after overnight incubation at 37℃ by multiplying the total CFU formed on the agar plate with the corresponding dilution factor and divided by 0.1 ml.

3.2.6 Agar Well-Diffusion Assay

In this assay, the preparation of bacterial inoculum, adjustment of bacterial suspension to the density of the 0.5 McFarland Standard and inoculation of agar plates were performed precisely according to CLSI protocols.

A sterile cotton-wool swab was dipped into bacterial suspension adjusted to 0.5 McFarland Standard and turned against the wall of the universal bottle to remove excess liquid. The inoculum was spread evenly over the entire surface of the BHI agar plate by swabbing in three directions. Then, three 4 mm-diameter wells were punched into the BHI agar, and 20 µl of larval extract (100 mg/ml) was loaded into one of the wells. The remaining wells contained 20 µl of sterile distilled water (negative control) and 20 µl of 5% formaldehyde solution (positive control), respectively. Plates were run in triplicates and repeated for ten times. The plates were incubated overnight at 37℃ and the radial zones of inhibition (mm) were measured on the following day.

3.2.7 Minimum Inhibitory Concentration (MIC) Assay

The adopted MIC method follows CLSI standard method of microdilution for aerobic bacteria. One set of microdilution consisted of ten wells including positive and negative control. Positive control well (the ninth well) contained 100 µl sterile Mueller Hinton broth and 5 µl bacterial suspension diluted to 0.5 x 10^2 cell/ml, while negative control well (the tenth well) contained 100 µl sterile larval extract (100 mg/ml) and 5 µl bacterial suspension.

In a 96-well flat-bottom microtiter plate, all wells except the tenth well, were filled with 100 µl of sterile Mueller Hinton broth. The tenth well (negative control well) was filled with 100 µl of sterile larval instead.

Firstly, 100 µl of sterile larval extract was pipetted into the first well (2^{-1}) and mixed thoroughly. Then, a separate and sterile pipette was used to transfer 100 µl of mixture in the first well into the second well (2^{-2}), and mixed thoroughly. Again, 100 µl of the mixture was transferred into the third well (2^{-3}) and mixed thoroughly. This serial dilution was continued to the eighth well (2^{-8}). Lastly, 100 µl was removed from the eighth well and discarded. The final concentration of larval extract is now one-half of the original concentration in each well.

Then, 5 µl of diluted bacterial suspension (0.5 x 10^2 cell/ml) was added into all wells and mixed thoroughly. Microdilution was performed in triplicates and repeated five times for each individual bacterial species. Before and after an overnight incubation at 37 ℃, microtiter plates were agitated and the optical density of each well at 625 nm wavelength was measured in order to determine the MICs of larval extract for each bacterial species.

3.2.8 Robustness of Larval Extract

After 13-month of storage at -70 ℃, 200 mg larval extract was re-suspended in 1 ml sterile distilled water and assayed for antibacterial activity against the seven selected bacteria using TB Assay with five replicates.

3.2.9 Heat Stability of Larval Extract

Reconstituted larval extract (200 mg/ml) was transferred into sealed microcentrifuge tubes and incubated in a boiling water bath (100 ℃) for five minutes or autoclaved at 121 ℃ for 20 minutes. Then, the microcentrifuge tubes were briefly cooled on ice and centrifuge at 8,000 x g for five minutes to pellet the denatured proteins. The collected supernatant was then assayed for antibacterial activity against the seven selected bacteria using TB Assay and repeated five times.

3.2.10 Freeze-Thaw Stability of Larval Extract

Larval extract was cycled from -70 ℃ to room temperature ten times, allowing for freezing and thawing of the samples. Then, the larval extract was assayed and repeated five times for antibacterial activity using TB Assay.

3.2.11 Statistical Analysis

Data were expressed as arithmetric mean ± standard deviation of 30 samples for TB assay, CFU assay and agar well-diffusion assay in the determination of the antibacterial activity of larval extract; five samples for MIC assay and TB assay in the determination of the physiochemical properties of larval extract. Standard deviation was used to describe the variability between samples. Statistical analyses were performed using SPSS for Windows version 11.0. The significance of differences between sample and control values was assessed using two-tailed unpaired Student's *t*-tests with significance

set at $P \leq 0.05$. On the other hand, differences between the potency values of larval extract against each bacterial species were analyzed using a one-way ANOVA with Tukey's HSD (Honestly Significant Difference) post hoc test for multiple comparisons at $P \leq 0.05$.