CHAPTER 3

EXPERIMENTAL

3.1 Source and Authentication of the Plant Materials

The aerial parts of *A. indica* were collected at Nakhonsawan, Thailand (January, 2009). Leaves, stems and fruits of *C. javanicum* and *B. retusa* were collected from Huay Kaew Arboretum, Huay Kaew Road, Chiang Mai, Thailand (December, 2009). The plants were identified by J. F. Maxwell, Chiang Mai University, Thailand. Voucher specimens of *A. indica* (No. 1), *C. javanicum* (No. 2) and *B. retusa* (No. 3) were deposited in the herbarium of Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand**.**

3.2 General Techniques

3.2.1 Chemicals

Commercial grade organic solvents were redistilled prior to use for extraction, as eluents for thin layer chromatography and column chromatography. Organic solvents used for antioxidant activity assessment were analytical reagent grade including ABTS (2,2'-azino-bis(3-ethylbenzothaizoline-6-sulfonic acid)), DPPH (1,1-diphenyl-2-picrylhydrazyl), gallic acid and ascorbic acid were purchased from Sigma (St. Louis, USA). Trolox and quercetin were obtained from Aldrich (Milwaukee, USA). Potassium persulfate were obtained from UNILAB (AU). Absolute ethanol (99%) was purchased from Merck (Damstadt, Germany).

3.2.2 Chromatography

3.2.2.1 Thin Layer Chromatography

Thin layer chromatography (TLC) was used to identify and evaluate the crude extracts and the fractions of medicinal plants. The adsorbents used were silica

gel 60GF precoated aluminium plate (0.25 mm) and silica gel RP18 F-254 precoated aluminium plates (E. Merck). The TLC chromatograms were viewed under an UV viewer (wavelength 254 and 365 nm) and by exposing to anisaldehyde reagent.

3.2.2.2 Column Chromatography

Column chromatography was used to isolate and purify the fractions of crude medicinal plant extracts.

Adsorbent: Silica gel 60 with particles sizes 0.063-0.200 mm (E. Merck) was used throughout the experiments.

Packing method: Slurry packing.

Sample loading: The sample was dissolved in a small amount of suitable organic solvent, mixed with a small quantity of celite with, air dried and added gently onto the top of column.

Elution: After loading of the sample, the column was eluted with suitable solvent system using gradient elution technique.

Examination of eluates: Fractions were examined by TLC under ultraviolet light at wavelengths of 254 and 365 nm and by exposing to anisaldehyde reagent.

3.2.2.3 Spectroscopy

3.2.2.3.1 Mass spectra (MS)

The high resolution mass spectra were performed on a Q-TOF 2^{TM} mass spectrometer with a Z-spray^{TM} ES source (Micromass, Manchester, UK). Electron impact mass spectra were measured with Agilent-HP 5973 Mass Spectrometer (Department of Chemistry, Faculty of Science, Chiang Mai University).

3.2.2.3.2 Proton and Carbon-13 nuclear magnetic resonance spectra (^{1}H-NMR and ^{13}C-NMR)

^{1}H and ^{13}C NMR spectra of the isolated compounds were measured with a Bruker AVANCE 400 NMR spectrometer in deuterated chloroform ($CDCl_3$) and

deuterated methanol (CD_3OD) , operating at 400 and 100 MHz, respectively. The chemical shifts were recorded in ppm by reference to TMS signal.

3.3 Analysis of essential oil

The leaves of the plant were cleaned, cut into small pieces and accurately weighed, then subjected to hydro-distillation in a Clevenger-type apparatus for 8 h. The oily layer obtained was separated and dried over anhydrous magnesium sulfate. The essential oil was kept in the dark. Then the essential oil was analysed using GC and GC-MS.

The essential oil was analysed on a Hewlett-Packard GC-MSD 6850 series 2 mass spectrometer fitted with a HP-5 (Hewlett-Packard 19091S-433E) cross-linked fused silica capillary column (30 m, 0.25 mm i.d.), coated with 5% phenyl methyl siloxane (0.25 µm film thickness). The analytical conditions were: the oven temperature was programmed from 75°C for 0.50 min, isothermal, then heating to 270°C and isothermally for 35 min at 260°C. Injector temperature was 270°C. Samples were injected automatically by splitting and the split ratio was 1:100. The mass spectrometer had a delay of 2.40 min to avoid the solvent peak and then scanned from m/z 35 to m/z 550. Ionization energy was set at 70 eV. The carrier gas was He at a flow rate of 1.0 mL/min. The identification of volatile components were accomplished by comparison of their GC retention indices (RI) as well as their mass spectra (NIST, NISTREP) with corresponding data of authentic compounds or published spectra.

3.4 Antioxidant activity

The antioxidant activities of the crude medicinal plant extracts and the essential oils were determined by ABTS and DPPH methods, total phenolic and flavonoid contents.

3.4.1 ABTS Method

The antioxidant activity of the crude extracts and essential oils were investigated using the ABTS radical cation scavenging assay which was conducted according to the method of Roberta *et al.* (1999) (60), compared with trolox standard solutions (concentration ranging from 0.5-2.5 mg/mL) in Figure 3.1. For the ABTS assay, 20 µL of the test sample was mixed with 2.0 mL of diluted ABTS solution (A_{734nm} = 0.700 ± 0.020) and the absorbance was determined at 734 nm after incubation for 5 min at room temperature. Appropriate solvent blank was run in each assay. All determinations were carried out at least three times, and in triplicate. Inhibition of free radical by $ABTS^{\bullet+}$ in percent (I%) was calculated by the equation below:

$$I\ (\%) = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The percentage inhibition of the absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of trolox for the standard reference data.

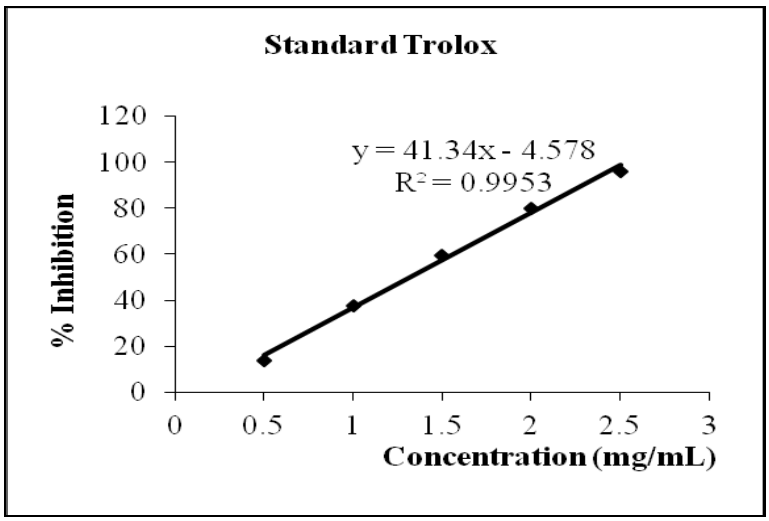


Figure 3.1: A linear regression (R^2 = 0.9953) of trolox (ABTS assay)

3.4.2 DPPH Method

The antioxidant activity of the test sample was determined by the DPPH radical scavenging assay. This modified method was described by Brand-Williams *et al.* (1995) (61). The DPPH 6.6 mg/mL (in ethanol) was prepared and stored in the dark before use. Various concentrations of trolox standard solutions and extract solutions were prepared using ethanol as solvent. This experiment was carried out with samples in the various concentrations. To each well of 96-well microtitre plate, 180 µL of ethanolic DPPH solution and 20 µL of the test sample were added. The total volume for each reaction mixture in each well was 200 µL. The plates were then incubated at 37ºC for 30 min to check for the colorimetric change (from deep violet to light yellow) when DPPH was reduced. The absorbance of each well was measured at 540 nm. The DPPH solution was used as the negative control. Trolox was used as the reference standard (Figure 3.2). Radical scavenging capacity was calculated by using the formula below:

$$\% \text{ Inhibition} = [(Ac - As) \times 100] / Ac,$$

Where Ac is the absorbance of the control and As is the absorbance of the test sample after incubation for 30 min. The values of % inhibition were obtained from the above equation. For the 50% Inhibitory Concentration (IC_{50}) evaluation of the test sample, graphs showing the concentration of the test samples versus % Inhibition (% DPPH reduction) were plotted. A linear regression (R^2) of standard Trolox was also used to calculate the radical scavenging capacity.

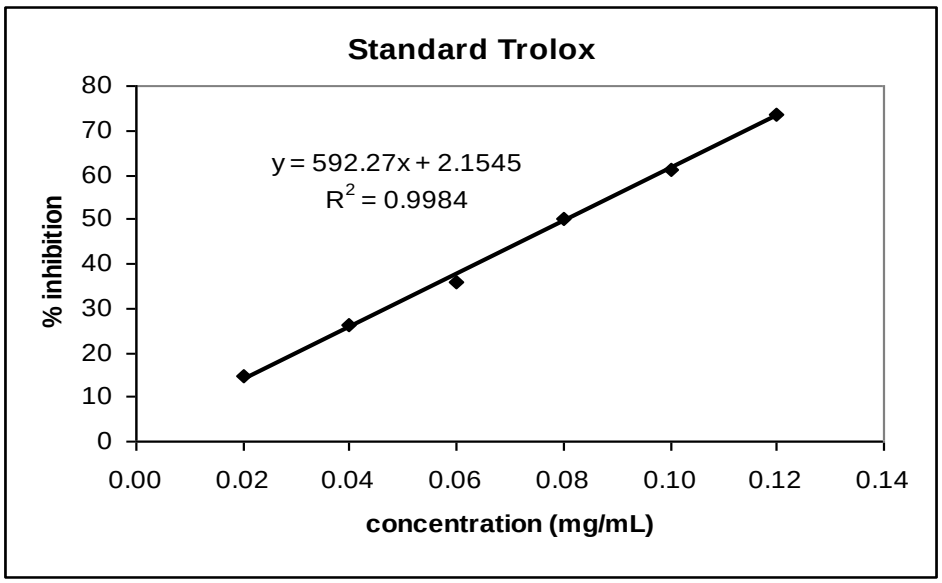


Figure 3.2: Linear regressions (R^2) of trolox (DPPH assay)

3.4.3 Total phenolic contents

The concentration of total phenolics was measured by the method described by Singleton and Rossi (1965) (62) with some modification. Briefly, an aliquot (1 ml) of appropriately diluted test sample or standard solution of gallic acid (0.004, 0.01, 0.02, 0.04, 0.06 and 0.08 mg/mL) was added to a 25 ml volumetric flask containing 9 ml of double distilled water. A reagent blank using double distilled water was prepared. One milliliter of Folin and Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solution was added with mixing. The solution was then immediately diluted to volume (25 ml) with double distilled water and mixed thoroughly. After incubation for 90 min at 23 ºC, the absorbance versus prepared blank was read at 750 nm. Total phenolic contents of the test sample was expressed as % gallic acid (w/w) of dry plant material by comparison with the gallic acid was used as reference standard. All samples were analyzed in three replications.

3.4.4 Total flavonoid contents

A modified method (63) was used for this purpose: diluted solution (1 mL) containing flavonoids, 5% (w/w) $NaNO_2$ (0.7 mL) and 30% (v/v) ethanol (10 mL) were mixed for 5 min, and then 10% $AlCl_3$ (w/w, 0.7 mL) was added and mixed altogether. Six minutes later, 1 mol/L NaOH (5 mL) was added. The solution was then diluted to 25 mL with 30% (v/v) ethanol. After standing for 10 min, the absorbance of the solution was measured at 430 nm with a spectrophotometer. A standard curve was plotted using quercetin as a standard. Different concentrations of quercetin were prepared in 80% ethanol and their absorbances were read at 430 nm by means of a spectrophotometer (UV-Vis spectrophotometer, Series Jasco, Model 7800, Japan). The results were expressed in % quercetin (w/w) of dry plant material by comparison with the quercetin was used as reference standard, which was made under the same conditions.

3.5 Biological Activity

3.5.1 Antimicrobial activity

The determination of the inhibitory effect of the essential oil or each crude medicinal plant extract on test bacteria was carried out by agar well diffusion method (64). *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were grown in Mueller-Hinton agar for 24 h, the culture suspensions were adjusted by comparing against 0.5 McFarland. *Candida albican, Aspergillus flavus* and *Trichophyton mentagrophyte* were grown in Sabouraud dextrose agar for 7 day, the culture suspensions were adjusted by comparing against 1.0 McFarland. Petri dishes with 20 mL of nutrient agar were prepared, previously inoculated with 200 µL of the culture suspension. The wells (9.0 mm in diameter) were made and the essential oil was diluted with ethanol then 150 µL of test concentration (50 mg mL^{-1}) was added to the wells and the same volume (150 µL) of ethanol was used as a control. The inoculated plates were incubated for

24 h. After incubation, the diameter of the inhibition zone was measured. The measurement was made basically from the edge of the zone to the edge of the well.

The zone of inhibition was calculated by measuring the minimum dimensions of the zone of no microbial growth around the disc and minimum inhibitory concentration (MIC) were determined by the standard disc diffusion method (Pelczar et al., 1993). The microorganisms used were *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27553).

The strains were maintained in agar conservation at room temperature. The strains inoculum were diluted in sterile 0.85% Saline to obtain turbidity visually comparable to a McFarland Nº 0.5 standard (10^{7-8} CFU/mL). Sterile nutrient agar plates were prepared and incubated at 37ºC for 24 h to check for any contamination. Sterile filter paper discs (Whatman No.1) of 6 mm diameter were placed in appropriate position on the surface of the plate with quadrants marked at the back of the petri dishes. Ten µL of the oil was carefully added into the sterile filter paper discs by means of steriled dropping automatic pipette. The *in vitro* antibacterial activities of different concentration of the essential oil at 10, 20, 30, 40 and 50 mg/mL were studied by disc diffusion method (65) against *S. aureus*, *E. coli* (ATCC25922), *P. aeruginosa*. The Petri dishes were incubated at 37ºC for 24 h and the diameters of the zone of inhibition were measured in mm. An average of three independent determinations were recorded.

3.5.2 Cell Toxicity on Cell Lines

3.5.2.1 Cytotoxicity activity on human cell lines

The cytotoxicity of the oil was assayed by using three cancerous human-cell lines: KB cell line (epidermoid carcinoma of oral cavity, ATCC CCL-17), MCF 7 cell line (breast adenocarcinoma, ATCC HTB-22) and NCI-H 187 cell line (small cell lung carcinoma, ATCC CRL-5804). This assay was performed using the method described by Brien *et.al*. (2000) (66). In brief, cells at a logarithmic growth phase were harvested and diluted to $7x10^4$ cells/mL for KB and $9x10^4$ cells/mL for MCF-7 and NCI-H 187, in fresh medium. Successively, 5 µL of test sample (the oil) was diluted in 5% DMSO, and 45 µL of cell suspension were added to 384-well

plates, incubated at 37ºC in 5% CO_2 incubator. After the incubation period (3 days for KB and MCF-7, and 5 days for NCI-H187), 12.5 µL of 62.5 µg/mL Resazurin solution was added to each well, and the plates were then incubated at 37ºC for 4 hours. Fluorescence signal was measured using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. Percent inhibition of cell growth was calculated by the following equation: %Inhibition=[1-(FU_T/ FU_C)] x 100. Whereas FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively. Dose response curves were plotted from 6 concentrations of 2-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be derived using the SOFTMax Pro software (Molecular Devices, USA).

3.5.2.2 Cytotoxic activity on Vero Cells

The cytotoxicity against primate cell line (Vero) of the oil was assayed by using Green fluorescent protein (GFP) detection described by Hunt *et.al.* (1999) (67). In brief, The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N-1 plasmid (Clontech). The cell line was maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 0.8 mg/mL geneticin, at 37ºC in a humidified incubator with 5% CO_2.

The assay was carried out by adding 45 µL of cell suspension at $3.3x10^4$ cells/mL to each well of 384-well plates containing 5 µL of test compounds previously diluted in 0.5% DMSO, and then incubating for 4 days in 37 ºC incubator with 5% CO_2. Fluorescence signals were measured by using SpectralMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signal at day 4 was subtracted with background flurescence at day 0. The percentage of cytotoxicity was calculated by the following equation, where FU_T and FU_C represent the fluorescence units of cells treated with test compound and untreated cell, respectively.

$$\% \text{ cytotoxicity} = [1-(FU_T / FU_C)] \times 100,$$

IC_{50} values were derived from dose-response curves, using 6 concentrations of 2-fold serially diluted samples, by the SOFTMax Pro sofeware (Molecular device). Ellipticine and 0.5% DMSO were used as a positive and negative control, respectively.

3.6 Extraction

Three medicinal plants were used in this investigation.

(1) *Acalypha indica* Linn. (*A. indica*).

Part of plant used: aerial parts.

(2) *Bridelia retusa* (L.) A. Juss. (*B. retusa*).

Part of plant used: leaves, stems and fruits.

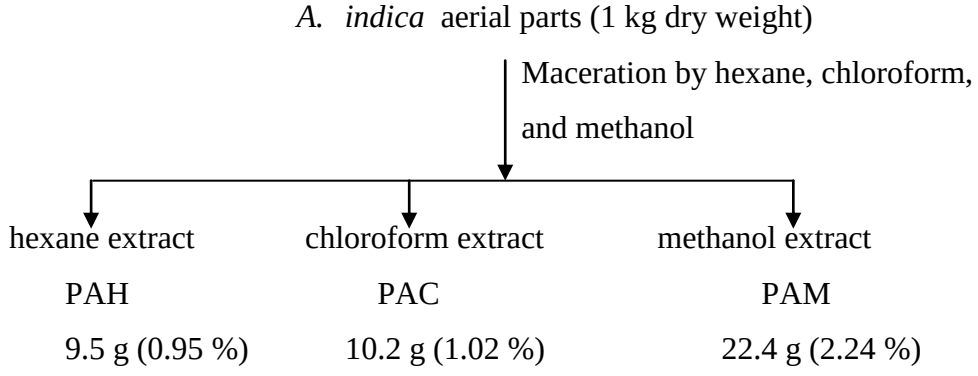
(3) *Cleidion javanicum* BL. (*C. javanicum*).

Part of plant used: leaves, stems and fruits.

All plant materials in 1-3 were dried at 40 ºC for 8 hrs and then ground into powder. All dried plant powders were successively extracted with hexane, chloroform and methanol by maceration. The extracts were filtered through Whatman No. 5 filter papers. Each filtrate was concentrated by using rotatory evaporator to yield crude extract. All crude extracts were kept in the dark at 4 ºC for further isolation as shown in scheme 3.1, 3.11, 3.12, 3.13, 3.22, 3.23 and 3.24.

3.6.1 Isolation of *A. indica*

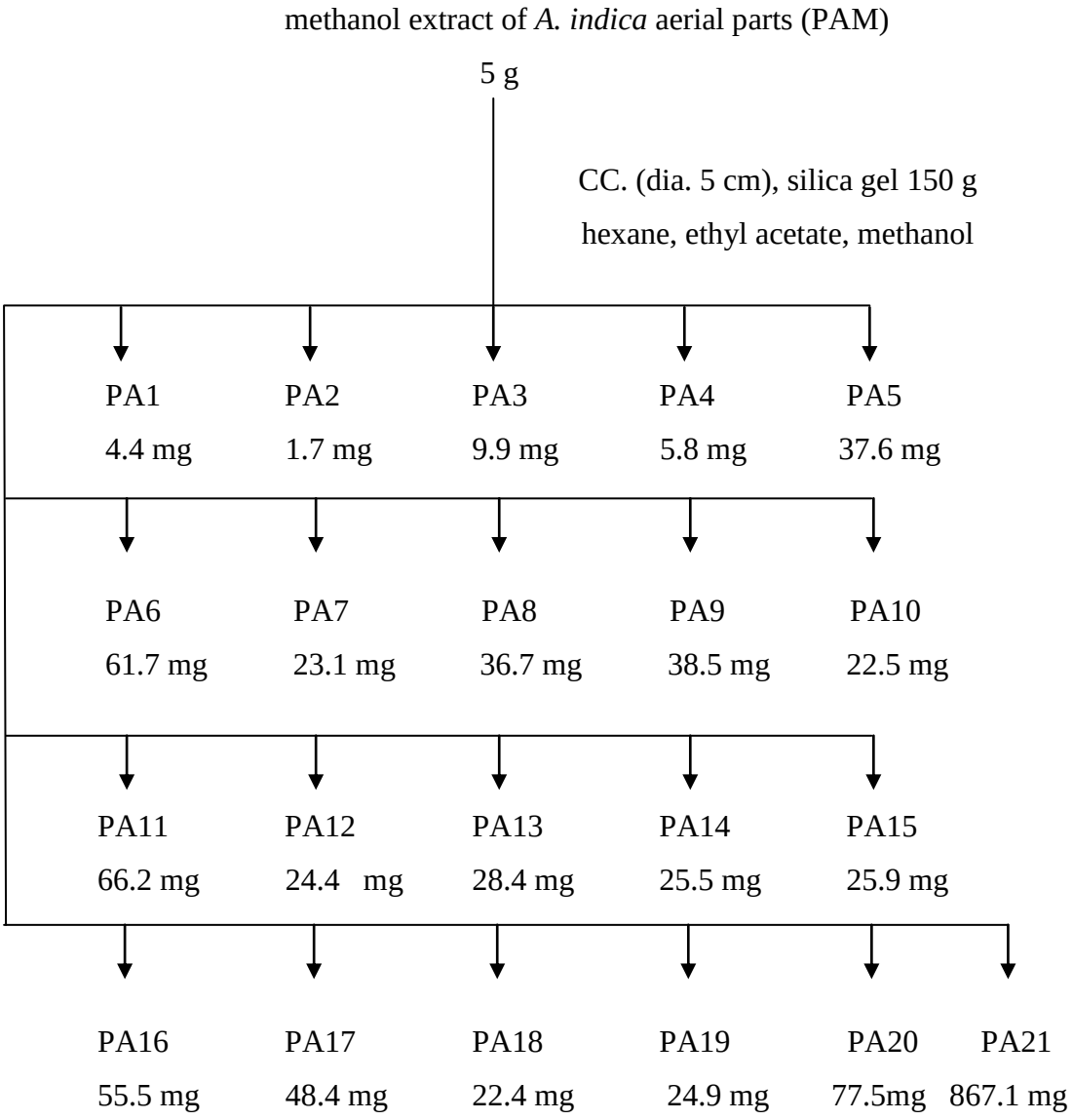
The extraction sequence is shown in Scheme 3.1



Scheme 3.1 Extraction scheme of *A. indica*

3.6.1.1 Isolation of methanol extract of *A. indica* (aerial parts)

The methanol extract, PAM (5 g), was fractionation by column chromatography (diameter 5 cm) over silica gel 60 (150 g) and eluted with a gradient of hexane, ethyl acetate and methanol respectively. Each 100 mL fraction of the eluate was collected and their composition was monitored by TLC profiles were grouped into 21 major fractions PA1-PA21 as shown in scheme 3.2.



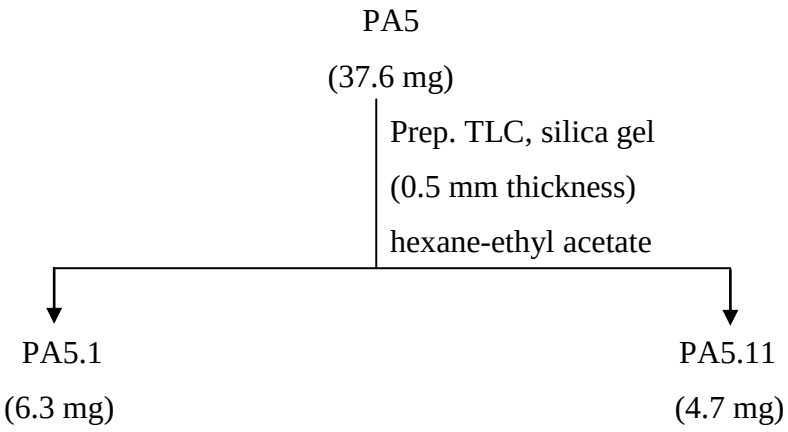
Scheme 3.2 Isolation scheme of methanol extract of *A. indica* aerial parts (PAM)

3.6.1.1.1 Isolation of PA1.1, PA2.1, PA3.1 and PA4.1

The fractions PA1, PA2, PA3 and PA4 were evaluated by TLC analysis. TLC profiles were grouped into 4 fractions, PA1.1, PA2.1, PA3.1 and PA4.1 respectively.

3.6.1.1.2 Isolation of PA5.1

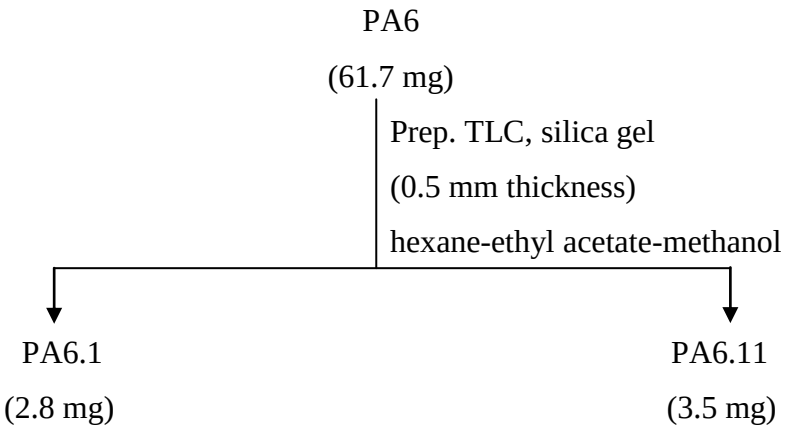
The fraction PA5 (37.6 mg) was purified using preparative TLC on silica gel 60 to yield PA5.1. The fractionation process of PA5.1 is shown in Scheme 3.3.



Scheme 3.3 Isolation scheme of compound PA5.1

3.6.1.1.3 Isolation of PA6.1

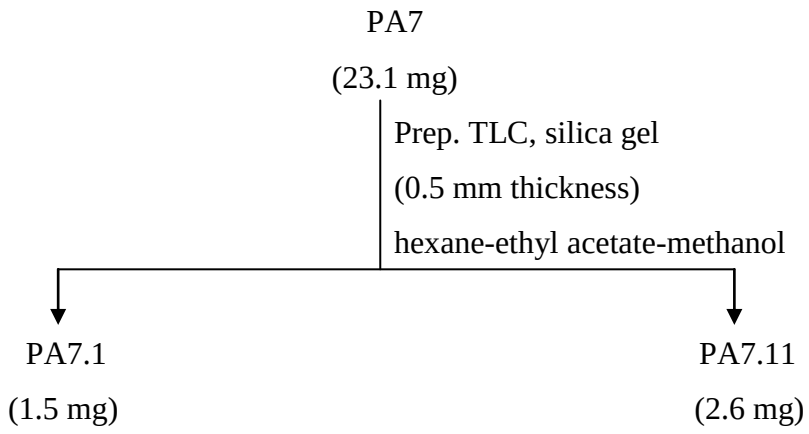
The fraction PA6 (61.7 mg) was purified using preparative TLC on silica gel 60 to yield PA6.1. The fractionation process of PA6.1 is shown in Scheme 3.4.



Scheme 3.4 Isolation scheme of compound PA6.1

3.6.1.1.4 Isolation of PA7.1

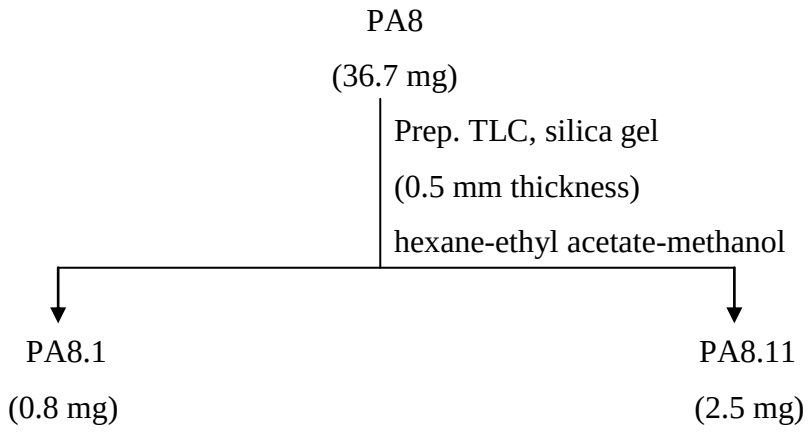
The fraction PA7 (23.1 mg) was purified using preparative TLC on silica gel 60 to yield PA7.1. The fractionation process of PA7.1 is shown in Scheme 3.5.



Scheme 3.5 Isolation scheme of compound PA7.1

3.6.1.1.5 Isolation of PA8.1

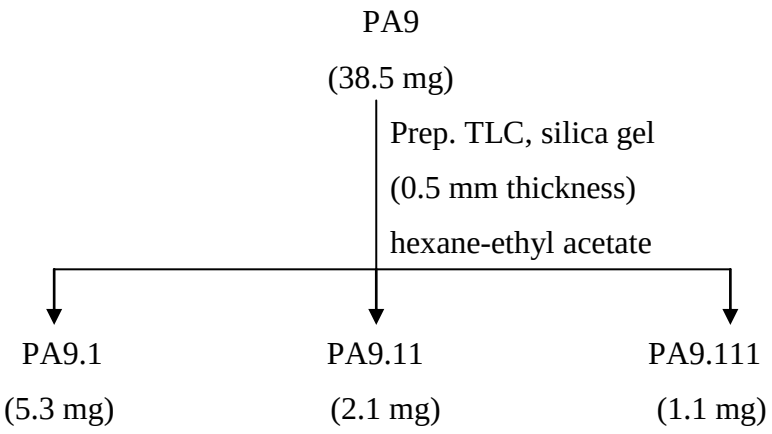
The fraction PA8 (36.7 mg) was purified using preparative TLC on silica gel 60 to yield PA8.1. The fractionation process of PA8.1 is shown in Scheme 3.6.



Scheme 3.6 Isolation scheme of compound PA8.1

3.6.1.1.6 Isolation of PA9.1

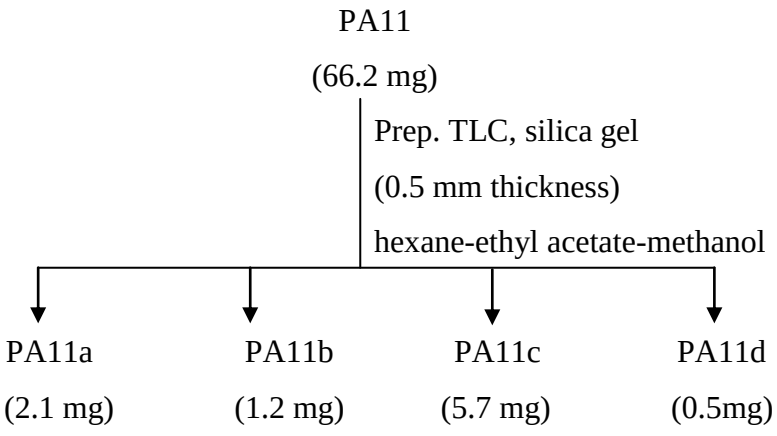
The fraction PA9 (38.5 mg) was purified using preparative TLC on silica gel 60 to yield PA9.1. The fractionation process of PA9.1 is shown in Scheme 3.7.



Scheme 3.7 Isolation scheme of compound PA9.1

3.6.1.1.7 Isolation of PA11a, PA11b, PA11d

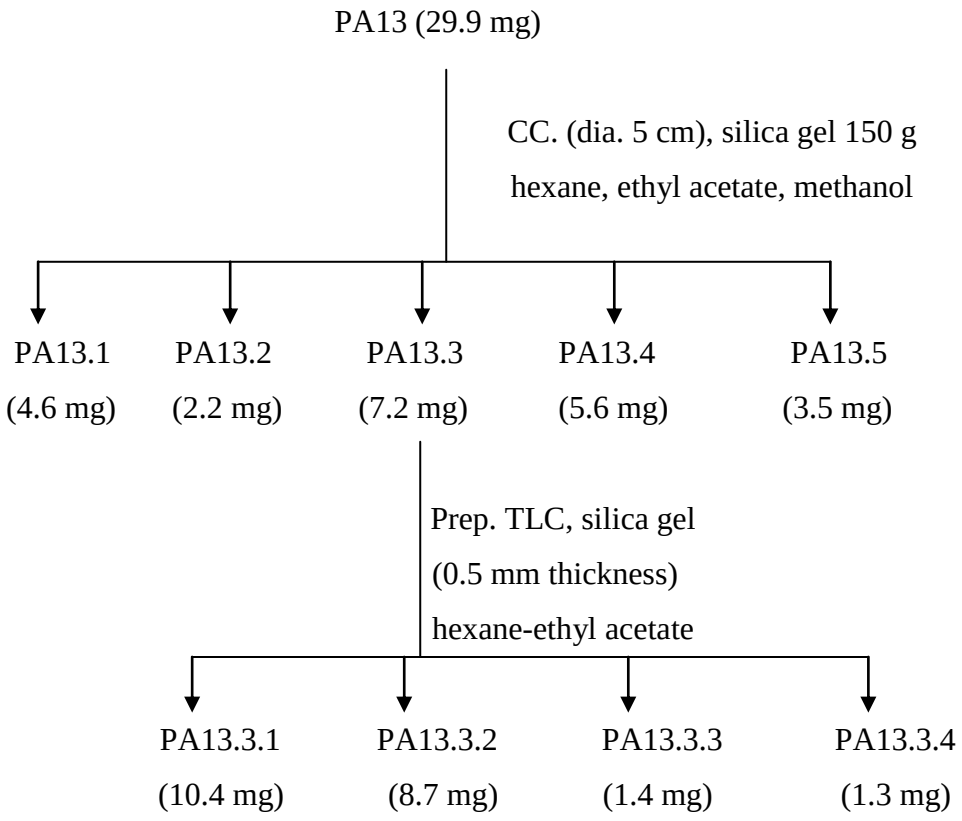
The fraction PA11 (66.2 mg) was purified using preparative TLC on silica gel 60 to yield PA11a, PA11b and PA11d. The fractionation process of PA11a, PA11b and PA11d is shown in Scheme 3.8.



Scheme 3.8 Isolation scheme of compound PA11a, PA11b and PA11d

3.6.1.1.8 Isolation of PA13.3.1, PA13.3.2, PA13.3.3, PA13.3.4

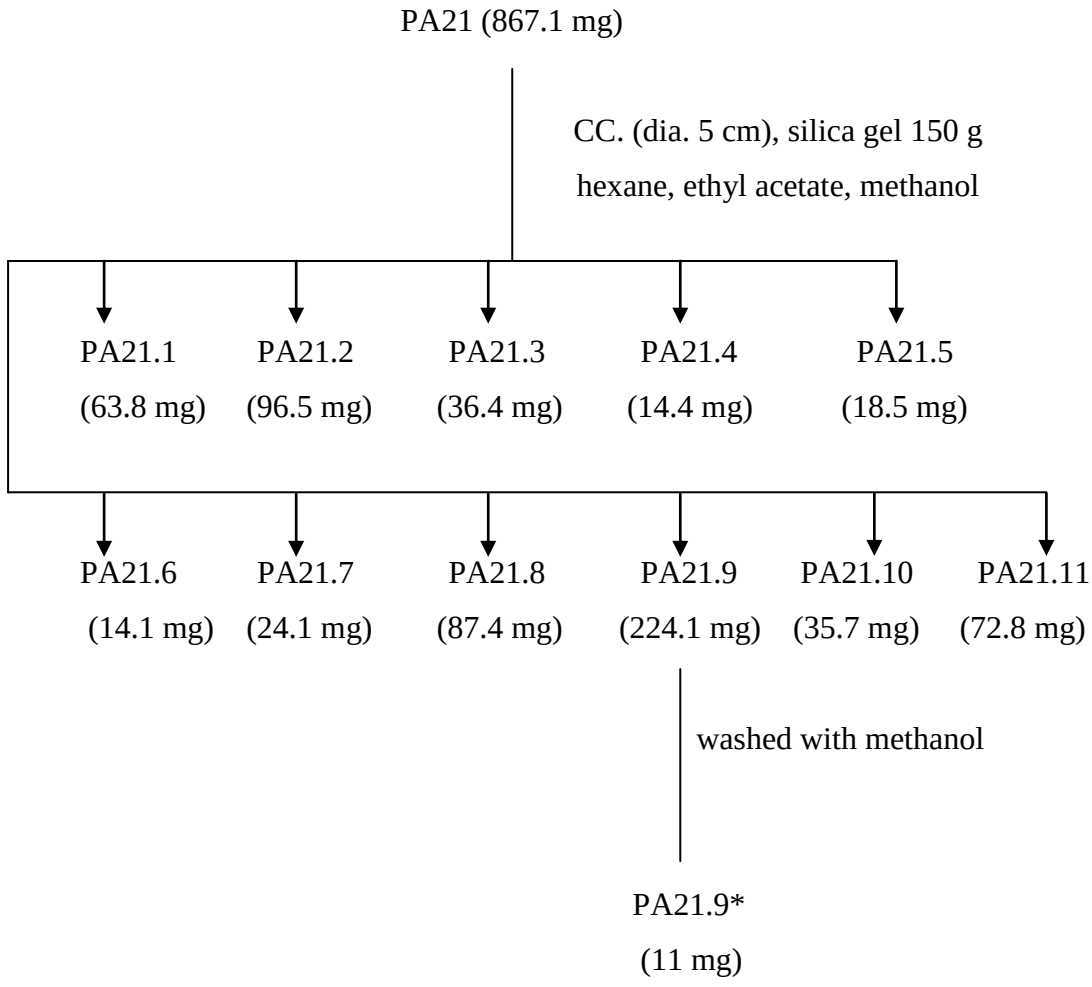
The fraction PA13 (29.9 mg) was further purified by silica gel 60 (150 g) and eluted with hexane, ethyl acetate and methanol. Each 100 ml fraction of the eluate was collected and combined according to their TLC patterns, yielding 5 fractions, PA13.1 to PA13.5.The fraction 13.3 was further isolated using preparative TLC to give PA13.3.1, PA13.3.2, PA13.3.3, PA13.3.4 as shown in Scheme 3.9.



Scheme 3.9 Isolation scheme of compound PA13.3.1, PA13.3.2, PA13.3.3, PA13.3.4

3.6.1.1.9 Isolation of PA21.9*

The fraction PA21 (867.1 mg) was refractionated over silica gel 60 (150 g) and eluted with a gradient of hexane, ethyl acetate and methanol. Each 100 ml fraction was collected and combined according to their similar TLC patterns were grouped into 11 fractions, PA21.1 to PA21.11. as shown in Scheme 3.10. Among these, a potion of PA21.9 was further washed with methanol to obtain PA21.9* (5 mg) as white crystalline powder.



Scheme 3.10 Isolation scheme of compound PA21.9*

The sample PA21.9* white crystalline powder (5 mg) was subjected to development of a reversed phase HPLC method for the determination of *L*-quebrachitol in *Acalypha indica* Linn.

3.6.1.1.10 Determination of *L*-quebrachitol by HPLC

Preparation of Standard Solution:

A *L*-quebrachitol standard stock solution was prepared by transferring accurately weighed 10 mg of *L*-quebrachitol reference standard into a 10 mL volumetric flask. Then 10 mL of diluent (methanol) was added followed by sonication for 15 min or until dissolved. The volume was completed with the same diluent and then filtered through 0.45 µm membrane filter. Working standard solutions were prepared by dilution from this stock standard solution.

Preparation of Samples:

Two milligrams of *L*-quebrachitol (sample) was weighed accuratedly into a 2-mL volumetric flask and dissolved in methanol (HPLC grade). Then the solution was adjusted to volume with methanol and mixed well. The solution was sonicated for 15 min, then filtered through a 0.45 µm membrane filter and kept for further RP-HPLC analysis.

HPLC Method:

A series of L-Quebrachitol working standard solutions containing 3.9, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0 and 1000.0 µg/mL of *L*-quebrachitol were prepared by dilution from the stock standard solution. These solutions were analyzed by the developed RP-HPLC method. Twenty µL aliquots of standard and sample solutions were injected in triplicated into a reversed phase C_{18} column (4.6 mm x 250 mm) and *L*-quebrachitol was detected by photodiode array detector at 206 nm. A mobile phase consisting of filtered and degassed mixture of methanol:acetonitrile:tetrahydrofuran (79.5:20.4:0.1 v/v) was used at a flow rate of 0.3 mL/min under ambient temperature (25ºC). Under these conditions the peak corresponding to *L*-quebrachitol was identified at 9.442 min (average retention time). A calibration was constructed by plotting L-Quebrachitol concentrations against peak areas. *L*-quebrachitol in the

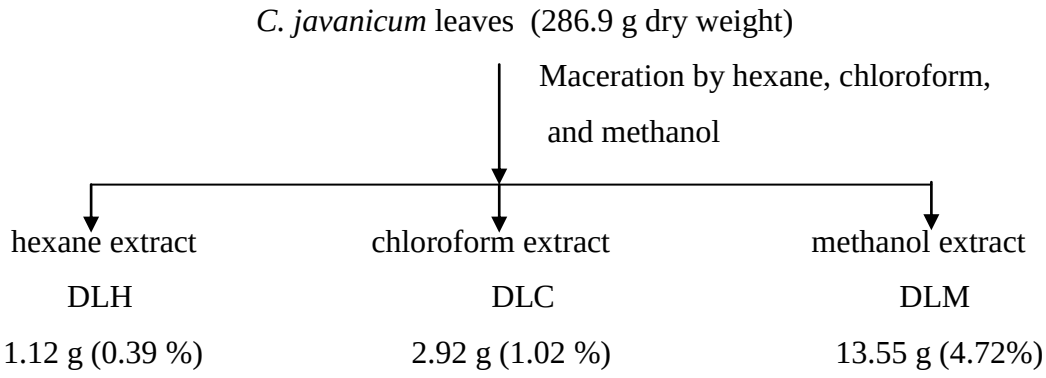
sample was determined by reference to the calibration curve, using standard addition method.

Method Validation:

The HPLC method was validated in terms of system suitability, precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), and accuracy according to USP pharmacopoeia and International Conference on Harmonization (ICH) guidelines (68). System suitability of the assay method was determined as the system suitability parameters such as theoretical plates, tailing factor, resolution, asymmetry, retention time and area. Precision of the assay method was determined using nine concentrations. Precision of the method was evaluated by repeatability (intraday) and intermediate precision (interday) test and evaluated by the calculated of relative standard deviation (RSD), where values of less than 2% were considered acceptable. Linearity of the assay was determined over nine nine different *L*-quebrachitol concentrations (3.9, 7.8**,** 15.6, 31.2, 62.5**,** 125.0, 250.0, 500.000 and 1000.0 µg/mL) each analyzed in triplicate. The response, represented by the average peak area, was plotted against concentration and least square regression analysis was applied. LOD and LOQ of the method were calculated using the slope (S) of the calibration curve, obtained from linearity assessment. The method accuracy was evaluated as the percent recovery of added standard from the accuracy samples, which were prepared by the addition (spiking) of known amounts of *L*-quebrachitol reference standard to the sample test. The standard addition method was investigated by plot the signal against the concentration with the initial unknown concentration set at 0. Extrapolate the line connecting the measured responses back to 0 response and read the concentration value off the (negative) x-axis. The main assumption is that the response is linear in the working region (69).

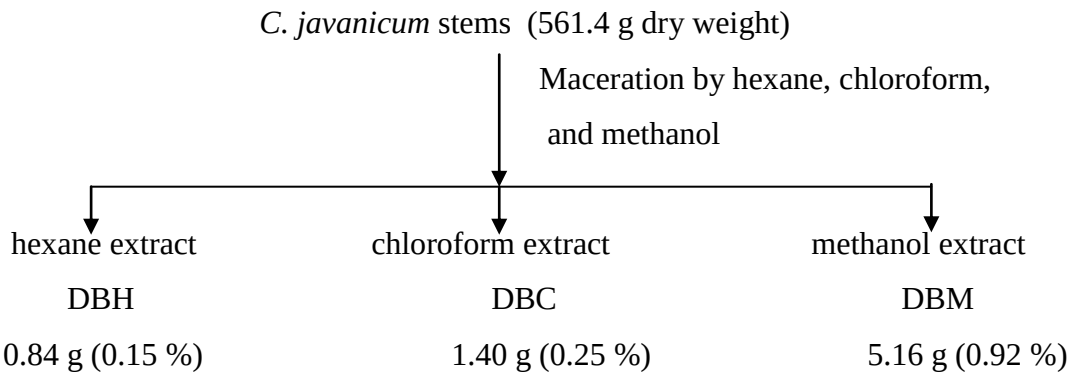
3.6.2 Isolation of *C. javanicum*

The leaves extraction sequence is shown in Scheme 3.11



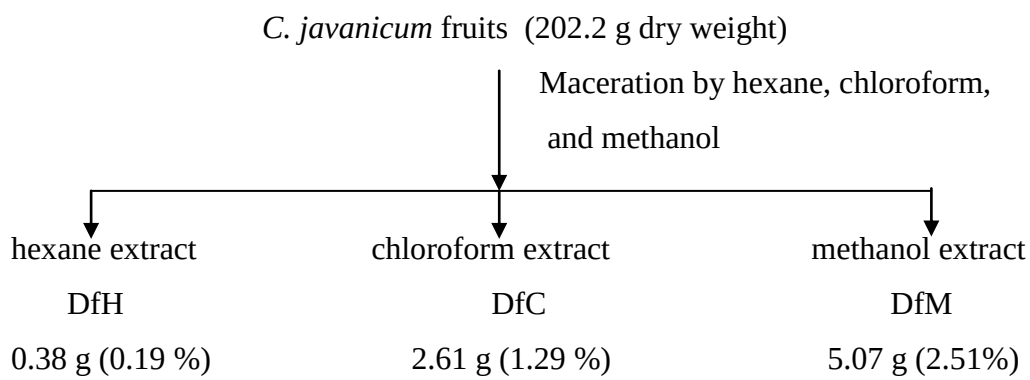
Scheme 3.11 Extraction of *C. javanicum* leaves

The stems extraction sequence is shown in Scheme 3.12



Scheme 3.12 Extraction of *C. javanicum* stems

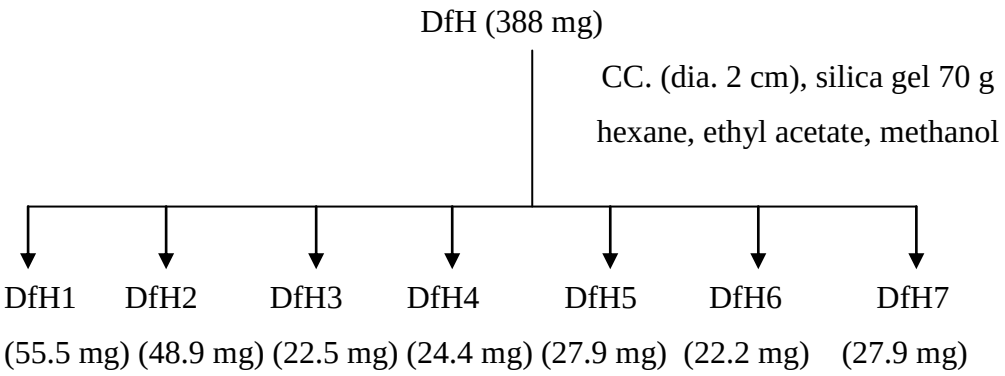
The fruits extraction sequence is shown in Scheme 3.13



Scheme 3.13 Extraction of *C. javanicum* fruits

3.6.2.1 Isolation of the hexane extract of *C. javanicum* fruits

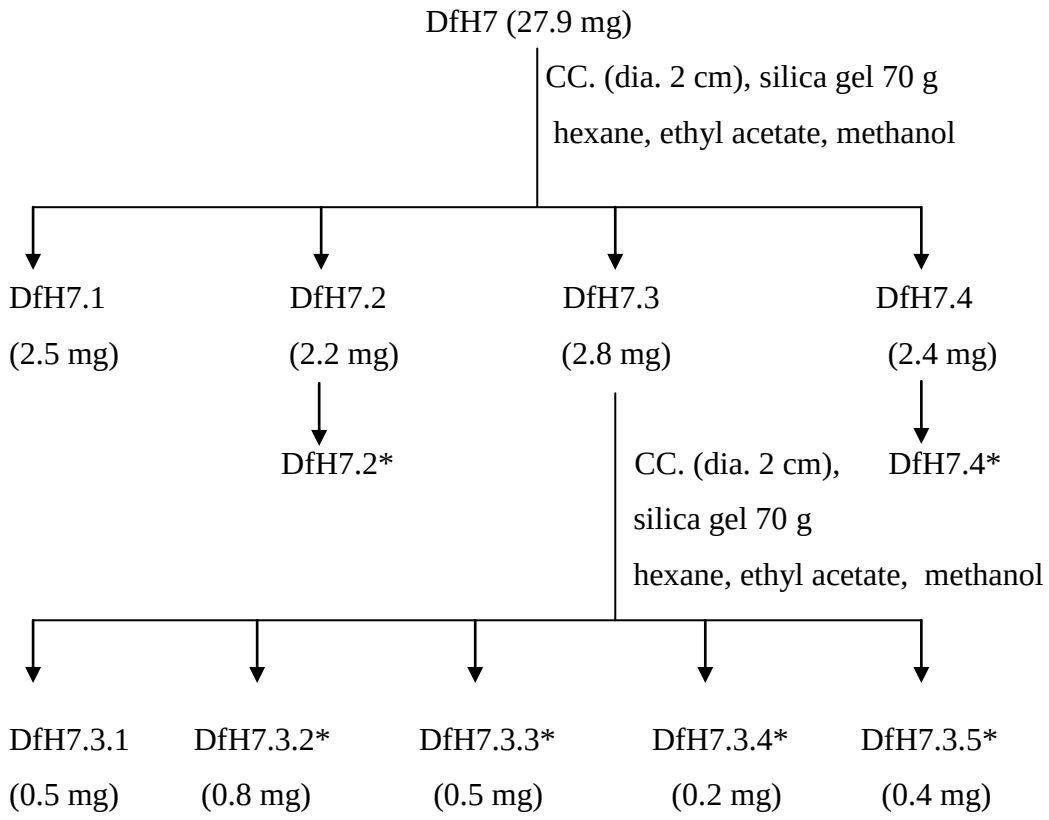
The hexane extract, DfH (388 mg), was fractionation by column chromatography (diameter 2 cm) over silica gel 60 (70 g) and eluted with a gradient of hexane, ethyl acetate and methanol respectively. Each 50 mL fraction of the eluate was collected and evaluated by TLC, those fractions showing similar TLC profiles were grouped into 7 fractions, DfH1-DfH7 as shown in scheme 3.14.



Scheme 3.14 Isolation scheme of hexane extract of *C. javanicum* fruits (DfH)

3.6.2.2 Isolation of DfH7.2*, DfH7.3.2*, DfH7.3.3*, DfH7.3.4*, DfH7.3.5* and DfH7.4*

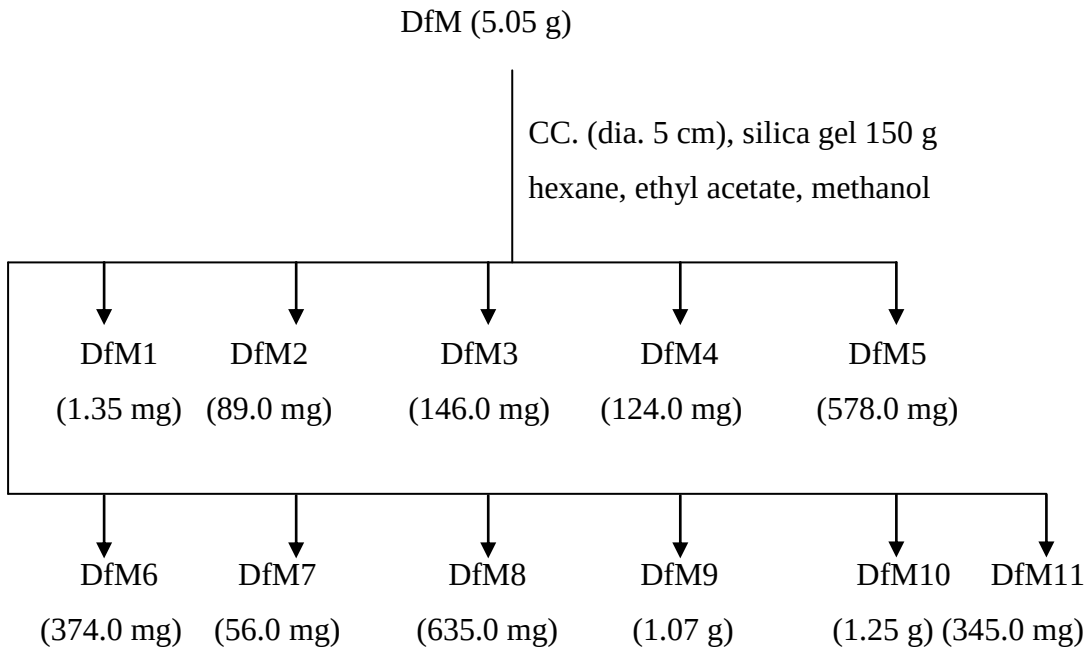
The fraction DfH7 (27.9 mg) was refractionated over silica gel 60 (70 g) and eluted with hexane, ethyl acetate and methanol. Each 50 ml fraction was collected and combined according to their TLC patterns to give 4 fractions, DfH7.1 to DfH7.4. The fraction DfH7.2 and DfH 7.4 giving white needle crystal to give yield DfH7.2* and DfH7.4*. The fraction DfH7.3 was selected for further purification. This fraction was subjected to column chromatography over silica gel 60 (70 g) and eluted with hexane, ethyl acetate and methanol. Each 50 mL fraction was collected and combined according to their TLC profiles to yield 5 fractions, DfH7.3.1-DfH7.3.5. The fraction DfH7.3.2, DfH7.3.3, DfH7.3.4 and DfH7.3.5 giving white needle crystal to give yield DfH7.3.2*, DfH7.3.3*, DfH7.3.4* and DfH7.3.5* as shown in Scheme 3.15.



Scheme 3.15 Isolation scheme of compounds DfH7.2*, DfH7.3.2*, DfH7.3.3*, DfH7.3.4*, DfH7.3.5* and DfH7.4*

3.6.2.3 Isolation of the methanol extract of *C. javanicum* fruits

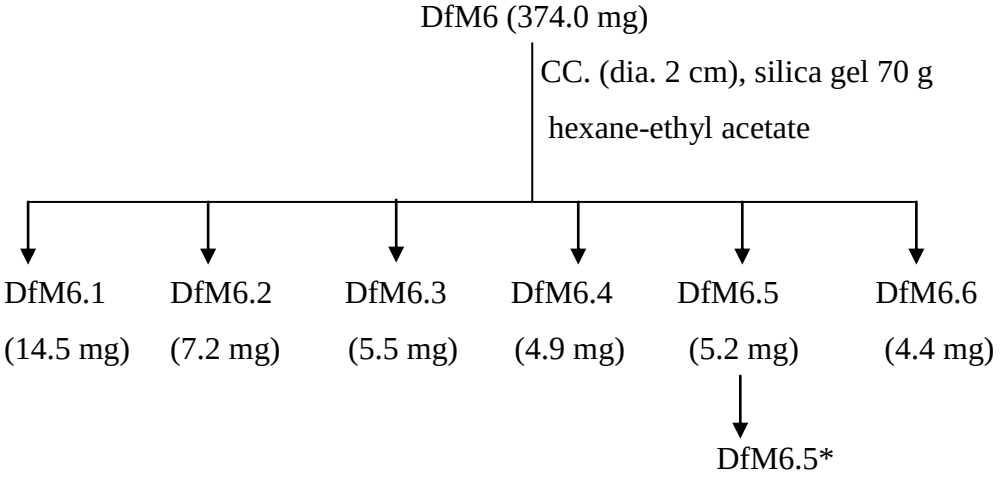
The methanol extract, DfM (5.05 g), was fractionation by column chromatography (diameter 5 cm) over silica gel 60 (150 g) and eluted with a gradient of hexane, ethyl acetate and methanol respectively. Each 100 mL fraction of the eluate was collected and evaluated by TLC, those fractions showing similar TLC profiles were grouped into 11 fractions, DfM1-DfM11 as shown in scheme 3.16.



Scheme 3.16 Isolation scheme of methanol extract of *C. javanicum* fruits (DfM)

3.6.2.4 Isolation of DfM6.5*

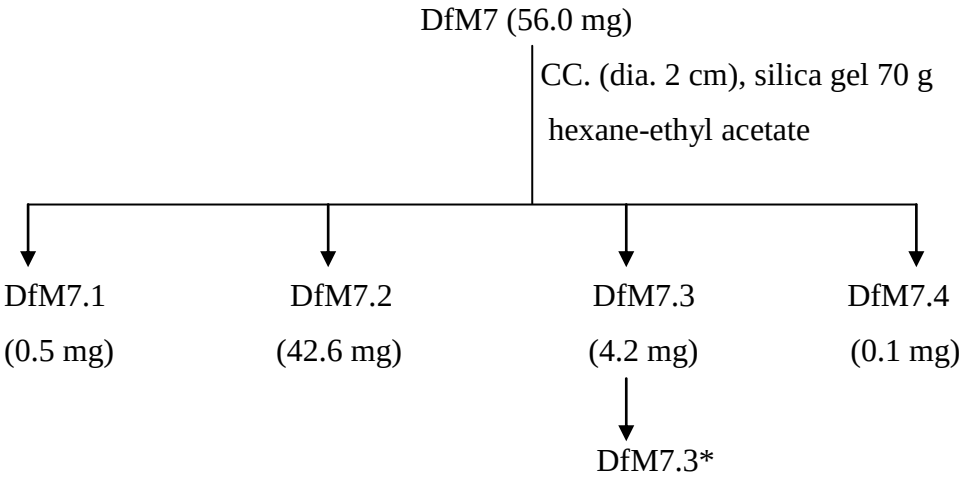
The fraction DfM6 (374.0 mg) was refractionated over silica gel 60 (70 g) and eluted with hexane and ethyl acetate. Each 50 ml fraction was collected and combined according to their TLC profiles to give 6 fractions, DfM6.1 to DfM6.6. The fraction DfM giving white crystal to give yield DfM6.5* as shown in Scheme 3.17.



Scheme 3.17 Isolation scheme of compound DfM6.5*

3.6.2.5 Isolation of DfM7.3*

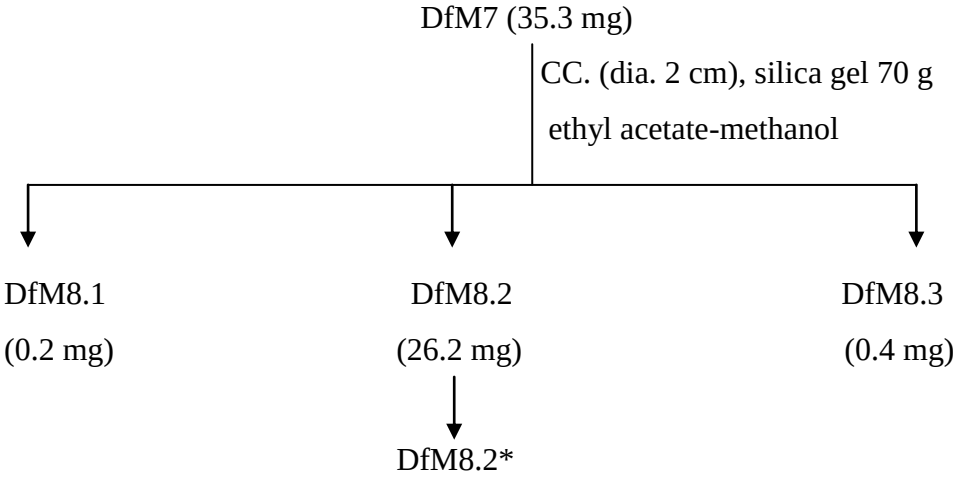
The fraction DfM7 (56.0 mg) was further purified by silica gel 60 (70 g) and eluted with hexane and ethyl acetate. Each 50 ml fraction was collected and combined according to their TLC profiles to yield 4 fractions, DfM7.1 to DfM7.4. The fraction DfM7.3 giving white crystalline powder to give yield DfM7.3* as shown in Scheme 3.18.



Scheme 3.18 Isolation scheme of compound DfM7.3*

3.6.2.6 Isolation of DfM8.2*

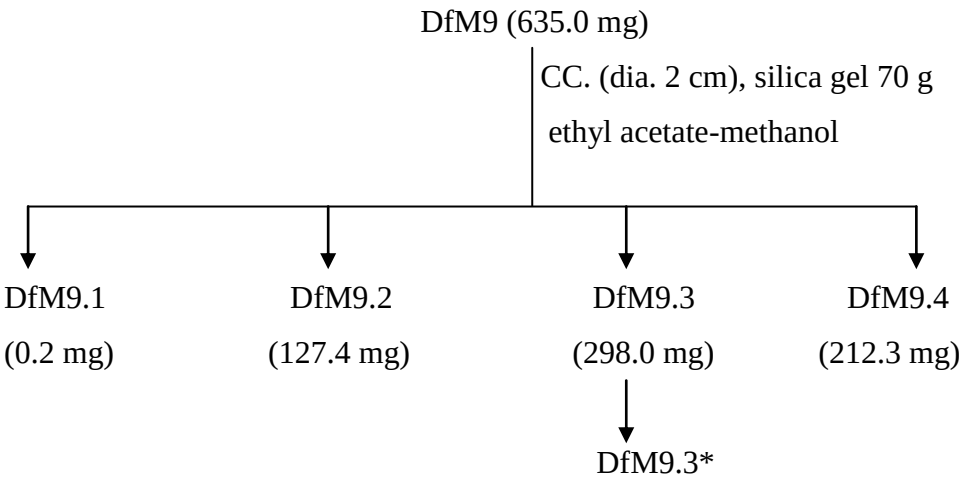
The fraction DfM8 (35.3 mg) was further purified over silica gel 60 (70 g) and eluted with ethyl acetate and methanol. Each 50 ml fraction was collected and combined according to their TLC profiles to give 3 fractions, DfM8.1 to DfM8.3. The fraction DfM8.2 giving white crystalline powder to give yield DfM8.2* as shown in Scheme 3.19.



Scheme 3.19 Isolation scheme of compound DfM8.2*

3.6.2.7 Isolation of DfM9.3*

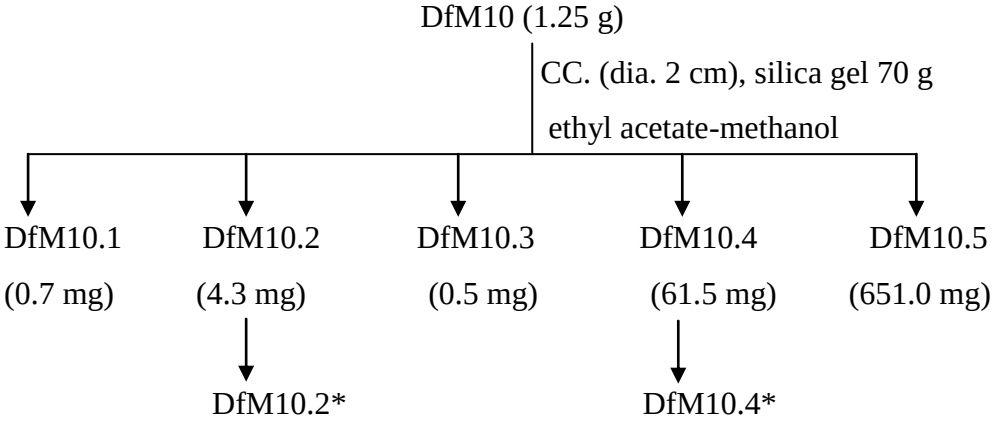
The fraction DfM9 (635.0 mg) was futher purified over silica gel 60 (70 g) and eluted with ethyl acetate and methanol. Each 50 ml fraction was collected and combined according to their TLC profiles to yield 4 fractions, DfM9.1 to DfM9.4. The fraction DfM9.3 giving white crystalline powder to give yield DfM9.3* as shown in Scheme 3.20.



Scheme 3.20 Isolation scheme of compound DfM9.3*

3.6.2.8 Isolation of DfM10.2 and DfM10.4*

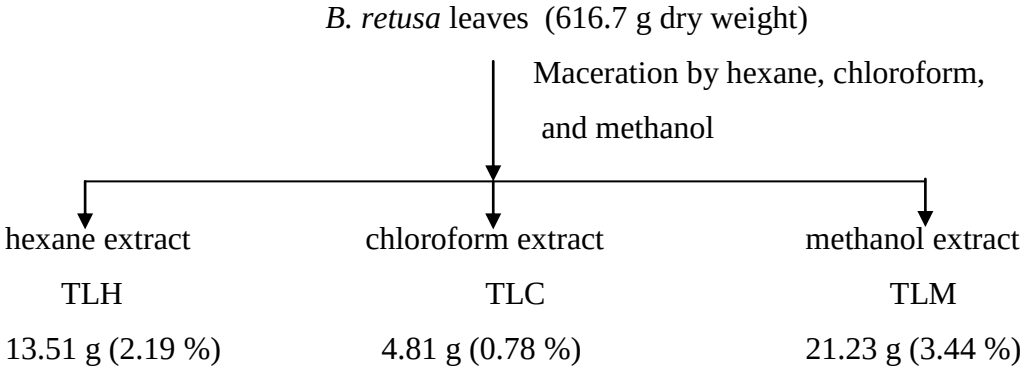
The fraction DfM10 (1.25 g) was refractionatedover silica gel 60 (70 g) and eluted with ethyl acetate and methanol. Each 50 ml fraction was collected and combined according to their TLC profiles to give 5 fractions, DfM10.1 to DfM10.5. The fraction DfM10.2 and DfM10.4 giving white crystalline powder to give yield DfM10.2* and DfM10.4* as shown in Scheme 3.21.



Scheme 3.21 Isolation scheme of compound DfM10.2* and DfM10.4*

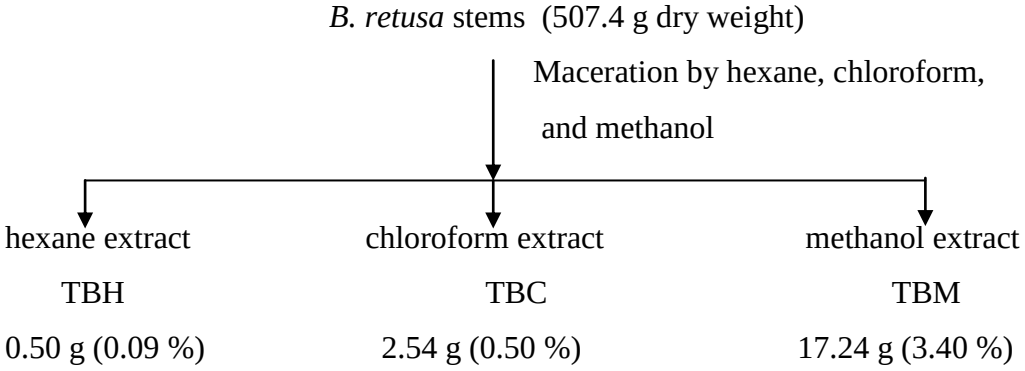
3.6.3 Isolation of *B. retusa*

The leaves extraction sequence is shown in Scheme 3.22



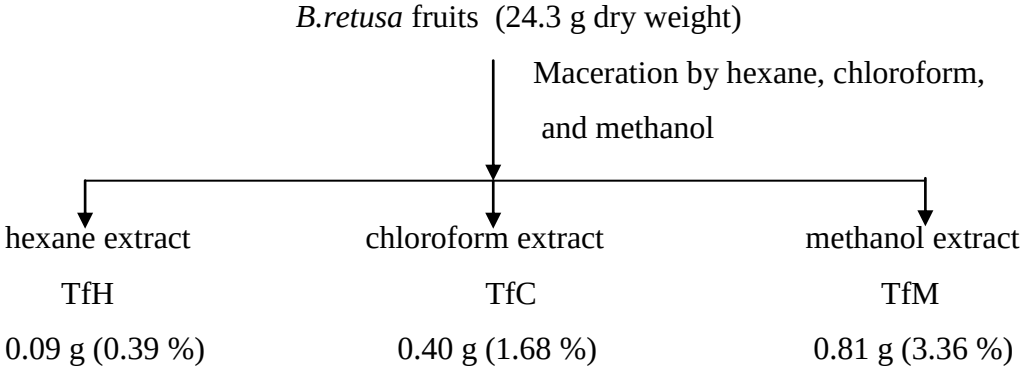
Scheme 3.22 Extraction of *B. retusa* leaves

The extraction sequence is shown in Scheme 3.23



Scheme 3.23 Extraction of *B. retusa* stems

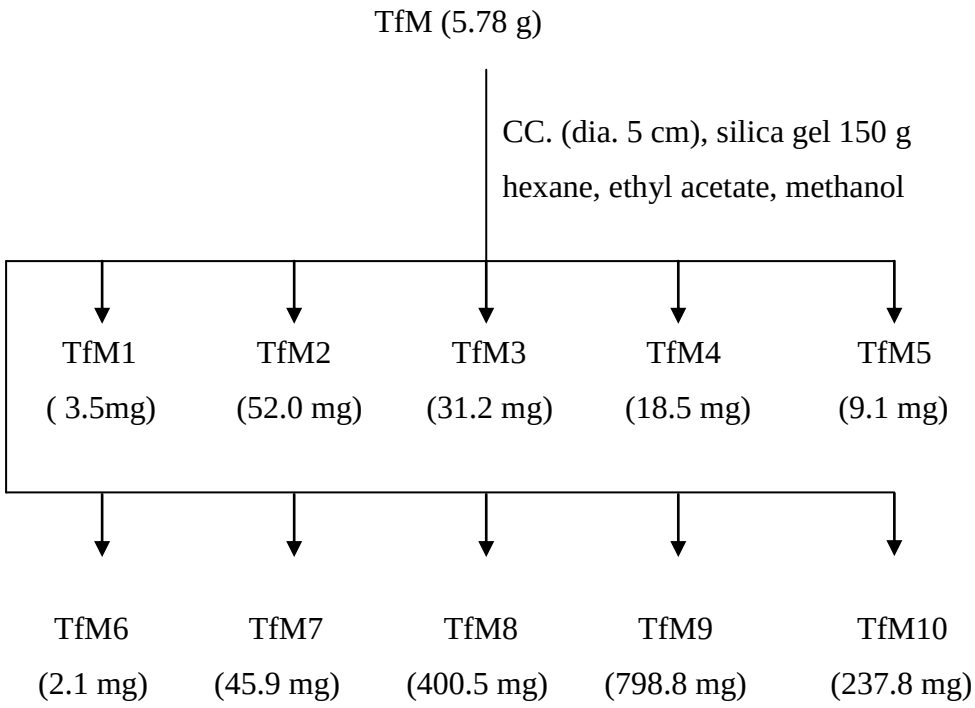
The extraction sequence is shown in Scheme 3.24



Scheme 3.24 Extraction of *B. retusa* fruits

3.6.3.1 Isolation of the methanol extract of *B. retusa* fruits

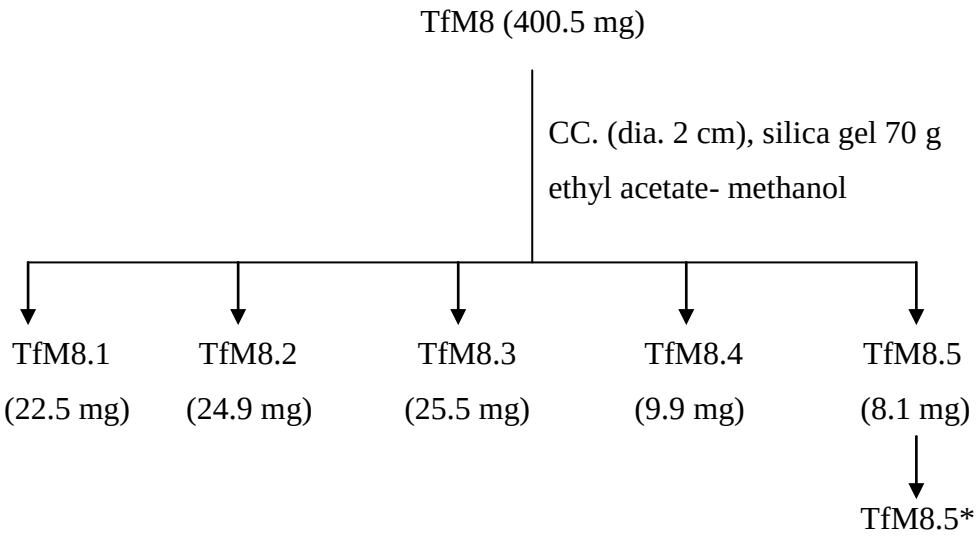
The methanol extract, TfM 5.78 g was fractionation by column chromatography (diameter 5 cm) with silica gel 60 (150 g) and eluted with a gradient of hexane, ethyl acetate and methanol respectively. Each 100 mL fraction of the eluate (100 ml each) were collected and evaluated by TLC analysis patterns to yield 10 fractions, TfM1-TfM10 as shown in scheme 3.25.



Scheme 3.25 Isolation scheme of methanol extract of *B. retusa* fruits (TfM)

3.6.3.1.1 Isolation of TfM8.5*

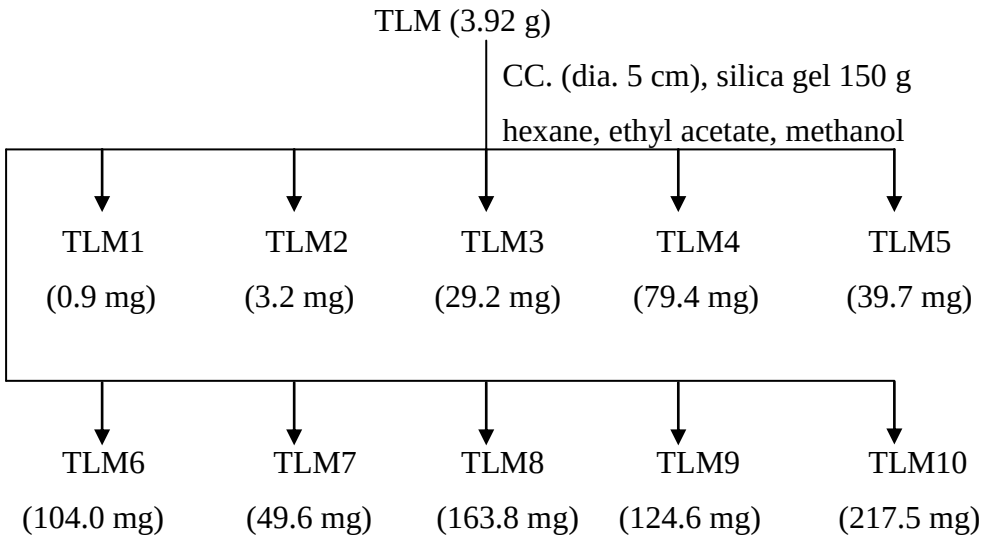
The fraction TfM8 (400.5 mg) was further purified over silica gel 60 (70 g) and eluted with ethyl acetate and methanol. Each 50 ml fraction was collected and combined according to their TLC profiles to give 10 fractions, TfM8.1 to TfM8.5. The fraction TfM8.5 giving white crystalline powder to give yield TfM8.5* as shown in scheme 3.26.



Scheme 3.26 Isolation scheme of compound TfM8.5*

3.6.3.2 Isolation of the methanol extract of *B. retusa* leaves

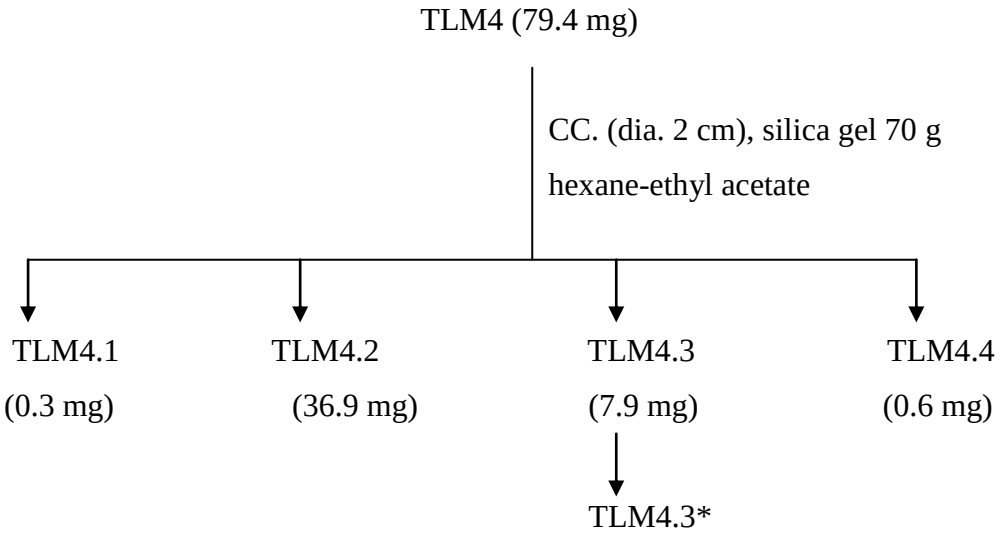
The methanol extract, TLM 3.92 g was fractionation by column chromatography (diameter 5 cm) over silica gel 60 (150 g) and eluted with a gradient of hexane, ethyl acetate and methanol respectively. Each 100 mL fraction of the eluate was collected and evaluated by TLC profiles to yield 10 fractions, TLM1-TLM10 as shown in scheme 3.27.



Scheme 3.27 Isolation scheme of methanol extract of *B. retusa* leaves (TLM)

3.6.3.2.1 Isolation of TLM4.3*

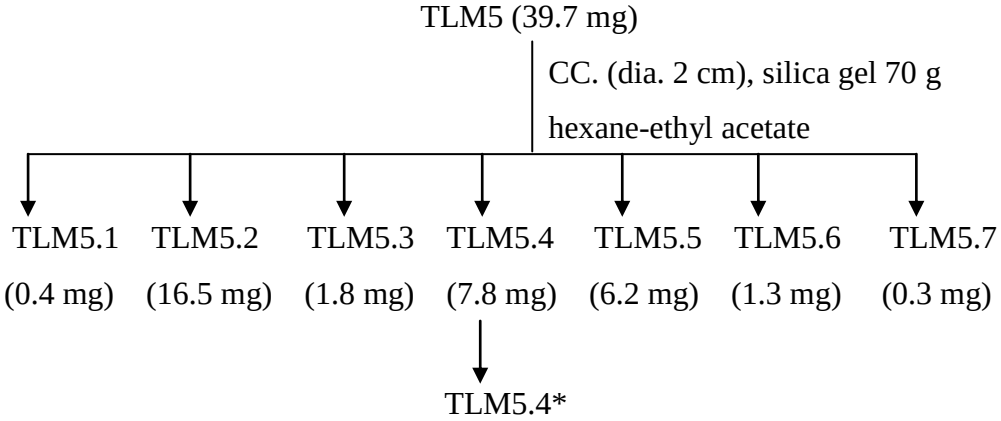
The fraction TLM4 (79.4 mg) was futher purified over silica gel 60 (70 g) and eluted with hexane and ethyl acetate. Each 50 ml fraction was collected and combined according to their TLC profiles to give 4 fractions, TLM4.1 to TLM4.4. The fraction TLM4.3 giving white crystalline powder to give yield TLM4.3* as shown in Scheme 3.28.



Scheme 3.28 Isolation scheme of compound TLM4.3*

3.6.3.2.2 Isolation of TLM5.4*

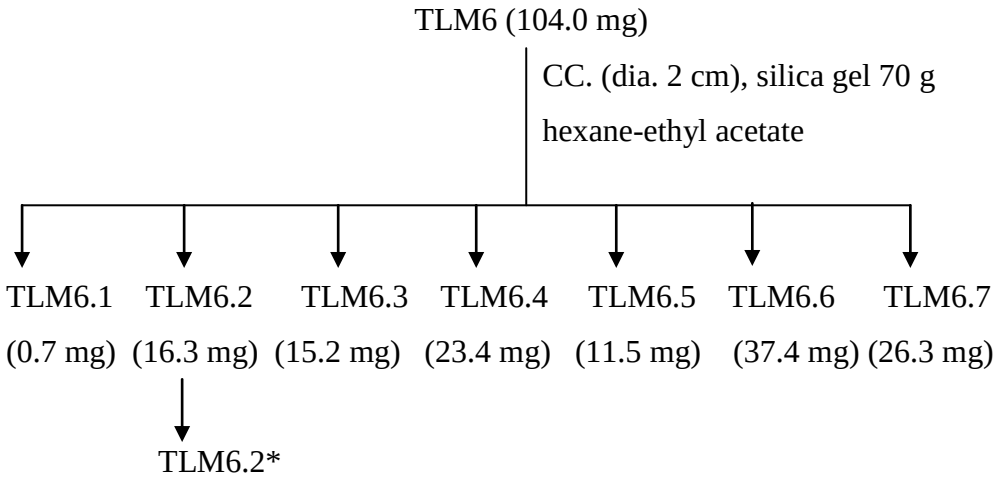
The fraction TLM5 (39.7 mg) was refractionated over silica gel 60 (70 g) and eluted with hexane and ethyl acetate. Each 50 ml fraction was collected and combined according to their TLC profiles to give 7 fractions, TLM5.1 to TLM5.7. The fraction TLM5.4 giving white crystalline powder to give yield TLM5.4* as shown in Scheme 3.29.



Scheme 3.29 Isolation scheme of compound TLM5.4*

3.6.3.2.3 Isolation of TLM6.2*

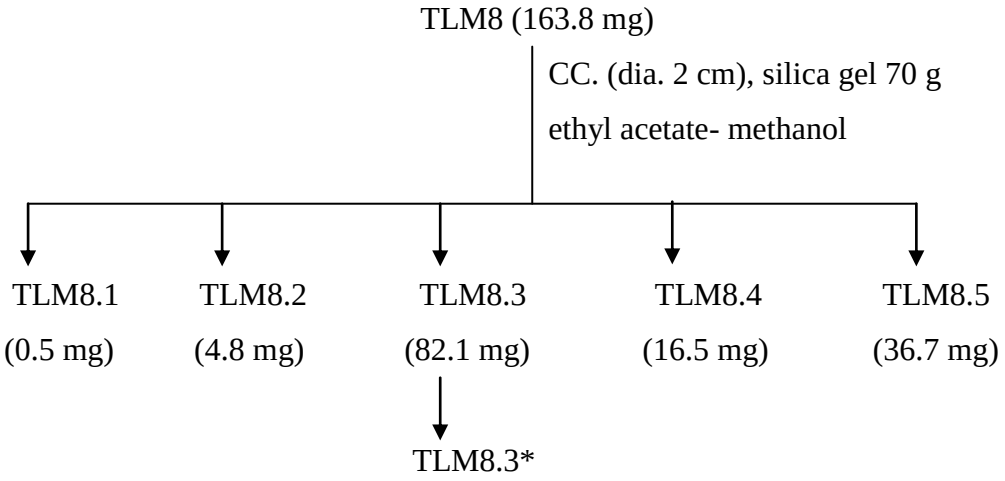
The fraction TLM6 (104.0 mg) was further purified over silica gel 60 (70 g) and eluted with hexane and ethyl acetate. Each 50 ml fraction was collected and combined according to their TLC profiles to give 7 fractions, TLM6.1 to TLM6.7. The fraction giving white crystalline powder to give yield TLM6.2* as shown in Scheme 3.30.



Scheme 3.30 Isolation scheme of compound TLM6.2*

3.6.3.2.4 Isolation of TLM8.3*

The fraction TLM8 (163.8 mg) was refractionated over silica gel 60 (70 g) and eluted with ethyl acetate and methanol. Each 50 ml fraction was collected and combined according to their TLC profiles to yield 5 fractions, TLM8.1 to TLM8.5. The fraction TLM8.3 giving white crystalline powder to give yield TLM8.3* as shown in Scheme 3.31.



Scheme 3.31 Isolation scheme of compound TLM8.3*