CHAPTER 2

Experimental

2.1 GENERAL EXPERIMENTAL PROCEDURES

^{1}H and ^{13}C-NMR spectra were recorded on a Bruker DRX 300 NMR spectrometer operating at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR. Liquid chromatography was performed using MCI gel CHP 20P (Supelco), Chromatorex ODS (100-200 mesh, Fuji Silysia Chemical LTD), Sephadex LH-20 (Amersham Biosciences), Toyopearl HW-40F (Tosoh corporation) and Silica gel (230-400 mesh, Merck) column. Semi-preparative HPLC was performed using Waters system composed of a quaternary pump, a manual injector and a photodiode array (PDA) detector fitted with a reversed-phase semi-preparative Symmetry C-18 column (7.8 i.d. x 300 mm, 7 μm). TLC was performed on pre-coated silica gel 60 F_{254} plates (0.2 mm thick, Merck) with $CHCl_3$–MeOH–H_2O (8:2:0.1; 7:3:0.5; 6:4:1 or 5:5:1.5 v/v) or C_6H_6–HCOOEt–HCOOH (1:7:1 v/v) and spots were detected by observing under UV illumination and by spraying with 10 % H_2SO_4 followed by heating or 2 % ethanolic $FeCl_3$ reagent. LC-MS analysis was performed at 30,000 resolutions using a Thermo Scientific LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Breman, Germany) fitted with an electrospray interface and chromatographed with 3 μm, 2.1 mm I.D. x 150 mm Hypersil GOLD RP C_8 or RP C_{18} column (Thermo Scientific, San Jose, USA) on a U-HPLC system comprising of a pump with a build-in degasser, PDA detector scanning from 200-600 nm and an auto-sampler (Thermo Fisher Scientific, San Jose, USA). All data were processed using Qual browser (Thermo Fisher

Scientific, San Jose, USA). IR spectra were recorded with a Spectrum 100 Fourier transform-infrared (FT-IR) spectrometer (Perkin-Elmer, CA, USA), equipped with a mid-infrared deuterated triglycine sulphate (DTGS) detector. The spectra were obtained from the scan range of 4000-450 cm^{-1} with a resolution of 4 cm^{-1} and a total accumulation of 16 scans. Portable and programmable temperature controller (4000 series™ High Stability Temperature Controller, Specac, Ltd) was used in the range of 50-120 °C. The 2D-IR correlation spectra were acquired by employing Softdoc software developed by Tsinghua University (Beijing, China). HPLC chromatograms were generated with Waters system composed of a quaternary pump (Waters 600E), an autosampler (Water 717 plus) and a PDA detector (Waters 2996 PAD) scanning from 190-400 nm using a reversed-phase Symmetry C-18 column (4.6 i.d. x 250 mm, 5 µm). The chromatograms were processed using Empower 2 software. Principal component analysis (PCA) was performed using the The Unsrambler X software (Camo, Norway). A microplate reader (ASYS UVM340) was used for the measurement of absorbance. The statistical analysis of the biological activities was performed using SigmaPlot 11.0 (San Jose, California). All reagents and solvents used were of analytical grade except HPLC and LC-MS analyses which used HPLC grade and LC-MS grade, respectively.

2.2 EXTRACTION, FRACTIONATION AND ISOLATION OF COMPOUNDS FROM *PHYLLAGATHIS ROTUNDIFOLIA*

2.2.1 Chemicals

The analytical grade MeOH and distilled water were used in open column chromatography whereas HPLC grade acetonitrile and purified water were used in HPLC.

2.2.2 Plant Materials

The leaves of *Phyllagathis rotundifolia* were collected from Pasoh Forest Reserve, Negeri Sembilan on 27th Aug 2008. The reference specimens (A624-A633) were identified by a FRIM botanist and deposited at the specimen collection of Genetic Laboratory, Forest Biotechnology Division, Forest Research Institute Malaysia (FRIM), Selangor, Malaysia.

2.2.3 Extraction

The oven-dried (40 °C) leaves of *P. rotundifolia* (330 g) were extracted five times by soaking in MeOH for three days each time at room temperature and yielded 60.8 g of crude extract.

2.2.4 Fractionation

The concentrated crude extract (60.8 g) was suspended in H_2O and partitioned consecutively with hexane and ethyl acetate to give hexane (10.5 g), ethyl acetate (11.1 g) and water (36.0 g) fractions.

2.2.5 Purification

The water and ethyl acetate fractions were each fractionated by open column chromatography employing MCI gel CHP 20P and eluting with MeOH at gradient of 0-100 % in H_2O. A mixture of granular white crystal was obtained from two fractions when the eluting solvent reached 100 % MeOH. The mixture was further purified by a gradient mode HPLC system using acetonitrile in water from 20-30 % at runtime 25 min and a flow rate of 2.88 mL/min to give 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**, ED-1, 8.1 mg) and 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**, ED-2, 6.5 mg). The remaining fractions were also fractionated and purified by a combination of column chromatography utilising MCI gel CHP 20P, Chromatorex ODS, Sephadex LH-20, and Toyopearl HW-40F. The ethyl acetate fraction yielded prunasin 6'-*O*-gallate (**1**, CG-1, 41.4 mg), prunasin 2',6'-di-*O*-gallate (**2**, CG-2, 8.6 mg), prunasin 3',6'-di-*O*-gallate (**3**, CG-3, 21.6 mg), prunasin 4',6'-di-*O*-gallate (**4**, CG-4, 8.5 mg), prunasin 2',3',6'-tri-*O*-gallate (**5**, CG-5, 34.6 mg), prunasin 3',4',6'-tri-*O*-gallate (**6**, CG-6, 24.9 mg), prunasin 2',3',4',6'-tetra-*O*-gallate (**7**, CG-7, 33.8 mg), 1,2,3-tri-*O*-galloyl-β-D-glucose (**10**, GT-3, 7.0 mg), 1,4,6-tri-*O*-galloyl-β-D-glucose (**11**, GT-4, 32.5 mg), 3,4,6-tri-*O*-galloyl-D-glucose (**12**, GT-5, 23.1 mg), 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**13**, GT-6, 27.0 mg), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**14**, GT-7, 21.3 mg), pterocarinin C (**17**, ET-3, 55.5 mg) and gallic acid (**21**, AR-1, 31.6 mg). Compounds 6-*O*-galloyl-D-glucose (**8**, GT-1, 13.9 mg) and 3,6-di-*O*-galloyl-D-glucose (**9**, GT-2, 19.2 mg) were isolated from the water fraction. Additionally, the water and ethyl acetate fractions both yielded 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**15**, ET-1, 331.8 mg) and praecoxin B (**16**, ET-2, 157.3 mg).

2.3 EXTRACTION, FRACTIONATION AND ISOLATION OF COMPOUNDS FROM *PHYLLAGATHIS PRAETERMISSA*

2.3.1 Chemicals

The analytical grade MeOH and distilled water were used in open column chromatography whereas HPLC grade acetonitrile and purified water were used in HPLC.

2.3.2 Plant materials

The leaves of *Phyllagathis praetermissa* were collected from Kuang, Selangor on 6th Nov 2007 and the reference specimen (FRI 54725) was identified and deposited at the specimen collection, Natural Products Division, Forest Research Institute Malaysia (FRIM), Selangor, Malaysia.

2.3.3 Extraction

Dried (40 °C) and ground leaves (600 g) of *P. praetermissa* were extracted by repeated soaking in MeOH for five times to yield 121.6 g of crude extract.

2.3.4 Fractionation

The concentrated extract (121.6 g) was suspended in water and partitioned consecutively with hexane (15.4 g) followed by ethyl acetate (4.6 g). After concentration, the water fraction yielded 98.3 g extract.

2.3.5 Purification

The water and ethyl acetate fractions were each chromatographed using MCI gel CHP 20P, starting with 100 % H_2O and increasing amount of MeOH (0-100 %). The resulting fractions were further purified by a combination of column chromatography utilising MCI gel CHP 20P, Chromatorex ODS, Sephadex LH-20, Toyopearl HW-40F and Silica gel. The ethyl acetate fraction yielded gallic acid (**21**, AR-1, 73.6 mg), its methyl ester (**22**, AR-2, 42.0 mg) and 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**13**, GT-6, 63.0 mg). Casuarinin (**18**, ET-4, 21.9 mg) and 3,6-di-*O*-galloyl-D-glucose (**9**, GT-2, 32.9 mg) were isolated from the water fraction. 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**15**, ET-1, 22.6 mg) and praecoxin B (**16**, ET-2, 40.4 mg) were found in both the water and ethyl acetate fractions. 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19,** ED-1, 6.8 mg) and 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20,** ED-2, 5.2 mg) were obtained by a semi-prep HPLC system of a mixture of granular crystal from the water and ethyl acetate fractions using MCI gel CHP 20P column. A gradient mode of acetonitrile in water from 20-30 % with a flow rate of 2.88 mL/min and run time of 25 min at room temperature was employed.

2.4 CHARACTERISATION OF COMPOUNDS ISOLATED FROM *P. ROTUNDIFOLIA* AND *P. PRAETERMISSA* BY LC-ESI-MS/MS

2.4.1 Chemicals

The MeOH, acetonitrile and H_2O used were of LC-MS grade. The mass calibration was performed using mixture of caffeine, sodium dodecyl sulphate, sodium taurocholate, tetrapeptide MRFA and Ultramark 1621.

2.4.2 Sample Preparation

The isolated compounds (**1-22**) were prepared in a stock solution of 1 mg in 1 mL of MeOH:H_2O (1:1). A serial dilution was done for each compound to give the concentration of 800 ppb. All the solution was filtered by 0.22 µm polytetrafluoroethylene (PTFE) filters prior to LC-ESI-MS^n analysis. Aliquots of 10 µL of each compound were injected to LC-MS for the analysis.

2.4.3 LC-UV Analysis

The analysis was performed using the Accela™ U-HPLC system (Thermo Scientific, San Jose, USA) equipped with a quaternary pump, a built-in degasser, a PDA detector and an auto-sampler. A Hypersil GOLD RP C_{18} column (3 µm, 2.1 mm I.D. x 150 mm) was used. The step-gradient and isocratic solvent composition at a flow rate of 200 µL/min over 35 min was as follow, acetonitrile:H_2O (2:98 to 2:98 over 2 min, 2:98 to 5:95 over 3 min, 5:95 to 10:90 over 3 min, 10:90 to 15:85 over 2 min, 15:85 to 20:80 over 2 min, 20:80 to 25:75 over 2 min, 25:75 to 30:70 over 2 min, 30:70 to 35:65 over 2 min, 35:65 to 40:60 over 2 min, 40:60 to 45:55 over 2 min, 45:55 to 50:50 over 2 min, 50:50 to 2:98 over 3 min then isocratic for 8 min). Sample injection volume was 10 µL. After acquisition of the UV spectra, the eluent was redirected to electrospray interface of a mass spectrometer.

2.4.4 LC-ESI-MS^n Analysis

Negative ion mode was employed in the analysis as this afforded the best detection limits for the compounds. Full scan and MS data were acquired at 30,000 resolutions using Orbitrap FTMS full scan and data dependent MS^n was acquired using FTMS with dynamic exclusion, the wideband activation and the repeat count was set at 2 in 30 s durations. In

this method, the most intense parent ion was isolated and further fragmented twice to generate MS^2 data. Then the most intense daughter ion was also isolated and further fragmented twice to produce MS^3 data. Spectral *m/z* from 100-1000 was recorded and the MS^n fragmentation was carried out with 35-40 % collision energy. The electrospray ionisation conditions were as follows: source accelerating voltage, 3.5 kV; capillary temperature, 285 °C; sheath gas flow, 40 arb; auxiliary gas, 20 arb.

2.5 MULTI-STEPS INFRARED MACRO-FINGERPRINTING OF *P. ROTUNDIFOLIA* AND *P. PRAETERMISSA*

2.5.1 Chemicals

The spectrophotometric grade potassium bromide (KBr) was used in IR analysis.

2.5.2 Sample Collection

A total of 30 individual plants for each species were sampled from four sampling sites, namely Pasoh Forest Reserve, Negeri Sembilan; Labis Forest Reserve, Johor; Ampang Forest Reserve, Selangor; and Bukit Lagong, Selangor. They were consisted of ten individual plants of *P. rotundifolia* each collected from Pasoh Forest Reserve (4278-4287; 27 Aug 2008), Sungai Batang, Labis Forest Reserve (6863-6877; 29 July 2009) and Takar Melor, Labis Forest Reserve (6878-6892; 29 July 2009) and ten individual plants of *P. praetermissa* each collected from Pasoh Forest Reserve (4201-4211; 2 June 2008), Ampang Forest Reserve (4212-4226; 11 June 2008) and Bukit Lagong (4227-4236; 25 June 2008). All the samples were collected at random from each locality. The leaves of each individual were removed and used for macro-fingerprinting in triplicate. The same samples

were used for the HPLC fingerprints.

2.5.3 Sample Preparation

The oven-dried leaves of each plant were cut into small pieces and then ground into powder. Each of the sample (2 mg) was mixed with 100 mg of KBr and the mixture was further ground and pressed into a 13 mm diameter disc. Similarly mixtures of *P. praetermissa* and *P. rotundifolia* were prepared on weight per weight basis from 0-100 % with an interval of 5 % (w/w). Each of the mixtures (2 mg) was further ground with KBr (100 mg) and pressed into discs. All the samples were analyzed in triplicate.

2.5.4 One and Two-dimensional Infrared Spectral Analysis

1D-IR spectra were recorded from a total of 16 scans in the 4000-450 cm^{-1} range with a resolution of 4 cm^{-1}. The second derivative spectra were obtained by means of Savitzky-Golay filter through 13 point smoothing. Savitzky-Golay smoothing aimed for minimum distortion by least squares fitting a cubic polynomial. For the measurement of 2D-IR spectra, each sample disc was put into the sample pool connected with a temperature controller. Dynamic spectra were collected at different temperatures ranging from 50-120 °C at an interval of 10 °C. The analysis for each sample was carried out in triplicate.

2.5.5 PCA Analysis

The 1D-IR spectra were subjected to principal component analysis (PCA) by The Unsrambler X software. The spectra were transformed to second derivative with 13 points smoothing. The data were transferred to excel spreadsheet prior to analysis.

2.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF *P. ROTUNDIFOLIA* AND *P. PRAETERMISSA*

2.6.1 Chemicals

The HPLC grade MeOH and acetonitrile, and purified H_2O were used in the analysis.

2.6.2 Sample Collection

The collection of samples used in this part has been described in the previous section on multi-steps IR macro-fingerprinting (Section 2.5.2).

2.6.3 Sample Preparation

The dried and ground samples (0.5 g) was each extracted by sonication in 3 mL MeOH for 60 min. The filtrates were filtered using 0.40 μm polytetrafluoroethylene (PTFE) filters before subjected to HPLC analysis. Aliquots of 10 μL from each sample were injected for the analysis.

2.6.4 HPLC Analysis

The chromatographic profiles were obtained using a reversed-phase C-18 column at flow rate of 1.0 mL/min at room temperature and the extract was eluted with a gradient system of acetonitrile (A), 0.1 % H_3PO_4 (B) and methanol (C). The elution profile was 0-5 % A in B (0-5 min), 5-10 % A in B (5-10 min), 10-15 % A in B (10-15 min), isocratic 15 % A in B (15-30 min), 15-18 % A in B (30-40 min), 18-20 % A in B (40-45 min), isocratic 20 % A in B (45-55 min), 20-25 % A in B (55-60 min), 25-100 % A in B (60-85 min), and 100 % B-100 % C (85-90 min).

2.6.5 PCA analysis

All the HPLC chromatograms were subjected to principal component analysis (PCA) using The Unsrambler X software. The data were transferred to excel spreadsheet prior to analysis by The Unsrambler X software. All the data were normalized followed by baseline correction before subjected to PCA.

2.7 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) OF *P. ROTUNDIFOLIA* AND *P. PRAETERMISSA*

2.7.1 Chemicals

The solvents used were LC-MS grade MeOH, acetonitrile and H_2O. The mixture of caffeine, sodium dodecyl sulphate, sodium taurocholate, tetrapeptide MRFA and Ultramark 1621 were used for the mass calibration in the LC-MS analysis.

2.7.2 Sample Collection

The samples used in this part have been described in the previous section on multi-steps IR macro-fingerprinting (Section 2.5.2). The representative samples of *P. rotundifolia and P. praetermissa* collected from each locality were analyzed in triplicate.

2.7.3 Sample Preparation

Exactly 1.0 g each of the dried and ground leaves was extracted in 4 mL MeOH with sonicated for 60 min. The filtrates were filtered using 0.22 µm polytetrafluoroethylene (PTFE) filters before subjected to LC-MS analysis. An aliquot of 1 µL was injected for LC-MS analysis.

2.7.4 LC-MS^n analysis

The chromatographic analysis was performed using the Accela™ U-HPLC system (Thermo Scientific, San Jose, USA) equipped with a quaternary pump, a built-in degasser, a PDA detector and an auto-sampler. A Hypersil GOLD RP C_8 column (3 µm, 2.1 mm I.D. x 150 mm) was used. The mobile phase consisted of acetonitrile (A) and water with 0.1 % formic acid (B) at a flow rate of 200 µL/min, with a combination of step-gradient and isocratic elution; A:B as follows: 0 min, 0:100; 12 min, 1:99; 13 min, 15.4:84.6; 15 min, 15.4:84.6; 100 min, 16:84; 150 min, 18:82; 205 min, 20:80; 255 min, 28:72; 265 min, 30:70 and maintained for 5 min. The eluent from the LC column was directed into the ESI probe. The MS^n analysis was performed on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ionisation probe according to the method described in Section 2.4.

2.8 NEUROPROTECTIVE ACTIVITY

2.8.1 Chemicals

The chemicals used for the analysis were dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, hypoxanthine aminopterin thymidine (HAT) medium, phosphate buffered saline (PBS), accutase enzyme, hydrogen peroxide (H_2O_2), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analytical grade DMSO.

2.8.2 Culture of NG108-15 Hybridoma Cells

The cell lines were cultured using dulbecco's modified eagle medium (DMEM) with 10 % heat-inactivated fetal bovine serum (FBS), 50 IU/mL penicillin, 50 µg/mL streptomycin, 0.25 µg/mL amphotericin B and hypoxanthine aminopterin thymidine (HAT) medium consisting of 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine as complete growth medium. The cells were cultured in 5 % CO_2 atmosphere with 95 % humidity at 37 °C (CO_2 incubator chamber, RSBiotech).

2.8.3 Preincubation Treatment

Confluent NG108-15 cells were rinsed with phosphate buffered saline (PBS) and then detached by accutase enzyme. A density of 5 x 10^3 cells/well was prepared and plated into 96-well plate. Cells were incubated in 5 % CO_2 at 37 °C for 48 hours. After that, the cells were pretreated with 20 µL of the compounds at concentrations of 6.25-100 µM for 2 hours followed by 20 µL of 2 mM hydrogen peroxide (H_2O_2) with 10 hours incubation.

2.8.4 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Mitochondrial dehydrogenases converted the MTT to insoluble purple formazan crystals whereas the formation of purple formazan crystals was proportional to cell viability. 20 µL of 5 mg/mL MTT solution was added to each well and incubated for 4 hours. The remaining solution was discarded and then 150 µL of DMSO was added to each well. Each plate was shaken to dissolve the formazan crystals. Once the crystals have dissolved completely, the absorbance values were measured at 570 nm (650 nm as reference wavelength) using a microplate reader (ASYS UVM340). The percentage of cell viability was calculated according to the equation below.

$$\text{Percentage of cell viability} = (A_0/A_C) \times 100\ \%$$

Where, A_0 is the absorbance of treated cells

Ac is the absorbance of control cells

2.8.5 Statistical Analysis

Results were given as mean ± SE value. Three independent experiments were conducted for each sample in triplicate. One way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons were carried out and the statistical significant limit was set at *($p < 0.01$).

2.9 *IN VITRO* CYTOTOXICITY AGAINST CANCER CELL LINES

2.9.1 Chemicals

The chemicals used in the analysis were RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, phosphate buffered saline (PBS), accutase enzyme, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analytical grade DMSO.

2.9.2 Cell Culture

The cervical epidermoid carcinoma cells (CaSki), colon carcinoma cells (HCT 116) and breast carcinoma cells (MCF 7) were purchased from the American type culture collection (ATCC) and were cultured using RPMI-1640 medium with autoclaved 10 % heat-inactivated fetal bovine serum (FBS), 50 IU/mL penicillin, 50 µg/mL streptomycin and 0.25 µg/mL amphotericin B. The cells were cultured in 5 % CO_2 atmosphere with 95 % humidity at 37 °C (CO_2 incubator chamber, RSBiotech).

2.9.3 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

The same protocol was used in the cytotoxicity assay of the three cell lines (CaSki, HCT 116 and MCF 7). First, the cells were rinsed with phosphate buffered saline (PBS) before detached with accutase enzyme. A density of 5 x 10^3 cells/well was prepared and 180 µL was pipetted into each well of 96-well plate. The plated cells were then incubated for 24 hours in 5 % CO_2 at 37 °C. After incubation, the cells were pretreated with 20 µL of the samples at various concentrations (0.01-100 µM) and further incubated for 72 hours. Then, 20 µL of 5 mg/mL MTT solution was added to each well and incubated for another 4

hours. The MTT solution was discarded and then 150 µL of DMSO was added to each well. After the plates were shaken, the absorbance values were measured at 570 nm with the reference of 650 nm using a microplate reader (ASYS UVM340). The percentage of cell viability was calculated according to the equation below.

$$\text{Percentage of cell viability} = (A_0/A_C) \times 100\ \%$$

Where, A_0 is the absorbance of treated cells

A_c is the absorbance of control cells

2.9.4 Statistical Analysis

Results were given as mean ± SE value. Three independent experiments were conducted for each sample in triplicate. One way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons were carried out and the statistical significant limit was set at *($p < 0.05$).

2.10 INHIBITION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

2.10.1 Chemicals

The chemicals used in the analysis were Mueller-hinton broth (MHB), McFarland standard, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analytical grade DMSO.

2.10.2 Bacterial Isolates

Three MRSA clinical isolates from Hospital Universiti Kebangsaan Malaysia (HUKM) that included a reference MRSA strain (ATCC 33591) and two clinical MRSA isolates (N441 and U949) and one MSSA reference strain (ATCC 25923) were used in the experiment. The isolates were maintained on Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, Lancashire, England) at -20 ^{o}C. Prior to the assay, isolates were sub-cultured overnight at 37 ^{o}C in Mueller-hinton broth (MHB) and adjusted to obtain turbidity comparable to that of 0.5 McFarland standards.

3.10.3 Minimum Inhibitory Concentration (MIC) Value Determination Assay

The experiment was carried out in triplicate to evaluate the potential of compounds as inhibitory agent against test isolates using double-broth micro-dilution method involving 96 wells microtitre plate as described by Mohtar *et al.* in 2009. Briefly, serial two fold dilutions of the test compounds dissolved in DMSO were prepared in concentrations of 1000-15.625 µg/mL prior addition of 100 µl overnight microbial suspension (10^8 cfu/mL) followed by incubation at 37 ^{o}C for 24 hours. The highest concentration of DMSO remaining after dilution (5 %, v/v) caused no inhibition of bacterial growth. The minimum inhibitory concentration (MIC) value was defined as the lowest concentration producing no visible growth (absence of turbidity and or precipitation) as observed through naked eye. For further confirmation, 20 µl (1 mg/mL) of MTT reagent was added to the bacterial suspension in the selected wells, followed by 20 min incubation at 37 ^{o}C. The colour of reagent-bacterial suspension will remain clear or yellowish for cidal activity as opposed to dark blue indicating growth.