2.0 MATERIALS AND METHODS

2.1 Plant material and extract preparation

In the current study, aqueous and ethanolic extracts were derived from *Tinospora crispa* plant. The *T. crispa* plant in dried form was kindly provided by Assoc. Prof. Dr. Mahmood from the Immunology Laboratory of University of Malaya.

2.1.1 Aqueous extracts preparation

The dried form of plant was mixed with sterile distilled water in a ration of 1:20 (100 g in 1 L solvent), and was stirred and heated for about 4 hours. After cooling, the extract was filtered by using Whatman No.1 filter paper. The filtrate was collected and frozen in ice cube container. The frozen ice cube was freeze-dried (i.e. lyophilisation) to obtain concentrated, aqueous extracts in powder form.

2.1.2 Ethanol extracts preparation

The dried plant material was mixed and macerated with absolute ethanol at a 1:20 ratio (100 g in 1 L solvent) for 7 days. Then the extract was filtrated through Whatman No 1 filter paper and then followed by rotor- evaporated the supernatant by using the BUCHI Switzerland Rotary Evaporator to remove the ethanol and to obtain concentrated, oily extract. The crude extracts were then kept at -20 ºC in sterile universal bottles.

2.1.3 Sterility proofing of the extracts

With reference to tests done by Sule & Agbabiaka (2008), we made some modifications to the sterility proofing of the extracts by introducing 2ml of the extract into 10 ml of Muller Hinton broth, and incubated at 37 ^{0}C for 24 hours. The absence of

turbidity or clearness of the broth after the period of incubation signifies the presence of a sterile extract

2.2 Identification of bacterial strains

The eight Methicillin resistant *Staphylococcus aureus* (MRSA) pure isolates used in this study were kindly provided by Assoc. Prof. Dr. Yassim from the Microbiology Laboratory of University Malaya Medical Centre and a reference strain *Staphylococcus aureus* ATCC 25923 were obtained from the Molecular Bacteriology Laboratory, Faculty of Medicine. All samples were cultured and sub-cultured again for purity on Columbia Horse Blood Agar plates (Biomedia Laboratories Sdn. Bhd.). Colony morphology and Gram staining was carried out to confirm the identity of working strains as mentioned in the Textbook of Diagnostic Microbiology (Hindler & Jorgensen, 2007).

2.3 Antimicrobial susceptibility testing

2.3.1 Disc diffusion test

Disc diffusion method for antimicrobial susceptibility testing was carried out based on recommendations given by the Clinical Laboratory Standards Institute, CLSI (Hindler & Jorgensen, 2007; Jorgensen & Turnidge, 2007).

2.3.2 Preparation of impregnated discs

A stock solution of each plant extracts was prepared by dissolving 100 mg of extract with one ml of their respective solvents (sterile distilled water and 99.9% dimethyl sulfoxide. Ten, 30 µl, 50 µl of a final concentration of 100 mg/ml have then used to impregnate in sterilized 6 mm blank discs (Oxide, UK). Distilled water and

dimethyl sulfoxide-loaded discs were used as negative controls for aqueous and ethanolic extracts respectively. All impregnated discs were ensured to be fully dried in 45 ºC incubator for 18 to 24 prior to the application on bacterial lawn (Zaidan et al., 2005). The standard antibiotic disc used as positive controls was vancomycin (30 μg; Becton-Dickinson, USA) for all *S. aureus* strains.

2.3.3 Inoculums and inoculation procedure

The inoculum density was standardized to achieve a final concentration of 1.5 x 10^8 CFU/ml by the growth method. Three to five single colonies from an agar plate culture were suspended in four to five ml of Mueller Hinton broth and incubated at 37 ºC until visibly turbid (0.5 McFarland standard) (Jorgensen & Turnidge, 2007). Furthermore, the inoculum suspension was used within 15 minutes of standardization, which is a very important factor to avoid any change of the size of inoculums or lose their viability (Wanger, 2007). A sterile cotton swab was dipped into the standardized bacterial inoculum suspension, and then it was streaked over the whole dried surface of 90 mm Mueller-Hinton agar (MHA; Becton-Dickinson, USA) plates twice. The agar plate was rotated about 60 degrees each time to ensure that the inoculum was distributed the entire agar surface. In order to expel the excess moisture from the inoculated plates their lids were left ajar for less than 15 minutes.

2.3.4 Application of impregnated discs

The discs which had been impregnated with plant extracts using sterile forceps were applied on the inoculated Mueller Hinton agar once it has completely dried. The disks were pressed gently to ensure uniform contact with agar surface. Furthermore, each one of the test plates was comprised of no more than five discs which placed about equidistance to each other to avoid the overlapping of inhibition zone. Three treated

discs, one positive control, which is a standard commercial antibiotic disc, and the last one negative control. Then, the plates were inverted and incubated for 24 hours at 37°C. The diameter of inhibition zone either around the treated discs or around the control discs were measured for the antibacterial activity assessment. If present, their diameters were measured to the nearest whole millimetre with a ruler. All tests were carried out three times to ensure the reliability, and the average of the three replicates for each extract, and antibiotic were calculated.

2.3.5 Minimum Inhibitory Concentration (MIC)

The highest dilution of the extracts that inhibit the growth (no visible bacterial growth when compared with control tube) but not kill the organism was defined as MIC. For the active plant extracts which showed inhibition zone in some test plate from the disc diffusion method were further tested to determine MIC values by broth macrodilution method based on recommendations given by the Clinical Laboratory Standards Institute, CLSI (Hindler & Jorgensen, 2007; Jorgensen & Turnidge, 2007).

Broth dilution procedure (macrodilution) was carried out for quantitative measurement to investigate *in vitro* the antimicrobial property of plant extract against the test bacterial isolate.

2.3.6 Preparation of extract dilutions

A stock solution of plant aqueous extract was prepared by dissolving 100 mg of extract in 1 ml of sterile distilled water. Likewise, for the ethanol extract, 100 mg of extract was dissolved in 1 ml of 10 % Tween-20 rather than the original solvent (i.e. Dimethyl sulfoxide), and so the initial concentration of the plant extract (100 mg/ml) was diluted using double fold serial dilution by transferring 1ml of the sterile plant extract (stock solution) into 1ml of sterile Mueller Hinton broth to obtain 50 mg/ml

concentration. The above process was repeated several times to obtain other dilutions: 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml 3.125 mg/ml 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml, 0.2 mg/ml, and finally 0.1 mg/ml.

The concentrations were prepared to a volume of 0.5 ml in separate microcentrifuge tubes at double the intended concentrations so that addition of equal volumes of bacterial inocula in next steps would result in the desired final concentrations in each tube.

2.3.7 Inoculation procedure

The bacterial inoculum were prepared with Mueller-Hinton broth (MHB; Becton-Dickinson, USA) which was similar to the disc, then incubated at 37 ºC for 18-24 hours, and the bacterial concentration was adjusted to a 0.5 McFarland standard (1.5 x 10^8 CFU/ml). The suspension was then diluted 1:100 with sterile broth to obtain a cell number of approximately 10^6 CFU/ml. Next, 0.5 ml of the standardized bacterial suspension was then added to the tubes containing the previously prepared 0.5 ml of diluted extracts, resulting in a recommended final cell count of about 5 x 10^5 CFU/ml. A tube containing broth, extract solvent either distilled water or 10% (v/v) Tween-20, and the inoculums was known as positive growth control. On the other hand, a tube containing broth without inoculum, and extract solvent served as the negative control. All the tubes were incubated overnight at 37 ºC.

2.3.8. Determination of MIC values

In order to find out if there was any bacterial growth, the turbidity of the solution in each tube was observed on the next day. To ensure the presence or absence of bacterial growth in the tubes, a standard loop of the suspensions in each tube was inoculated on 3mm MHA and incubated overnight at 37 ºC. The plates were observed

following incubation to confirm absence or growth of bacteria. The lowest concentration of extract dilution showing no visible growth was recorded as the MIC value. The tubes were further incubated another 24 hours and plated again to observe for absence/growth after 48 hours incubation period. Likewise, the MIC value after 48 hours incubation was recorded too.

2.4 Antibacterial effects of several plant solvents

Several common plant solvents for the antibacterial susceptibility test were assessed before a particular solvent was chosen for the MIC assay as well as a medium to study the acute toxicity. The purpose of carrying out this investigation was to ensure that the chosen solvent did not contain antibacterial property that could interfere with the MIC assay. The plant solvents examined were sterile distilled water, DMSO, absolute ethanol, and 10% Tween-20, while the bacterial strains used were MRSA strains and *S. aureus* ATCC 25923.

The steps of this assay were performed by following the same procedure of the MIC assay. Five hundred µl of each solvent was added to five hundred µl standardized bacterial suspension that was adjusted similarly to the MIC assay (final cell count of approximately 5×10^5 CFU/ml). The tubes were incubated and then streaked onto MHA to observe the presence of bacterial growth.

2.5 Acute toxicity study

Adult male and female *Sprague Dawley* rats (8- 10 weeks old) were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur (Ethics Approved number: PM 07/05/2008 MAA (a) (R)). The rats which weighed between 180-200 g were given tap water and standard pellet diet *ad libitum*, for a minimum of five days before the start of the treatment to allow for acclimatization.

Furthermore, when the rats were conveyed to the Experimental Animal Unit, they were kept in separate cages.

2.5.1 Acute oral toxicity study

In order to determine a safe dosage for the plant extract (aqueous and ethanol extract) a study of the acute toxicity was undertaken. Thirty six *Sprague Dawley* rats (18 males and 18 females) were equally assigned into 3 groups for each extract labelled as a vehicle (10% Tween-20, 5 ml/kg); 2 g/kg and 4 g/kg of plant extract preparation, respectively. The amount of the plant extract dosage given to each rat was initially based on the calculated animal's body weight. (Douds, 1997). Prior to testing, all animals were fasted overnight and food was withheld for a further 3 to 4 hours after dosing. The acute oral toxicity study was carried out based on the OECD Guideline for Testing of Chemicals 420 (2001).

2.5.1.1 Mortality and behavioural observation

The animals were observed for 30 min and 2, 4, 8, 24 and 48 h after the administration for mortality or behavioural changes indicative of toxicity. Signs of mortality were continuously observed and twice daily for up to 14 days. The animals were fasted and sacrificed on the following day (15^{th} day), and subjected to necropsy for gross observation, liver, renal function tests, and histological examination.

2.5.1.2 Body weight analysis

The individual weights of each rat were recorded before administering the plant extract as well as on the day of termination. This was done in order to determine if there were any variances in the body weight of each rat as suggested by OECD guidelines.

2.5.1.3 Liver and renal function analysis

Prior to termination, all animals were fasted. On the day of the termination, the animals were anaesthetised with diethyl ether in a chamber. Blood from each rat was drawn from the jugular vein and collected in separate BD Vacutainer® blood collection tubes with clot activator (Becton-Dickinson, USA). All the samples were sent immediately to the Clinical Diagnostic Laboratory of the University Malaya Medical Centre for liver and renal function tests. The results were compared to their respective control groups for the following parameters: total protein, albumin, globulin, alanine aminotransferase (ALT), and aspartate aminotransferase (APT), alkaline phosphatise (AP), creatinine, and urea levels.

2.5.1.4 Gross necropsy

Gross necropsy was performed on all terminated rats after blood collection.

2.5.1.5 Histological examination

For histological examination, the livers and kidneys of each rat were then excised and fixed in the freshly made 10% neutral buffered formalin for 6 hours. After the fixation, all tissue from the organs collected were trimmed appropriately and kept in a cassette for overnight with 10 % buffered formalin. Then the tissue processed in an automated tissue machine to undergo dehydration, clearing and impregnation for 16 hours. The tissues were embedded in paraffin, sectioned by microtome and stained with haemotoxylin and eosin (H and E) stain. The slides were then observed and analyzed under the light microscope with magnification of x10, x40 and x100 (oil immersion). The purpose of the doing histology section is to observe for any sign of histopathological changes in the organs.

2.6 Statistical analysis

Data was expressed in mean ± S.E.M. Comparisons of body weights and liver function parameters were compared against the animals' respective vehicle groups using one-way analysis of variants (ANOVA) and Bonferroni's post hoc test with SPSS Statistics 18.0 software. The treatment group were significantly different when *p* value is less than 0.05.