CHAPTER III

MATERIAL AND METHODS

3.1 Chemical Reagents and Plant Materials

Methanol, chloroform and hexane were GC-grade solvents and purchased from Merck (Darmstadt, Germany). Potassium hydroxide was supplied by Sigma (Aldrich, Germany).

The *Clinacanthus nutans* plant was supplied by a nursery farm in Seremban, Negeri Sembilan and immediately processed.

3.2 Instruments

The GS-MS analysis was performed using a Perkin-Elmer GC claurus 500 system coupled with a mass spectrometer. It equipped with Elite-1 fused silica capillary column (30 m × 0.25 mm ID × 1 μm df, composed of 100% Dimethyl poly siloxane).

3.3 Plant Extraction

Methanol, hexane and chloroform will be used in this investigation as extraction solvents using *Clinacanthus nutans* as a raw material. The usage of variety of solvents allows selecting the one with greatest ability to extract anticancer substances in *Clinacanthus nutans.*

The fresh leaves were rinsed with water, then dried and cut into small pieces. The extraction was implemented by weighing 3 g of the cut leaves triplicate and each was soaked into 20 mL of solvent for 1 day at room temperature. The three mixture design that use for extraction is shown in the Figure 3.1. The solvents used were prepared as in Table 3.1. The solution was then filtered using Whatman filter paper No 1 before rotary

evaporator at 40 °C. The crude extracts obtained were then stored at 4 °C prior to GCMS analysis.

Table 3.1: Composition of the solvents used for the extraction.

Chloroform % (v/v)	Hexane % (v/v)	Methanol % (v/v)	Total volume (ml)
100 (20.0 ml)	0 (0 ml)	0 (0 ml)	20.0
0 (0 ml)	100 (20.0 ml)	0 (0 ml)	20.0
0 (0 ml)	0 (0 ml)	100 (20.0 ml)	20.0
50 (10.0 ml)	50 (10.0 ml)	0 (0 ml)	20.0
50 (10.0 ml)	0 (0 ml)	50 (10.0 ml)	20.0
0 (0 ml)	50 (10.0 ml)	50 (10.0 ml)	20.0
33.33 (6.7 ml)	33.33 (6.7 ml)	33.33 (6.7 ml)	20.1

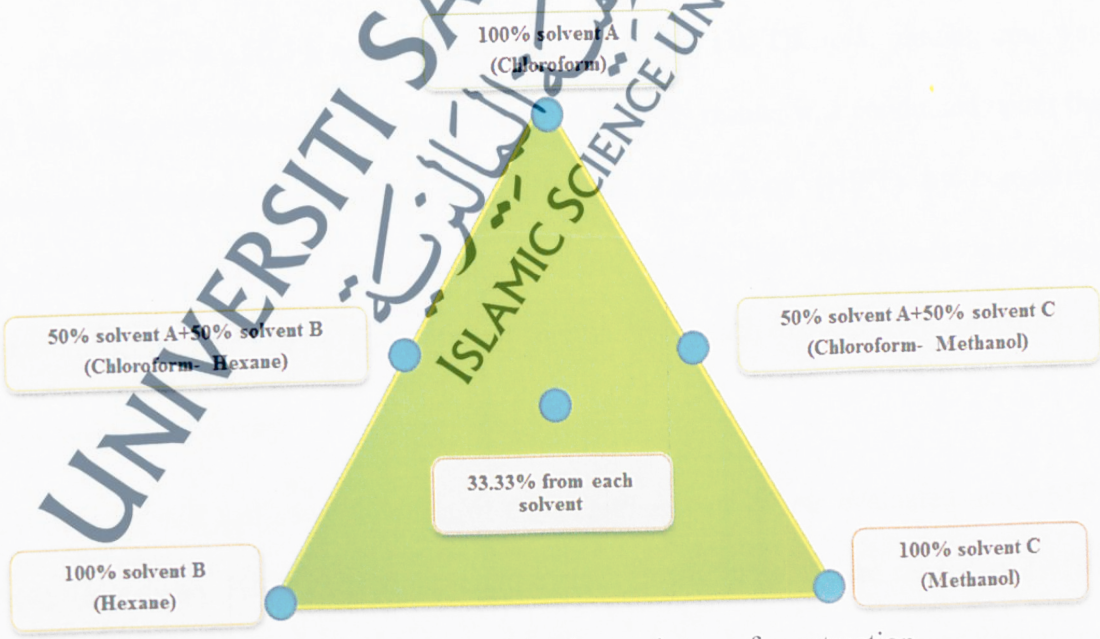

Figure 3.1: The design of the three mixtures for extraction.

3.4 GC-MS Analysis

The Gas chromatography–mass spectrometry (GS-MS) analysis was performed according to the method described by (Hema, Kumaravel, Gomathi, & Sivasubramaniam, 2010).The crude extracts of *Clinacanthus nutans* (20 mg) was dissolved in 2 mL of hexane and 20 µL of 2 N potassium hydroxide in methanol. Then, the mixture was vortexed for 1 min, centrifuged at 10,000 rpm for 10 min and the supernatant (1 mL) was transferred to amber vial to be analyzed using Gas chromatography–mass spectrometry. The compounds detection was performed using an electron ionization system functioning at ionization energy of 70 eV. The pure helium gas was the carrier gas at a constant flow rate of 1 mL/min. The injection volume was 0.5 µL, with a split ratio of 10:1 at injector temperature of 250 °C, where for the ion source was 280 °C. The oven temperature was set at 110 °C for 2 min and then increased to 200 °C with a ratio of 10 °C/min. Then, it was increased to 280 °C for 9 min with a ratio of 5 °C/min. Mass spectra were taken at 70 eV; a scan interval of 0.5 S and fragments from 45 to 450 Da. The total running time was 60 min. The identification and interpretation of GC-MS results was conducted using the database of National Institute of Standards and Technology (NIST) by comparing fragmentation pattern of common metabolites, taking the compounds with high probability more than 70 %. The names of compounds in each extracts were compared.

3.5 Cytotoxicity Assay

Cytotoxicity study of seven extracts of *Clinacanthus nutans* was evaluated using MTT assay (Mosmann, 1983) with slight modification. Colon 38 cells were seeded at 2×10^3 cells/well of a 96-well tissue culture plate. The plates were incubated for sufficient time

to attain attachment at about 40 % confluence. The media was aspirated off and replaced with fresh media (200 µL) containing different extract of different concentrations (0.1 to 100 µg/mL) while the last row was a blank control and the plates were incubated at 37 °C and 5 % CO_2 for 72 hours. Following incubation with the extracts, the media was aspirated off and the cells washed three times using PBS buffer to ensure that no contamination, and then replaced with a fresh media. MTT solution (20 µL) in a total volume of 200 µL was added to every well and mixed gently with the media, which was later incubated for 4 hours at 37 °C with 5 % CO_2. Thereafter, the MTT-containing medium was then removed carefully and replaced with DMSO (200 µL per well) to dissolve the formazin crystals. The plates were then be incubated at room temperature in the dark for about 30 minutes and then read in a microtiter plate reader at 570 nm. The concentration of extract needed to inhibit cell growth by 50 % (IC_{50}) was estimated from the dose-response curves for each extract and each cell line.