CHAPTER III

METERIALS AND METHODS

Source and Authentication of the plant materials

The Fresh leaves of *C. hystrix, A. marmelos,* and *C. aurantifolia* were purchased from Local Markets in Chiang Mai Province, Thailand and *F. limonia* was purchased from Phitsanulok Province, Thailand. Voucher specimens of *C. hystrix* (QBG No. 36963)*, A. marmelos* (QBG No. 33029)*,* and *C. aurantifolia* (QBG No. 36964) are deposited in Queen Sirikit Botanic garden, Chiang Mai Province, Thailand and *F. limonia* (No.30252) is deposited in the Biology herbarium, Biology department, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand for references.

General Techniques

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 ($K_2S_2O_8$) were obtained from UNILAB (AU). Absolute ethanol (99%) was purchased from Merck (Damstadt, Germany).

2. Chromatography

2.1 Thin Layer Chromatography

Thin layer chromatography (TLC) was used to identify and evaluate the crude extracts and the fraction 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).

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. Spectroscopy

3.1 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 ($CDCl_3$), operating at 400 and 100 MHz, respectively. The chemical shifts were recorded in ppm by reference to TMS signal.

3.2 Refractometer

Refractive index of the oil was determined using a hand held refractometer (ATAGO N3, Atago Co. Ltd., Japan) at 28 °C.

3.3 The optical rotation

The optical rotation was measured with a Jasco Dip-370 Digital Polarimeter using sodium lamp as light source. The length of the Polarimeter cell was 1.0 dm and sample tube was 100 mn. About 0.1250 g of the essential oil was accurately weighed into volumetric flask and then the volume was made up to 25 mL with dichloromethane. The optical rotation of the essential oil (0.5g $100mL^{-1}$) was measured with Polarimeter at 29 °C.

Analysis of essential oil

The leaves of the plant was cleaned, cut into small pieces and accurately weighed, then subjected to water distillation in Clevenger-type apparatus for 3 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 by using GC and GC-MS.

The essential oil was analysed on a Hewlett-Packard GC 6890/ MSD 5973 on a HP-5 capillary column (30m × 0.25 mm, 0.25 μm film thickness). The injector temperature was 250 °C, the essential oil of 0.2 μL was injected, using split mode (split ratio, 250:1). Helium was the carrier gas at a flow rate of 1.0 mL min^{-1}. The oven temperature was initially at 50 °C (3 min) and was then gradually increased at a rate of 7 °C min^{-1} up to 250 °C and held for 9 min. The ion source temperature of the MS unit was 230 °C, and the mass spectra were recorded at an ionizing voltage of 70 eV. The constituents were further identified and authenticated using their MS data compared to the wiley7n.l mass spectral library, as well as by comparison of their retention indices with literature values (86-88).

Analysis of mineral metal ions

A standard stock solution 1000 ppm of mineral metal ions was prepared from a standard mineral metal ions solution (AAS standard, 1000 ppm Merck, Germany). Working standard solutions were prepared by appropriate dilution of this stock standard solution. All solutions were prepared with Milli-Q deionised water.

The leaves of *C. hystrix, F. limonia*, *A. marmelos*, *C. aurantifolia* were dried, ground. Each dried plant sample was subjected to wet digestion. About 5.000 g of each sample was accurately weighed into a Kjeldahl digestion flask. About 15 mL of nitric acid : perchloric acid (3:1) was added. The sample was digested by addition of small quantity (~ 1 ml) of nitric acid (68 % w/v) until organic matters were eliminated and white fumes were observed. Then the sample solution was filtered through Whatman No. 42 paper. The filtrate was made up to 50 mL with deionized water. The mineral metals ions calcium, manganese, zinc, copper, iron, magnesium and sodium) in the sample solutions were determined by atomic absorption spectrometry (AAS) using standard addition method (89).

The mineral metals ions were determined by means of an atomic absorption spectrometer (AAS; PerkinElmer AAnalyst 100, USA) using a mixture of acetylene-air flame and hollow cathode lamps as light sources. The instrumental parameters are presented in Table 3.1.

Table 3.1 AAS parameters

Mineral metal	AAS parameters		
	Wavelength (nm)	Lamp current (mA)	Integration time (s)
Ca	422.67	10	3
Na	589.0	10	3
Fe	248.33	10	3
Mg	285.21	10	3
Mn	279.48	10	3
Zn	213.68	7	3
Cu	324.75	10	3

Antioxidant activity

The antioxidant activities of the crude medicinal plant extracts and the essential oils were determined by ABTS and DPPH methods.

1. ABTS Method

The antioxidant activity of the essential oil was investigated using the ABTS radical cation scavenging assay which was conducted according to the method of Roberta *et al.* (90), compared with Trolox standards (concentration rang 0.5-2.5 mM). For the ABTS assay, 20 μL of essential oil (0.1 g mL^{-1}) 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 5 min incubation 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 following way:

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

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, vitamin C, quercetin for the standard reference data.

2. DPPH Method

A stock solution (5.0×10^{-4} mol L^{-1}) of $DPPH^{\bullet}$ was prepared by dissolving the appropriate amount (10.0 mg) in 50 ml ethanol. This solution was kept at 4 ^{o}C and protected from light, and it was stable during a week. The $DPPH^{\bullet}$ working solution containing 1.0×10^{-4} mol L^{-1} was prepared by measuring 50 mL of the stock solutions, the volume was made up to 200 mL with ethanol. This working solution was freshly prepared daily and protected from light (91).

The test sample (20 µL) was added to 180 µL of 10 µL DPPH$^{\bullet}$ solution in a 96-well microtiter plate. The reaction mixture was incubated at 37 ^{o}C for 30 min, and then the absorbance of each well was measured at 540 nm. The DPPH$^{\bullet}$ solution was used as negative control. Trolox, vitamin C and quercetin were used as reference standards. For 50% inhibitory concentration (IC_{50}) evaluation of the essential oil, a graph showing concentration versus %DPPH$^{\bullet}$ reduction was plotted. The IC_{50} was then calculated from the calibration curve and activity was expressed as the percentage DPPH scavenging relative to the control using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Biological Activity

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 (92). *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 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 measurements were made basically from the edge of the zone to the edge of the well.

2. Anticancer activity

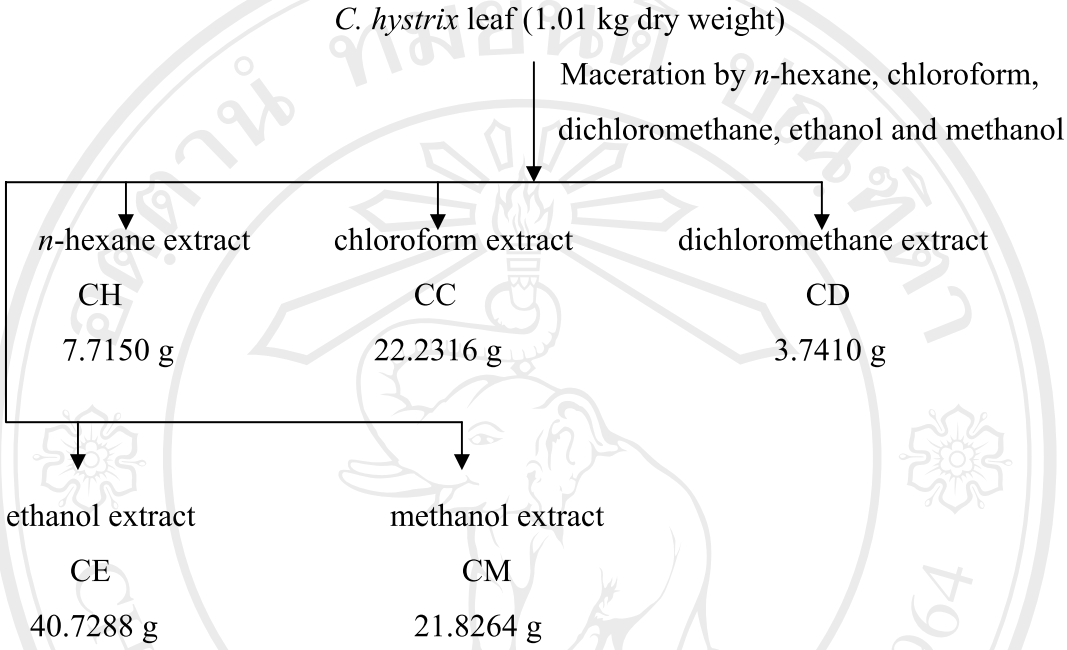
The anticancer activity of the essential oil or each crude medicinal plant extract was performed by using KB (Oral Cavity cancer), MCF 7 (Human breast adenocarcinoma) and NCI-H 187 (Human small cell lung carcinoma) and determined by resazurin microplate assay (REMA) following a modified method of the use of a fluorescent dye for mammalian cell cytotoxicity according to Brien *et al*. (2000) (93). Ellipticine and doxorubicin were used as positive controls. DMSO and sterile distilled water were used as negative controls. Cells at a logarithmic growth phase were harvested and diluted to10^5 cells/ml in fresh medium and gently mixed. Test compounds were diluted in culture medium in a ratio of 1:2 giving 8 concentrations. Five µl of the test sample and 45 µl of cells were put into 96 well microtiter plates with a total volume of 50 µl/well. Plates were incubated at 37 °C, 5% CO2, for 72 h for KB and MCF7 and 5 days for NCI-H187. After the incubation periods, 12.5 µl of resazurin solution was added to each well and the plates were incubated at 37 °C for 4 h. The plates were then processed for optical density absorbance analysis using a microplate reader at dual wavelengths of 530 and 590 nm.

Extraction

The leaves of *C. hystrix, F. limonia*, *A. marmelos*, *C. aurantifolia* were dried, ground and extracted with hexane, chloroform, dichloromethane, ethanol and methanol by maceration. The extracts were filtered through Whatman No. 5 paper. Removal of the solvent with rotary evaporator to give crude extracts and were kept in the dark at 4 °C until further investigation.

Isolation of *C. hystrix*

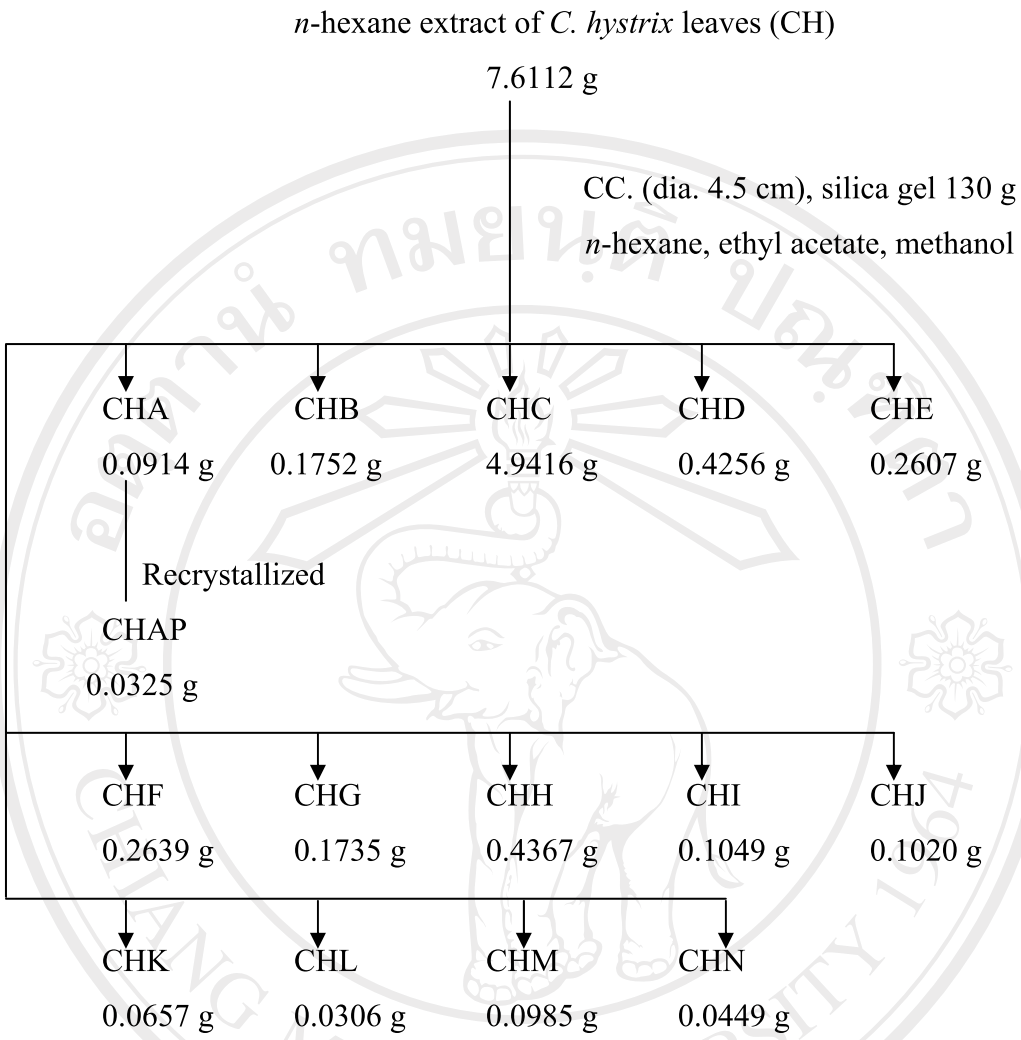
The extraction sequence is shown in Scheme 3.1



Scheme 3.1 Extraction scheme of *C. hystrix*

1. Isolation the *n*-hexane extract of *C. hystrix* leaves

The *n*-hexane extract, CH (7.6112 g), was fractionation by column chromatography (diameter 4.5 cm) with silica gel 60 (130 g) and the following solvents system of *n*-hexane, ethyl acetate and methanol respectively. Eighty-one fractions of the eluate (100 ml each) were collected and evaluated by TLC analysis patterns giving 12 fractions, CHA to CHN.



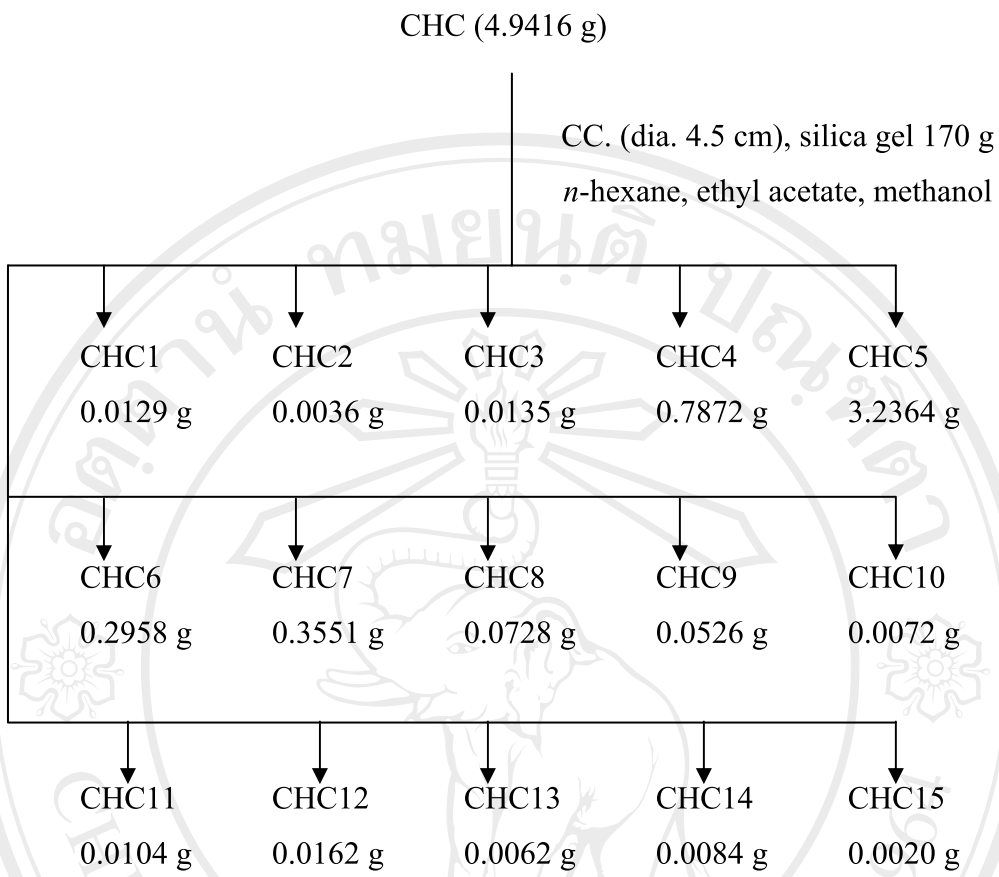
Scheme 3.2 Isolation scheme of *n*-hexane extract of *C. hystrix* leaves (CH)

Isolation of CHAP

A portion of fraction CHA (0.0941 g) was further purified by crystallization to yield white crystalline 0.0325 g.

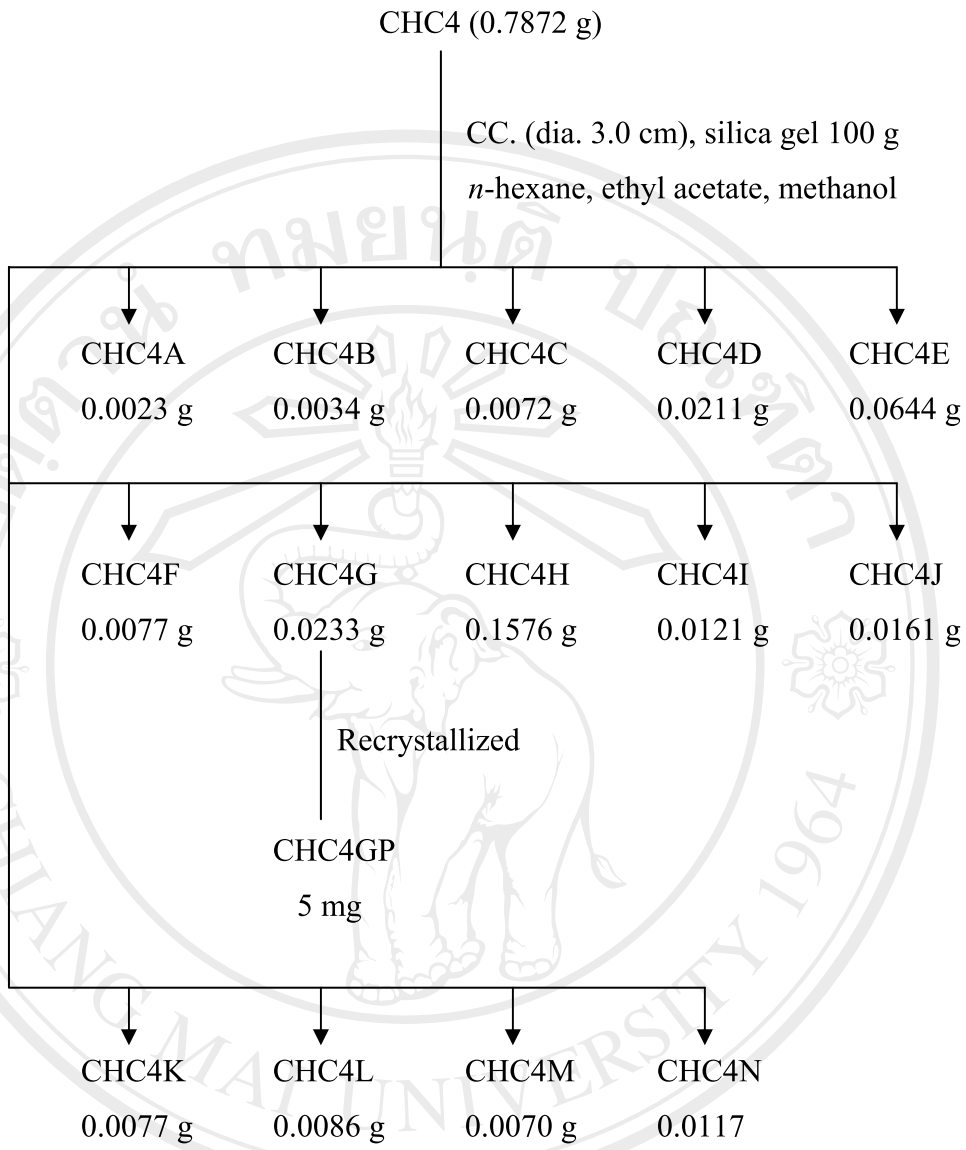
Isolation of CHC4GP

The fraction CHC (4.9416 g) was repeatedly purified by silica gel 60 (170 g) eluting with *n*-hexane, ethyl acetate and methanol. Each 100 ml fraction was collected and combined according to their TLC patterns 15 fractions, CHC1 to CHC15 as shown in Scheme 3.2.



Scheme 3.3 Isolation scheme of compound CHC4

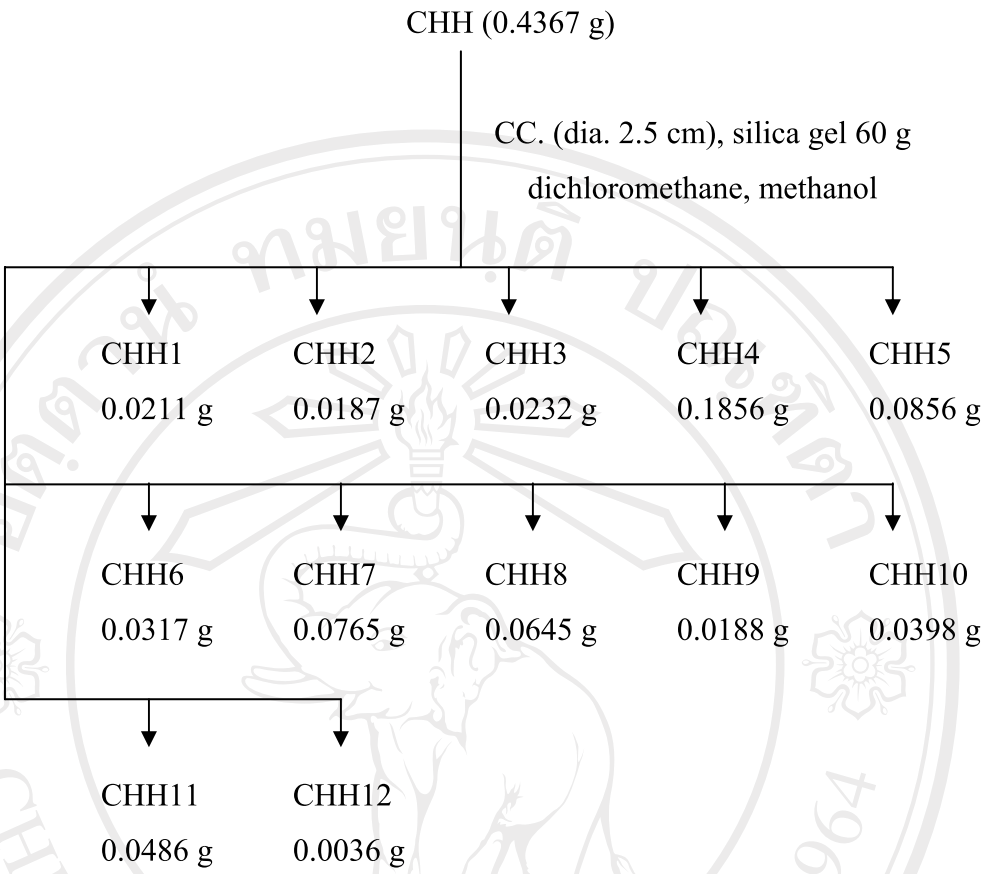
The fraction CHC4 (0.7872 g) was rechromatographed with silica gel 60 (100 g) and eluted with *n*-hexane, ethyl acetate and methanol. Each 50 ml fraction was collected and combined according to their TLC patterns 14 fractions, CHC4A to CHC4N as shown in Scheme 3.4. Among these, a potion of CHC4G was further recrystallized with ethyl acetate and methanol to obtain CHC4GP 5 mg as white crystalline powder.



Scheme 3.4 Isolation scheme of compound CHC4GP

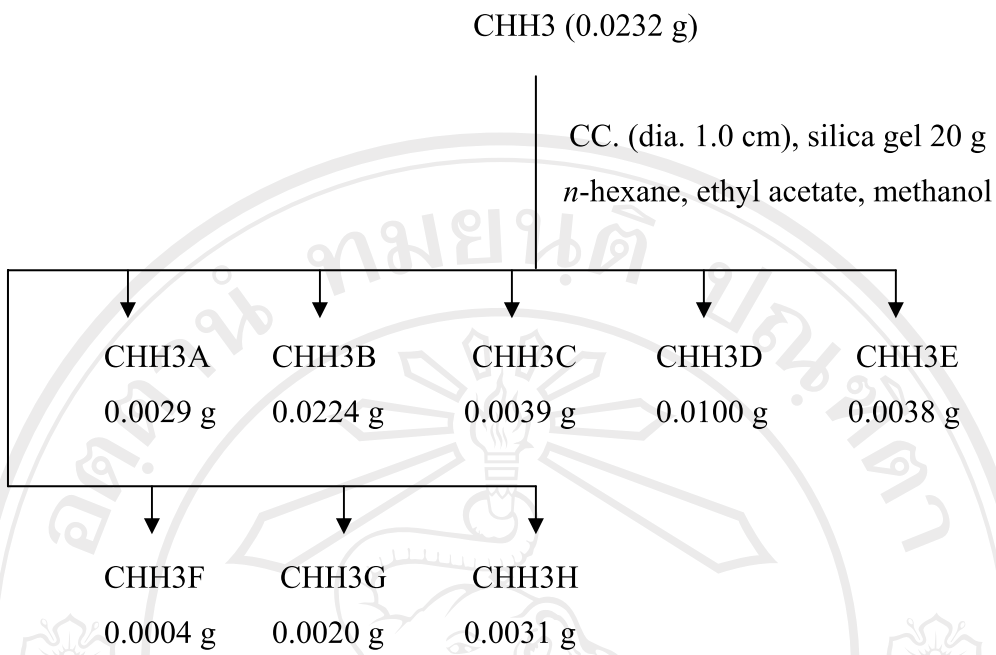
Isolation of CHH3G

The fraction CHH (0.4367 g) from Scheme 3.2 was separate by column chromatography (diameter 2.5 cm) with silica gel 60 (60 g) and the following solvents system of *n*-hexane, ethyl acetate and methanol. Fifty-five fractions of the eluate (50 ml each) were collected and evaluated by TLC chromatograms giving 12 fractions, CHH1 to CHH12 as shown in Scheme 3.5.



Scheme 3.5 Isolation scheme of compound CHH3

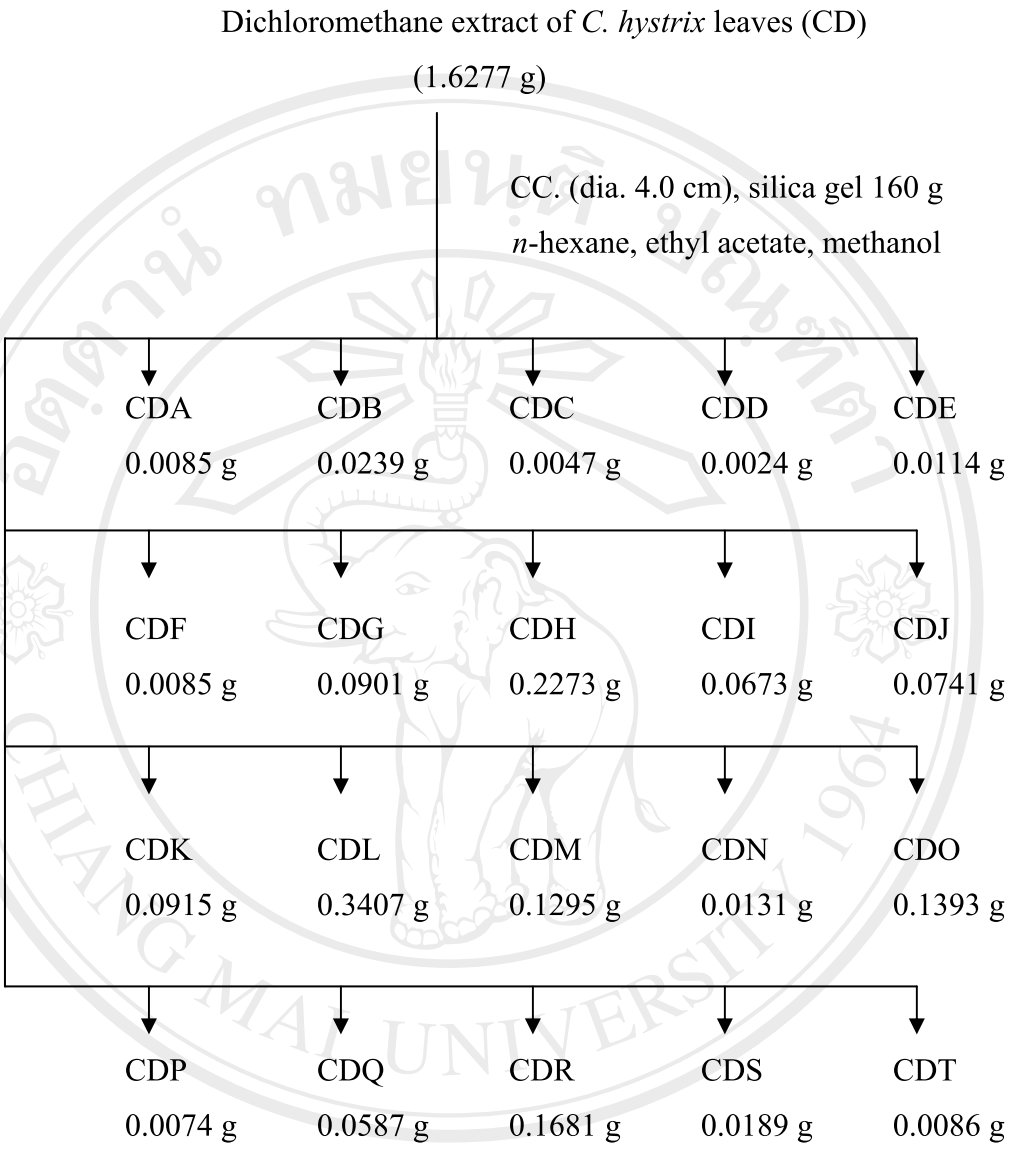
The fraction CHH3 (0.0232 g) was rechromatographed with silica gel 60 (20 g) eluting with *n*-hexane, ethyl acetate and methanol. Each 2 ml fraction was collected and combined according to their TLC patterns 8 fractions, CHH3A to CHC4H as shown in Scheme 3.6. Compound CHH3GP was obtained as yellow sticky (2 mg).



Scheme 3.6 Isolation scheme of compound CHH3G

2. Isolation of the dichloromethane extract of *C. hystrix* leaves

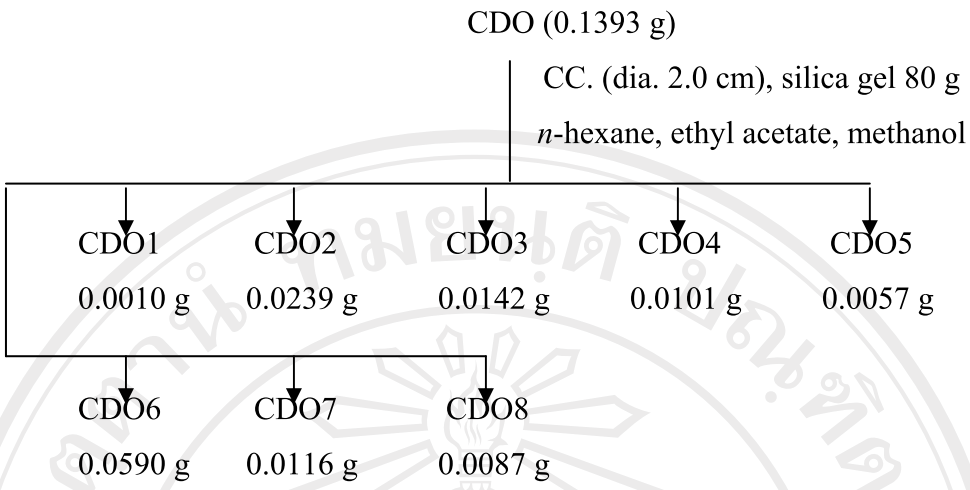
The dichloromethane extract, CD (1.6277 g), was initiated by open column chromatography (diameter 4.0 cm) on silica gel 60 (160 g) using the following solvents system of *n*-hexane, ethyl acetate and methanol. Eighty-six fractions of the eluate (100 ml each) were collected and evaluated by TLC analysis patterns giving 20 fractions, CDA to CDT as shown in Scheme 3.7. Compound CHD (0.2273 g) was obtained as yellow sticky liquid.



Scheme 3.7 Isolation scheme of dichloromethane extract of *C. hystrix* leaves (CD)

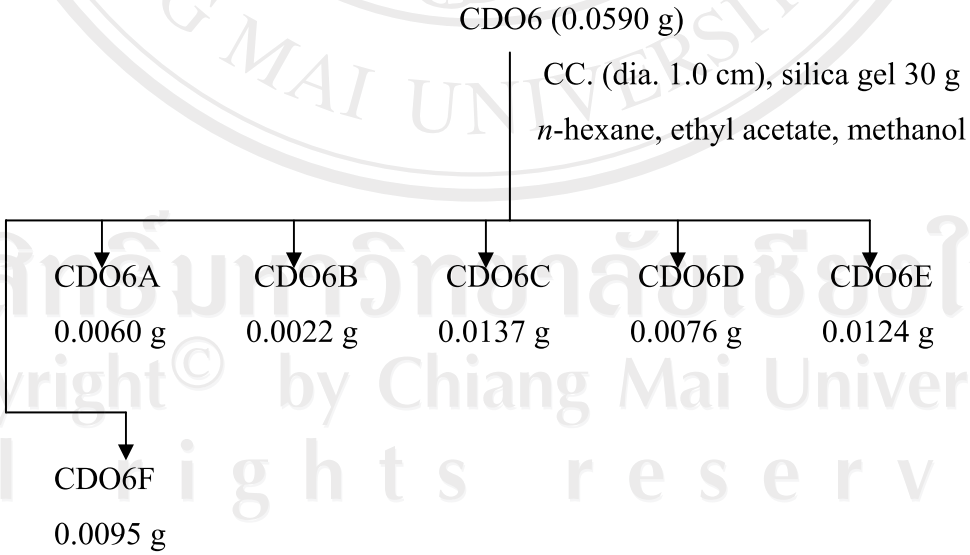
2.1 Isolation of CDO6B

The fraction CDO (0.1393 g) was fractonation by column chromatography (diameter 2.0 cm) with silica gel 60 (80 g) and the following solvents system of *n*-hexane, ethyl acetate and methanol. Fifty-nine fractions of the eluate (5 ml each) were collected and evaluated by TLC chromatograms giving 8 fractions, CDO1 to CCDO8 as shown in Scheme 3.8.



Scheme 3.8 Isolation scheme of compound CDO6

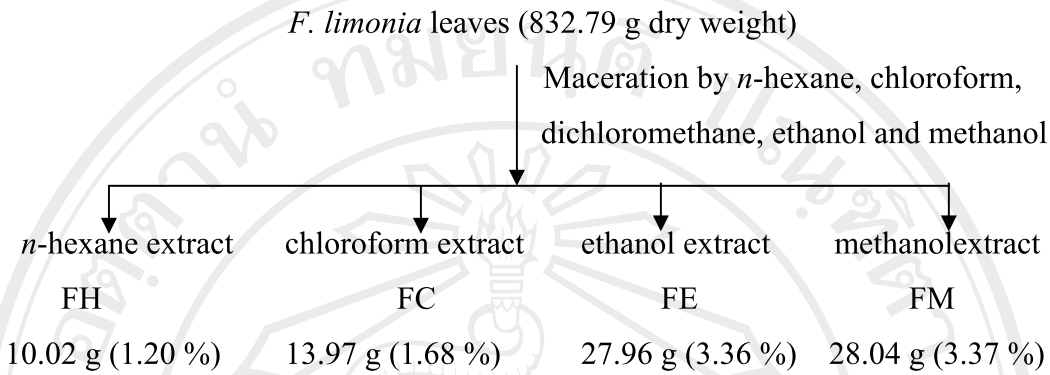
The fraction CDO6 (0.0590 g) was rechromatographed with silica gel 60 (20 g) eluting with *n*-hexane, ethyl acetate and methanol. Each 2 ml fraction was collected and combined according to their TLC patterns 8 fractions, CDO6A to CDO6F as shown in Scheme 3.9. Among these, a potion of CDO6B was further recrystallized with ethyl acetate and methanol to obtain CDO6B 2.2 mg as yellow sticky.



Scheme 3.9 Isolation scheme of compound CDO6B

Isolation of *F. limonia* constituent

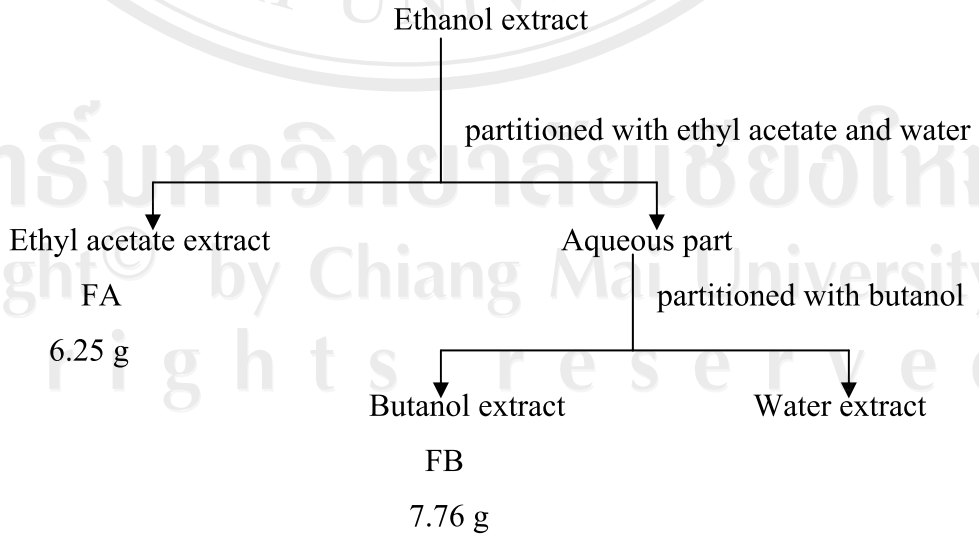
The extraction sequence is shown in Scheme 3.10



Scheme 3.10 Extraction of *F. limonia* leaves

1. Isolation of the ethanol extract of *F. limonia*

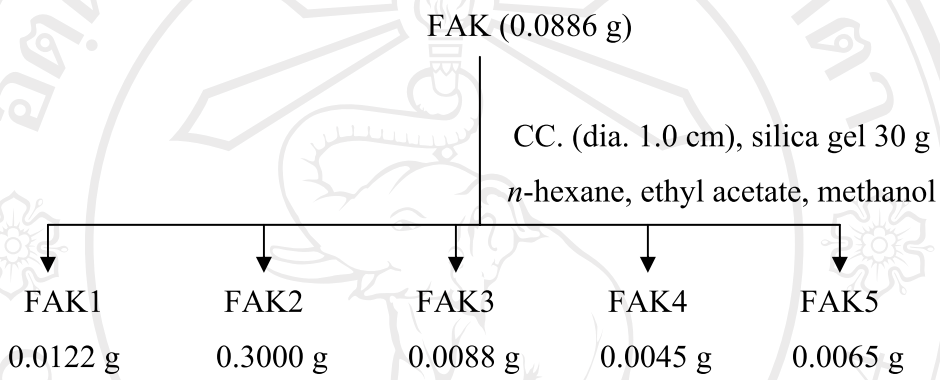
The ethanol extract 27.96 g was further partitioned with ethyl acetate and water (three times, approx. 1:1, 500 mL each). The organic layer was then separated and concentrated under reduced pressure to yield the ethyl acetate extract. The aqueous layer was further partitioned with butanol (three times, approx. 300 mL each) and then concentrated under reduced pressure to yield the butanol extract.



Scheme 3.11 Extraction scheme of ethyl acetate extract

Isolation of FAK1

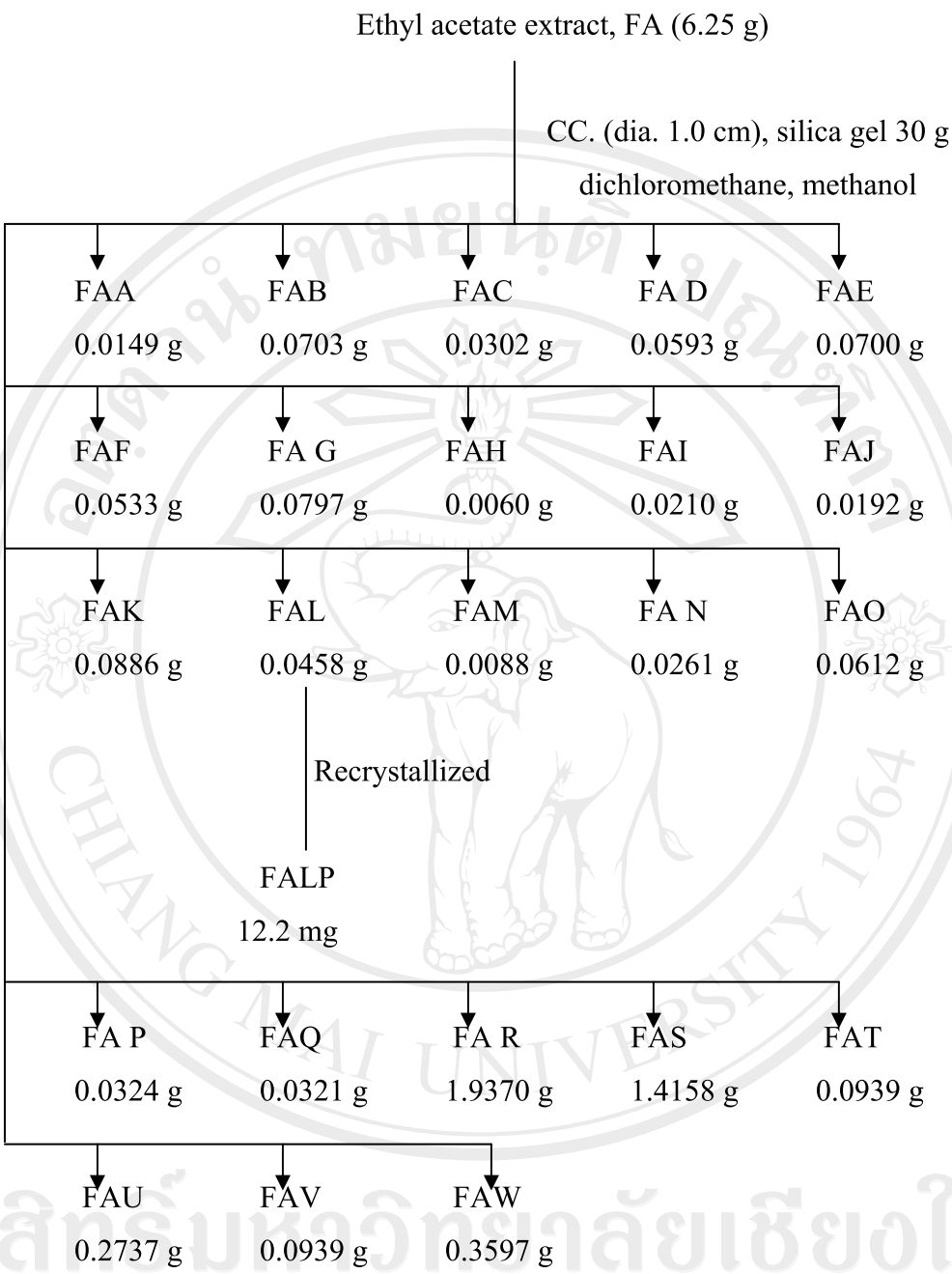
The fraction FAK (0.0886 g) was separated by column chromatography (diameter 1.0 cm) with silica gel 60 (30 g) and the following solvents system of *n*-hexane, ethyl acetate and methanol. Fifty-six fractions of eluate (2 ml each) were collected and evaluated by TLC chromatograms giving 5 fractions, FAK1 to FAK5 as shown in Scheme 3.12. Fraction FAK1 0.0122 g was obtained as yellow sticky.



Scheme 3.12 Isolation scheme of compound FAK1

Isolation of FALP

The ethyl acetate extract, FA (6.25 g), was rechromatographed with silica gel 60 (130 g) eluting with dichloromethane and methanol. Each 50 ml fraction was collected and combined according to their TLC patterns of 23 fractions, FAA to FAW as shown in Scheme 3.13. Among these, a potion of FAL was further recrystallized with dichloromethane and methanol to obtain AFLP 12.2 mg as white needles.



Scheme 3.13 Isolation scheme of compound FALP