# CHAPTER III

# MATERIALS AND METHODS

## 3.1 EXTRACTION

### 3.1.1 Preparation of fruitbodies

*Hericium erinaceus* fresh fruitbodies were obtained from Ganofarm Sdn. Bhd. in Tanjung Sepat, Selangor. The fruitbodies were freeze-dried at −50± 2 °C for 48 hours. The dried fruit bodies were blended in the commercial Waring blender and stored in airtight containers prior to assay.

### 3.1.2 Preparation of aqueous ethanol crude extract

The powdered freeze-dried fruit bodies of *H. erinaceus* was soaked in 80 % (v/v) aqueous ethanol for 3 days. The residue was then resoaked in 80 % (v/v) aqueous ethanol and the extraction and filtration process was repeated three times. The solvent containing extract was then concentrated under vacuum using a rotary evaporator.

### 3.1.3 Solvent- solvent extraction (fractionation)

The crude extract obtained was successively fractionated with hexane, ethyl acetate and water using a separating funnel. All the fractions (hexane, ethyl acetate and water) were filtered and concentrated under vacuum using a rotary evaporator to give hexane, ethyl acetate and water fractions

Figure 3.1 shows the flow chart of the extraction and fractionation procedures, process of biological investigations and isolation of active fractions of *H. erinaceus*.

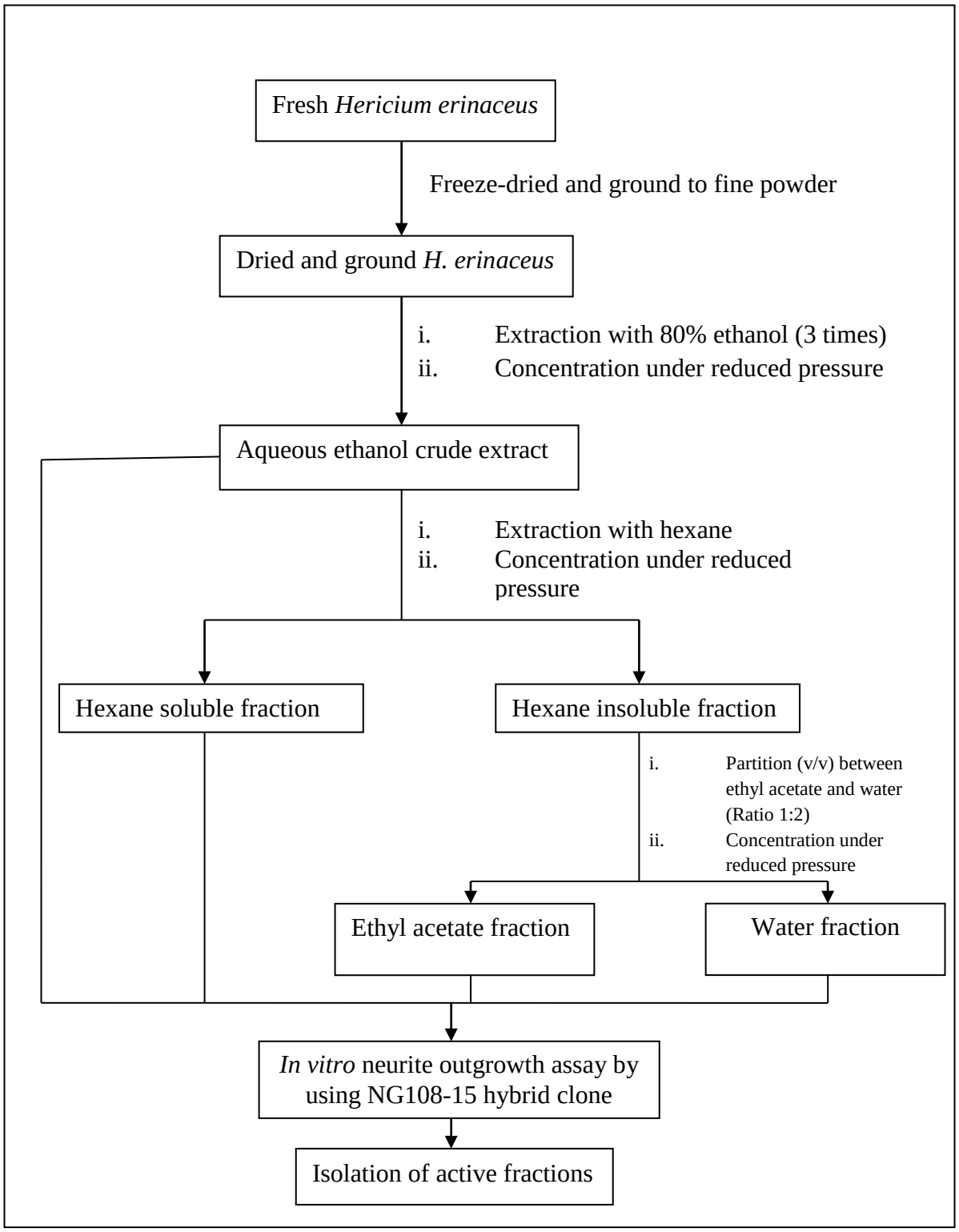


Figure 3.1: A schematic diagram showing the extraction and fractionation procedures, process of biological investigations and isolation of active fractions of *Hericium erinaceus*

## 3.2 NEURITE OUTGROWTH ACTIVITY ASSAY

### 3.2.1 Preparation of stock solutions

Each extract and fractions were dissolved in dimethylsulfoxide (DMSO) to form stock solutions 20 mg/ml for neurite outgrowth assay and kept at -20 ºC for future use. The concentration of samples was prepared according to the requirements for the assay by serial dilutions using the media or media with Tween 80.

### 3.2.2 Cell culture

The neural hybrid clone NG108-15 was chosen for this purpose. NG108-15 cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM).

### 3.2.3 Preparation of medium and buffer for cell culture

#### 3.2.3.1 Dulbecco's Modified Eagle's Medium (DMEM)

Basic medium

Final volume cell culture grade water (80 - 90 %) was measured. Water temperature should be 15-30 °C. Dry powder medium (13.38 g/l) was added slowly and allowing mixing time between additions (original package was rinsed with small amount of water to remove all traces of powder and added to solution). The solution was mixed for 30 minutes. 3.7 g/l of sodium bicarbonate ($NaHCO_3$) and hypoxanthine-aminopterine- thymidine (HAT) were added and stirred until dissolved. While mixing, the pH of the medium was adjusted to 6.9-7.1 using 1N NaOH or 1N HCl. Additional water was added to bring the solution to final volume and continue mixing for at least 30 minutes. The medium was sterilized by filtration using a membrane with a pore size of 0.22 microns and aseptically dispensed into sterile container.

Complete growth medium

Basic medium described above supplemented with 10 % (v/v) of fetal bovine serum, 100units/ml penicillin and 100 µg/ml streptomycin.

Revival medium

Complete growth medium described above supplemented with 20 % (v/v) instead of 10 % (v/v) fetal bovine serum.

Cryoprotectant medium

Basic growth medium described above supplemented with 10 % (v/v) dimethyl sulfoxide (DMSO) and 50 % (v/v) fetal bovine serum.

### 3.2.3.2 Phosphate Buffer Saline

1.52 g of sodium hydrogen phosphate ($Na_2HPO_4$), 0.58 g of potassium hydrogen phosphate ($KH_2PO_4$) and 8.5 g of sodium chloride (NaCl) were dissolved in 1L distilled water and pH was adjusted to 7.2. The solution was filtered with filter paper and autoclaved for 15 minutes at 121 °C, 15 psi. The solution was stored at room temperature.

### 3.2.4 Cell culture techniques

### 3.2.4.1 Revival of frozen cells

The vial containing frozen cells was thawed by gentle agitation in a 37 °C water bath. To reduce the loss of viability, the vial was thawed rapidly. Once the contents were thawed, the contents were transferred aseptically into a centrifuge tube by pipetting. 1 ml of revival medium was added and centrifuged at 1000 rpm for 5 minutes.

Supernatant was discarded and pellet was resuspended in 1 ml of revival medium. The suspended cells were pipetted into a cell culture flask containing 5- 10 ml revival medium. The culture was incubated at 37°C in a 5% $CO_2$ incubator at atmospheric pressure.

### 3.2.4.2 Subculture of cells

The culture was examined with an inverted microscope to check for any evidence of microbial contamination and to determine whether the majority of the cells were attached to the bottom of the flask. When the color of the medium changed from reddish to yellowish (the medium become acidic) or every 2- 3 days, the culture medium was removed and discarded. Trypsin-EDTA (1 ml) and phosphate buffer saline solution (3 ml) were added to the flask and cells were observed under inverted microscope until cell layer was detached from the bottom of the flask. The contents of the flask were transferred aseptically into a centrifuge tube containing 2 ml of complete growth medium by pipetting. The cells were centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in 3- 4 ml of complete growth medium. Appropriate amounts of suspended cell were added to a new culture flask containing complete growth medium. Cultures were incubated at 37 °C in a 5 % $CO_2$ incubator at air atmosphere.

### 3.2.4.3 Medium renewal

The medium was changed 3 to 4 times weekly.

### 3.2.4.4 Cryopreservation of cells

Cryopreservation of cells was carried out in accordance with subculturing procedure except that the pellet obtained after centrifugation was suspended in cryoprotectant medium instead of complete growth medium. The suspended cells were then transferred to a cryogenic vial and stored in liquid nitrogen vapor phase.

### 3.2.5 Effect of *Hericium erinaceus* on stimulation of neurite outgrowth activity of NG108-15

The neural hybrid cell NG 108-15 was cultured until 60-70 % confluent prior to assay. Cells were detached from the flask with 0.25 % (w/v) solution of trypsin in phosphate buffer saline solution and washed in culture medium. The cell pellet was obtained by centrifugation at 1000 rpm for 5 minutes. The density of the cells was counted by 0.4 % (w/v) of tryphan blue exclusion method in a haemocytometer. The cells were plated into 6 well plates coated with 2 x $10^{-5}$ % (w/v) of poly-D-lysine at a cell density of 10000 cells per well in medium containing 4 various concentrations (10, 25, 50, 100 µg/ml) of extract and fractions and subfractions of fruit bodies. Negative control is the well with the untreated cells whilst positive control is the well with the cells treated with Nerve Growth Factor (NGF). Plates were incubated at 37 °C in a 5 % $CO_2$ humidified incubator. Cells were observed for neurite outgrowth, branching of neurites after 24 hours.

### 3.2.6 Scoring of neurites

A cell was considered as positive for bearing neurites if it had at least one thin extension longer than one full diameter of its cell body. Specifically excluded were extensions associated with clearly different patterns of cell responses, such as broad,

sheet-like spreading of cells or the rare radially oriented processes apparently arising by "shrinkage" (Smalheiser and Schwartz, 1987). Cell clumps containing more than five cells were also not included in the results. If more relaxed criteria had been used, many short extensions would have been counted as neurites, and the assay would not have been useful to detect stimulatory effects upon neurite formation (Smalheiser and Schwartz, 1987). Duplicates were set up for each concentrations tested. Approximately 300 cells in each well were evaluated. Neurite formation was quantified by scoring the number of cells processing neurites and expressed as a percentage of the total number of cells counted.

$$\text{Neurite bearing cells (\%)} = \frac{\text{number of cells processing neurites}}{\text{total number of cells counted}} \times 100\ \%$$

The result was also expressed in percentage increase in neurite bearing cells in comparison to negative control.

$$\text{Percentage increase compared to negative control} = \frac{\text{neurite bearing cells of extract} - \text{neurite bearing cells of negative control}}{\text{neurite bearing cells of negative control}} \times 100\ \%$$

### 3.2.7 Statistical analysis

The means of data were subjected to a one way analysis of variance (ANOVA) and the significance of the difference between means was determined by the Duncan's multiple range test at 95 % least significance difference ($P<0.05$).

## 3.3 ISOLATION OF BIOACTIVE CONSTITUENTS

### 3.3.1 Column chromatography

The combined fraction of hexane and ethyl acetate was subjected to silica gel flash column chromatography. Column chromatography was performed by using Merck silica gel. The gel was packed onto the column. After the sample was introduced to the column, solvent with increasing polarity gradient was used to elute the column [developing solvent: chloroform (100 % chloroform) → chloroform-acetone mixtures (20 % Ac/$CHCl_3$, 40 % Ac/$CHCl_3$) → chloroform-methanol mixtures (10 % MeOH/$CHCl_3$, 30 % MeOH/$CHCl_3$) → methanol (100 % MeOH)]. Fractions were monitored by thin layer chromatography (TLC) and fractions that possess the same spots/bands on the TLC were combined and where necessary subjected to further separation. Nine fractions were obtained (E1-E9) from the flash column chromatography which monitored by TLC.

Figure 3.2 shows the flow chart of the isolation of active fractions of *H. erinaceus*.by using different types of chromatographic technique, process of biological investigations and identification of the active fractions.

### 3.3.2 Analytical thin layer chromatography

TLC was routinely used to detect and separate the various compounds. The fractions from column chromatography were examined by TLC using precoated glass plates, 0.25 mm thickness, silica gel F254 (Merck, Darmstadt, G.F.R). The TLC plates were spotted with a piece of fine glass capillary tube and then developed in saturated chromatography tanks with various solvent systems at room temperature. The spots were visualized by examination of the TLC plates under UV light, followed by applying iodine vapor.

### 3.3.3 Preparative thin layer chromatography

Subfraction E2 was successively subjected to preparative thin layer chromatography. The sample was dissolved by using chloroform. The TLC precoated (silica gel F254) glass plates (Merck, Darmstadt, G.F.R) with 0.25 mm thickness, 10 cm (width) x 10 cm (height) were used. A line was drawed (about 1 cm) from the bottom of the plate. ). The line on the TLC plates was spotted with a piece of fine glass capillary tube and then developed in saturated chromatography tanks (100 % $CHCl_3$) at room temperature. After the solvent reach the solvent front, the plates were taken out from the chromatography tanks. When the plates were dried enough, the bands were visualized by using UV light. The bands were marked lightly by using pencil. The bands were scrapped off onto a clean, white paper by using the edge of a spatula. The compounds were washed off from the silica gel by using chloroform. The solvent containing samples were filtered and concentrated under vacuum using a rotary evaporator.

### 3.3.4 High performance liquid chromatography (HPLC)

#### 3.3.4.1 HPLC samples and mobile phase preparation

The samples were dissolved in methanol/acetonitrile mixture. The samples were filtered through 0.45 μm Sartorius minisart PTFE-membrane syringe filter to remove any particular matter that might clog the column. The mobile phase that used was acetonitrile. The mobile phase was filtered by using 0.45 μm Sartorius PTFE-membrane and degassed before introduced to the system. All the solvents that used were in HPLC grade.

### 3.3.4.2 Analytical HPLC

Analytical HPLC analysis were carried out by using the instrument Waters Delta Prep consists of water Prep LC controller, quaternary pump, vacuum degasser, UV detector (water 2487, Dual λ Absorbance Detector). The separation profiles of the samples can be improved by changing the solvent system of the mobile phase, flow rate and column. The column that used for analytical HPLC was performance RP-18 encapped column 100-4.6 mm purchased from Merck. The analysis was carried out in isocratic mode at a flow rate of 1 ml/min, with column effluent being monitored at the wavelength of 214 nm and 254 nm.

### 3.3.4.3 Semipreparative HPLC

The samples were then further isolated out by using Chromolith Semiprep RP-18 column encapped 100-10 mm purchased from Merck. The separation was carried out in isocratic mode by using 100 % acetonitrile as mobile phase at a flow rate of 3 ml/min, with column effluent being monitored at the wavelength of 214 nm and 254 nm. The separated subfraction was collected manually.

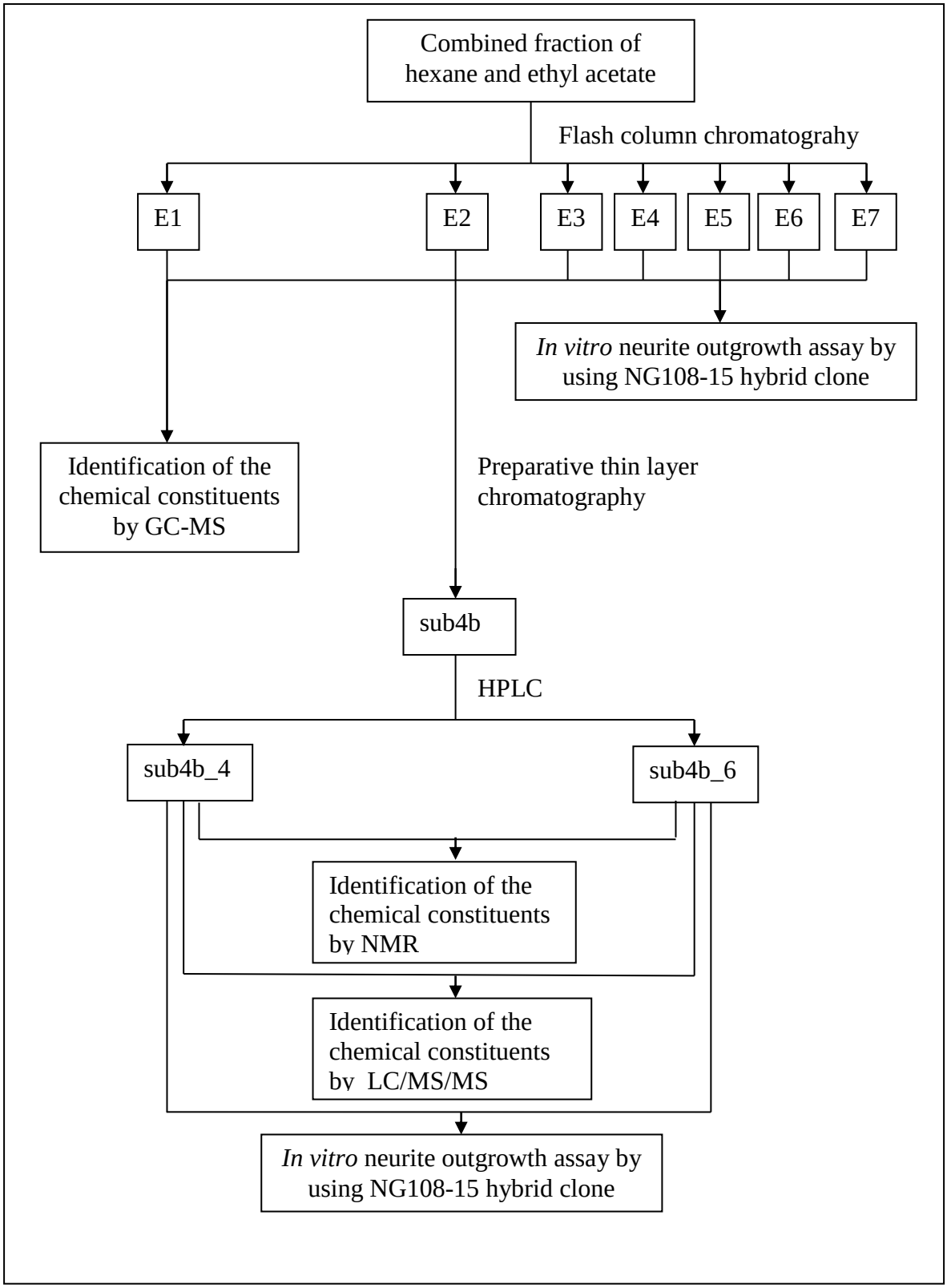


Figure 3.2: A schematic diagram showing the isolation of active fractions, process of biological investigations and identification of the active fractions.

## 3.4 IDENTIFICATION

### 3.4.1 Gas chromatography-mass spectrometry (GCMS)

GCMS analysis was performed on fraction E1 using Network Gas Chromatography System (Agilent Technologies 6890) and Inert Mass Selective Detector (Agilent Technologies 5975) (70eV direct inlet) on a HP-5MS (5 % phenyl methyl siloxane) capillary column (30 m x 250 μm x 0.25 μm) initially set at 150 ºC, then programmed to 300 ºC at 5 ºC $min^{-1}$ and held for 10 minutes at 300 ºC using helium as the carrier gas. The total ion chromatogram obtained was autointegrated by chemstation and the constituents were identified by comparison with the accompanying mass-spectra database (NIST 05 Mass Prectral Library, USA) wherever possible.

### 3.4.2 Nuclear magnetic resonance spectroscopy (NMR)

All the NMR experiments were performed on a JEOL 400MHz NMR spectrometer that install with the JEOL Delta software. Subfractions sub4b_4 and sub4b_6 were dissolved in deuterated chloroform and $^{1}H$, $^{13}C$ and $^{1}H$-$^{1}H$-COSY spectra were collected. The internal standard for $^{1}H$ NMR was TMS (δ: 0.00) and $^{13}C$ was $CDCl_3$ (δ: 77.00).

### 3.4.3 Liquid chromatography-mass spectrometry (LC/MS/MS)

LC/MS/MS analysis were carried out on subfractions sub4b_4 and sub4b_6 by using the instrument Applied Biosystems 3200Q Trap LCMS/MS with Shimadzu ultra pure liquid chromatography (UPLC) system. Full scan with MS/MS data collection was used. Positive ionization mode was set. The column that was used is Phenomenex Aqua C-18 with dimension 50.0 mm x 2.0 mm x 5.0 μM. Rapid screening was performed with 10 min run time.