CHAPTER III

EXPERIMENTAL

Source and Authentication of the Plant Materials

The aerial parts and subterranean parts of *C. odorata* were collected at Naresuan University, Phitsanulok, Thailand. The plant was identified as *Chromolaena odorata* by comparison with the herbarium specimens at the herbarium of the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand. The voucher specimens of this plant are kept at the Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University.

General Techniques

1. Chromatography

1.1 Analytical Thin Layer Chromatography

Adsorbent : Silica gel 60 F-254 precoated aluminium plate (E.Merck)
Silica gel 60 C-18 F-254 precoated aluminium plate (E.Merck)

Layer thickness : 250 μm.

Technique : One way, ascending.

Distance : 5 and 17 cm.

Temperature : Room temperature 25-30°C.

Detection : 1). Visual detection under daylight.
2). Ultraviolet light at the wavelengths of 254 and 365 nm.
3). Visual detection after exposed to iodine vapour.
4). Spraying with anisaldehyde-sulfuric acid solution and heating at 100-110°C for a few minutes.

1.2 Preparative Thin Layer Chromatography

Adsorbent :Silica gel 60 F-254 precoated glass plate (E.Merck)

Layer thickness : 1.0 and 2.0 mm.

Technique : One way, ascending.

Distance : 17 cm.

Temperature : Room temperature 25-30°C.

Detection : 1). Visual detection under daylight.

2). Ultraviolet light at the wavelengths of 254 and 365 nm.

3). Spraying with anisaldehyde-sulfuric acid solution and heating at 100-110°C for a few minutes.

1.3 Column Chromatography

1.3.1 Vacuum Column Chromatography

(Quick Column Chromatography)

Adsorbent : Silica gel 60 (No.9385) particle size 0.040-0.063 mm. (230-400 mesh ASTM).

Packing : Adsorbent was dry-packed into the column. The column was tapped and pressed down from the top of the adsorbent to insure tightly packing. The final height of the adsorbent was 5-7 cm.

Sample loading : The sample was dissolved in a small volume of dichloromethane and triturated with sufficient quantity of the adsorbent. The mixture was dried and put into the top of column.

Examination of elutes : Fractions were examined by TLC under ultraviolet light at wavelengths of 254 and 365 nm and by exposing to iodine vapour and anisaldehyde-sulfuric acid solution, respectively.

1.3.2 Flash Column Chromatography

Adsorbent : Silica gel 60 (No. 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM).

Packing : Adsorbent was wet-packed after being suspended in the eluent. The slurry of adsorbent was poured into the column, tapped and pressed down under air pump pressure, then allowed to settle overnight.

Sample loading : The sample was dissolved in a small volume of the eluent and loaded onto the top of the column.

Examination of eluates : Fractions were examined in the same manner as described in section 1.3.1

1.3.3 Gel Filtration Chromatography

Adsorbent : Sephadex LH-20.

Packing : The adsorbent was suspended in the eluent and left to swell for 24 hrs (before using), then poured into the column and allowed to settle tightly.

Sample loading : The sample was dissolved in a small volume of the eluent and loaded onto the top of the column.

Examination of eluates : Fractions were examined in the same manner as described in the section 1.3.1.

2. Spectroscopy

2.1 Mass spectra (MS)

The electron impact mass spectra (eims) of the isolated compounds were obtained on a Shimadzu QP 5000 GC/MS. The high resolution chemical ionization mass spectra (hrcims) and electron impact mass spectra (hreims) were recorded with a VG Autospec-oa-TOF high resolution mass spectrometer (Department of Chemistry, Faculty of Science, University of Wollongong, Australia).

2.2 Infrared (IR) absorption spectra

The spectra were obtained on a Nicolet 510 FT-IR spectrophotometer (Department of Chemistry, Faculty of Science, Chiang Mai University).

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

The nmr spectra were measured with a Varian Mercury VX 300 MHz spectrometer; 300 MHz for ^{1}H-nmr and 75 MHz for ^{13}C-nmr (Department of Chemistry, Faculty of Science, University of Wollongong, Australia).

2.4 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 0.1 dm and the volume of the cell was 1.0 ml.

3. Analysis of essential oil

The essential oil was analyzed by GC and GC/MS. The GC-FID analysis was performed on a Varian 3700 gas chromatography coupled to Shimadzu C-R3A integrator. Separation was achieved using hydrogen as carrier gas (ca. 1 ml/min) with a fused silica capillary column (25QC/BP5) obtained from SGE, Australia (25 m x 0.25 mm i.d., 0.25 μm film thickness). Injector and detector temperatures were 260 °C and 280 °C, respectively; oven temperature programme, 3 min isothermal at 40 °C, then at 4 °C/min to 280 °C (5 min isothermal). Programmed temperature Kovats retention indices (RI) were obtained by GC-FID analysis of an aliquot of the essential oil spiked with an *n*-alkane mixture containing each homologue from *n*-C_8 to *n*-C_{30}. GC-MS analysis was performed in both electron impact (EI, 70 eV) and chemical ionization (CI, *iso*-butane reagent gas) mode with a Shimadzu QP-5000 system, using helium as the carrier gas and a capillary column (DB-5MS) supplied by J&W Scientific, USA (30 m x 0.32 mm i.d., 0.25 μm film thickness).Gas chromatographic conditions were as above, with a helium flow of 1.4 ml/min.

4. Solvents

All of organic solvents used in this work, excluding the deuterated solvents for nmr spectra, were commercial grade that had to be redistilled prior to use.

Bioactivity Determination

1. Antimicrobial Activity

The determination of antimicrobial activity was performed by the disc diffusion method.

1.1 Microorganisms

The representative microorganisms were kindly provided by the Head of the Department of Microbiology, Faculty of Medical Science, Naresuan University. Three species of microorganisms were employed in the determination :

1). *Staphylococcus aureus* ATCC6538 representing Gram positive bacteria.

2). *Escherichia coli* ATCC6633 representing Gram negative bacteria.

3). *Candida albicans* representing fungi.

All of these microorganisms were subcultured in suitable media prior to use in order to intensify their activities. The incubation was carried out at 37°C, 24 hrs for bacteria and 48 hrs for fungi.

1.2 Media

1.2.1 Trypticase Soy Agar (TSA)

The medium used was BBL® Trypticase Soy Agar (Becton-Dickinson Microbiology System).

Formula per liter of purified water	
Pancreatic digest of casein	15.0 g
Papaic digest of casein	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

Final pH 7.3±0.2

The formula powder (40 g) was dispersed in 1 liter of purified water and stirred until well-suspended. The agar suspension, then, was heated to complete dissolution. The medium was sterilized by autoclaving at 121°C for 15 minutes. This medium was used for inoculating the bacteria during the assay.

1.2.2 Sabouraud Dextrose Agar (SDA)

The medium used was BBL® Sabouraud Dextrose Agar (Becton-Dickinson Microbiology System)

Formula per liter of purified water	
Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0 g
Dextrose	40.0 g
Agar	15.0 g
Final pH 5.6±0.2	

The formula powder (65 g) was dispersed in purified water and throughly mixed. The suspension was then boiled to completely dissolve the ingredients. The medium was used in subculturing and inoculating of yeast during the assay.

1.3 Sample preparation

The sample (solid extract) was dissolved in methanol and diluted to a concentration of 5 mg/ml. Twenty microliters of this solution was transferred to a 6.0 mm disc (Whatman® AA disc for antibiotic assay) and allowed to dry. Thus, the final concentration was 1 mg/disc. The 100% essential oil (20 µl) was transferred to a 6.0 mm disc and, then, the disc was placed on the medium. The determination was made in duplicate.

1.4 Bioassay

This bioassay was carried out by using aseptic technique. All glassware and materials had to be sterilized before use with an autoclave at 100°C for 20 minutes. Each microorganism was suspended in sterilized 0.85% saline solution and spread on the surface medium plate.

The sample discs were put on the microbe-spread medium plates and, then, incubated at 37°C for 24 hours for bacteria, or 48 hours for yeast. After incubation, if the test sample had antimicrobial activity, a clear zone appeared around the disc. The diameter of inhibition zone was measured and reported in the scale of millimeter.

2. Brine Shrimp Lethality Activity

The bioassay was applied using the microwell cytotoxicity assay method (125). This method is recognized as a simple bioassay for natural product researches with the advantages of being rapid, inexpensive and simple (no aseptic techniques are required). Activities of a broad range of known active compounds are manifested in toxicity to brine shrimp, a tiny crustacean. Brine shrimps have been utilized in various bioassay systems, for example, in the analysis of pesticide residues, mycotoxins, stream pollutants, anesthetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicants in marine environments (126). This *in vivo* lethality test has also been successfully employed for bioassay-guide fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba* (127), *cis*-annonacin from *Annona muricata* (128) and *ent*-kaur-16-en-19-oic acid from *Elaeoselinum foetidum* (129).

2.1 Brine shrimp

The eggs of brine shrimp (*Artemia salina*, Sanders® USA) were hatched in a shallow rectangular box filled with artificial sea water prepared from commercial sea salt (Aqua Marine, Thailand) 40 g/l and supplement with 6 mg/l dried yeast. The two unequal compartments of the plastic box with several holes on the divider was used for hatching. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 36 hours incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the light side whereas their shells were left in the other side.

2.2 Sample preparation

The test sample was dissolved in DMSO (5% of total volume of stock solution) and diluted with sea water to make a concentration of 2 mg/ml. The sample stock solution was diluted with an appropriate volume of artificial sea water and transferred to the microwells. The samples were tested at concentrations of 10, 100 and 1,000 μg/ml. All determinations were made in triplicate so that the total number of brine shrimps in each concentration was about thirty. Control microwells with DMSO were included in each experiment.

2.3 Bioassay

A suspension of nauplii (100 μl) containing about 10 organisms was transferred to each well. The plates were covered and incubated at room temperature (25-29°C) for 24 hours. Plates were then examined under the binocular stereomicroscope and the numbers of dead (non-motile) nauplii in each well were counted. One hundred microliters of methanol were then added to each well to immobilize the nauplii and after 15 minutes the total numbers of brine shrimp in each well were counted. The number of dead brine shrimps in the test wells was subtracted from those in the control wells. Analysis of the data was performed by probit analysis on a Finney computer program to determine the lethal concentration to half of the test organisms (LD_{50}).

3. Anti-inflammatory activity

The samples were first tested for anti-COX-2 activity at the concentration of 10^{-5} g/ml. Any positive samples which exhibited more than 50% of enzyme inhibition, were further tested for anti-COX-1 activity at the concentrations of 10^{-5} and 10^{-7} g/ml. If the sample showed selective inhibition of COX-2 over COX-1, then the IC_{50} ratios of COX-1/COX-2 were determined.

3.1 Materials

All tissue culture components were purchased from Gibco BRL (Gaithersburg, MD). Aspirin and calcium ionophore A23187 were purchased from Sigma (St. Louis, MO). ^{3}H-PGE_2 was from NEN Life Science (Boston, MA) and anti-PGE2 antibody was from Upstate Biotechnology (Upstate, NY) or Sigma (St. Louis, MO).

3.2 Cell culture and treatment

Immortalized mouse PGH-1 and PGH-2 null cells at the concentration of $1x10^5$ cells/ml in incomplete Dubelcco's Modified Eagle Meduim (DMEM) high glucose supplemented with hygromycin B (200 μg/ml), non essential amino acid (0.1 mM), L-glutamine (50 mg/L), ascorbic acid (0.05 mg/ml) and 10% fetal calf serum (FCS) were seeded into 96-well flat bottom tissue culture plates (83 μl/well). Cells were incubated 37°C in humidified incubator with 5% CO_2 for 72 hours, then washed with DMEM medium without FCS and preincubated for 30 minutes with 83 μl of serum-free DMEM medium containing vehicle or drugs. Following the preincubation period, the medium was removed and cells were immediately treated with serum-free medium containing vehicle or drugs and 20 μM arachidonic acid (AA) or 2 μM A23187 for 30 min. Culture supernatants were then collected from wells and analyzed for PGE_2 concentrations by radioimmunoassay (RIA).

3.3 PGE_2 Measurement

The RIA method used for measuring PGE_2 concentrations in the culture supernatant is based on the competition between PGE_2 in the samples and ^{3}H labeled PGE_2 for anti-PGE_2 antibody binding sites. The assay was performed on ice as following. To 1.5 ml microcentrifuge tubes 50 μl of culture supernatant, diluted 1:10 in DMEM or, for blank and zero % binding tube, 50 μl of DMEM were added. Then, 50 μl of anti-PGE_2 antibody in RIA buffer (0.1 mM phosphate buffer, pH 7.4, containing 0.9% sodium chloride, 0.1% sodium azide and 0.1% gelatin) was added to every tube accept for the blanks, in which 50 μl RIA buffer was added. Subsequently,

50 µl of ^{3}H-PGE_2 (1.12 µci/ml), was added to each tube, vortexed briefly and incubated overnight at 4 °C. Then, 100 µl at 2% charcoal-dextran suspension in RIA buffer was added to each tube. After 15 minutes incubation on ice, the tube were centrifuged at 3800 rpm at 4 °C for 10 minutes. Supernatants were then transferred to new 1.5 ml microcentrifuge tubes containing liquid scintillation cocktail, vortexed and counted for radioactivity. The resulting radioactive counts were used to calculate % binding of ^{3}H-PGE_2, which were then used for the estimation of PGE_2 concentrations from standard curves (130-131).

4. Cytotoxic Activity

The cytotoxicity tests were performed on KB (Human epidermoid carcinoma of cavity, ATCC CCL-17) and BC (Breast cancer cell line) which were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content according to Skehan *et al.* (132). Elliptine and doxorubicin were used as positive controls. DMSO was used as a negative control. Briefly, cells at a logarithimic growth phase were harvested and diluted to 10^5 cells/ml with fresh medium and gently mixed. Test compounds were diluted in distilled water and put into microtiter plates in a total volume of 200 µl. Plates were incubated at 37°C, 5% CO_2 for 72 hours. After the incubation period, cells were fixed by 50% trichloroacetic acid. The plates were incubated at 4°C for 30 minutes, and then the plates were washed with tap water and then air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B dissolved in 1% acetic acid for 30 minutes. After the staining period, SRB was removed with 1% acetic acid. Plates were air-dried before bound dye was solubilized with 10 mM Tris base for 5 minutes on a shaker. The optical density was read in a microtiter plate reader at a wavelength of 510 nm.

5. Antimalarial activity

Plasmodium falciparum (K1, multi drug resistant strain) was *in vitro* cultivated in RPMI 1640 medium containing 20 mM HEPES (N-2-hydroxy ethylpiperazine-N′-2-ethanesulfonic acid), 32 mM $NaHCO_3$ and 10% heat activated

human serum with 3% erythrocytes and incubated at 37°C in an incubator with 3% CO_2. Cultures were diluted with fresh medium and erythrocytes every day according to cell growth (133). Quantitative assessment of antimalarial activity *in vitro* was determined by microculture radioisotope techniques based upon the methods described by Desjardins *et al.* (134). Briefly, a mixture of 200 µl of 1.5% erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 µl of the medium containing a test sample dissolved in 1% DMSO (0.1% final concentration) for 24 hours employing the incubation conditions described above. Subsequently, 25 µl of [^{3}H] hypoxanthine (Amersham, USA) in culture medium (10 µCi) was added to each well and plates were incubated for an additional 24 hours. Levels of incorporated radioactively labeled hypoxanthine indicating parasite growth were determined using the Top Count microplate scintillation counter (Packard, USA). Inhibition concentration (IC_{50}) represents the concentration which indicates 50% reduction in parasite growth. The standard sample was dihydroartemisinine (DHA).

6. Anti-Herpes simplex virus type 1

Anti-herpes simplex virus type 1 (HSV-1) activity of plant or microbial extracts and synthetic compounds was tested against HSV-1 strain ATCC VR 260, using a colorimetric microtiter plate assay that determines host cell growth by measuring cellular protein content, as described by Skehan *et al.* (132). The growth of host cells, vero cell line ATCC CCL-81, infected with virus and treated with the plant extract (in DMSO) was compared with control cells, infected with virus only. Acyclovir and DMSO were used as positive and negative controls, respectively. The extracts were tested at non-cytotoxic concentrations (inhibit cell growth <50%).The extracts that inhibited virus more than 50% were tested further to determine the concentrations that inhibited viral activity by 50% (IC_{50}).

7. Antituberculosis activity

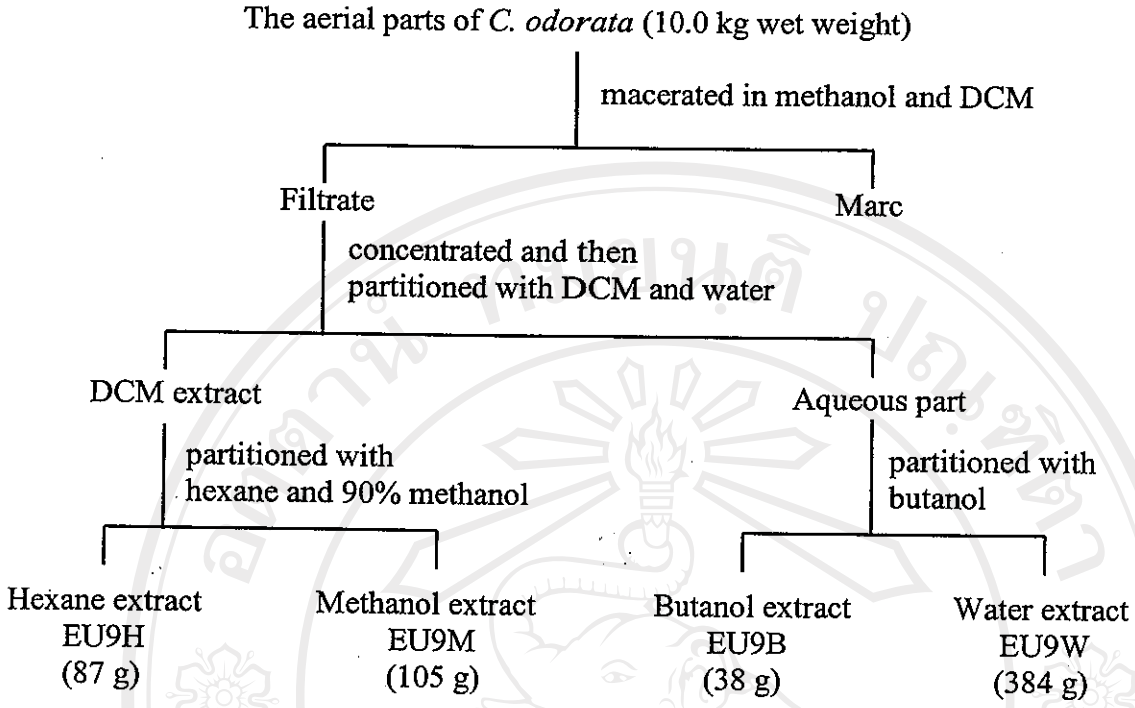
Antimicrobial susceptibility testing was performed in 96-well microplates (135). Outer perimeter wells were filled with sterile water to prevent dehydration in

experimental wells. Initial screened-sample dilutions were prepared in either dimethyl sulfoxide or distilled deionized water. The dissolved-screened samples were then diluted by Middlebrook 7H9 media containing 0.2% v/v glycerol and 1.0 gm/L casitone (7H9GC), and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC in the microplates. Frozen inocula (*M. tuberculosis* $H_{37}Ra$ was grown in 100 ml of 7H9GC containing 0.005% Tween 80. Culture was incubated in 500 ml plastic flask on a rotary shaker at 200 rpm and 37°C until they reached an optical density of 0.4-0.5 at 550 nm. Bacteria were washed and suspended in 20 ml of phosphate-buffered saline and passed through an 8-m-pore-size filter to eliminate clumps. The filtrates were aliquoted, stored at -80°C) were diluted 1: 100 in 7H9GC. Addition of 0.1 ml to the well resulted in final bacteral titers of about $5x10^4$ CFU/ml. Wells containing sample only were used to determine whether the tested-samples themselves could reduce the dye or not. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 6 of incubation, 20 µl of Alamar Blue solution and 12.5 µl of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 24 hours for a color change from blue to pink. If the B wells became pink by 24 hours, reagent was added to the entire plate. If the well remained blue, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24h post-reagent addition. Visual MICs were defined as the lowest concentration of sample that prevented a color change.

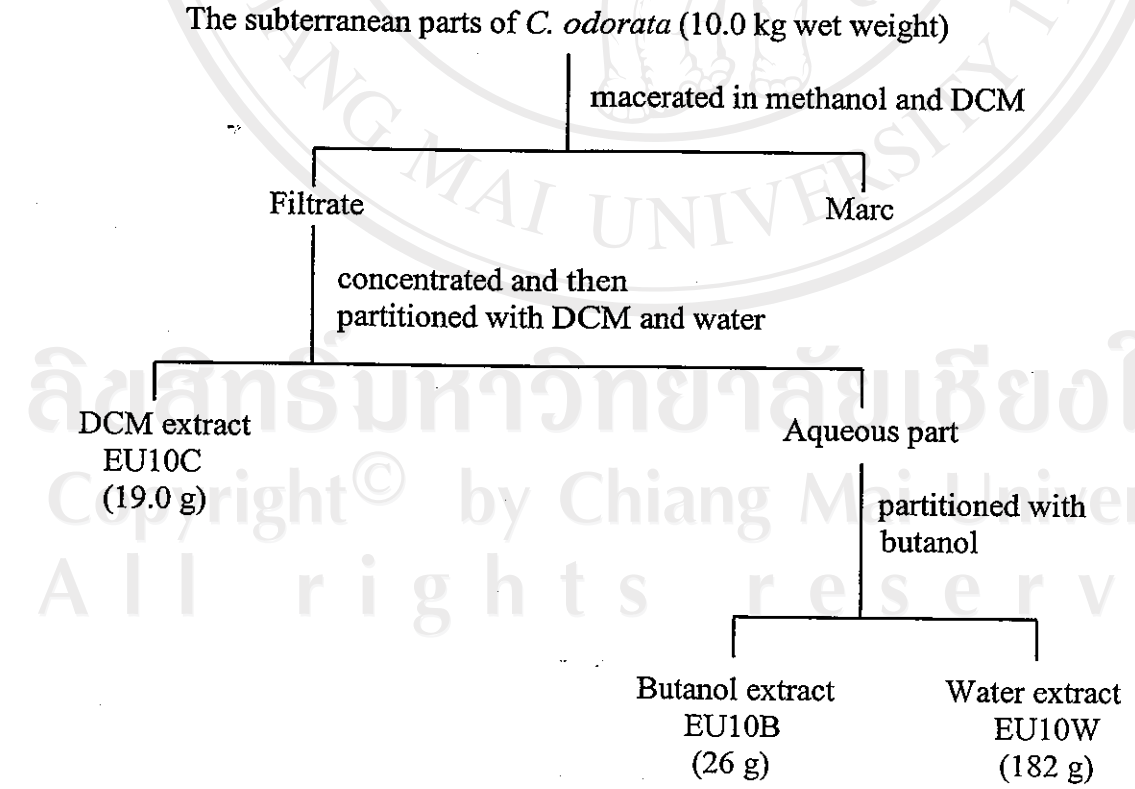
Extraction and Isolation

The aerial parts (coded EU9) and subterranean parts (coded EU10), each 10.0 kg wet weight, were cut or milled into small pieces and were repeatedly macerated in methanol 2 times (five days each) and once in dichloromethane (DCM) in order to increase the solubilization of non-polar components. The filtrates were then pooled and evaporated under reduced pressure at temperatures not over 50^{o}C to obtain crude extracts as syrupy masses. Both crude extracts, which were obtained from the aerial and subterranean parts, were further partitioned with dichloromethane and water (approx. 1:1, 1.5 L each). The organic layer was separated and concentrated under reduced pressure to yield the dichloromethane extract. The upper aqueous layer was further partitioned with butanol (approx. 500 ml) and then concentrated under reduced pressure to yield butanol and water extracts.

The dichloromethane extract from the aerial parts was dissolved in methanol (approx. 1,000 ml) and diluted with purified water to make 90%metahnol solution. It was then partitioned with hexane (three times, approx. 1,000 ml). Both fluid extracts were evaporated under reduced pressure to yield a hexane and 90%methanol extract. A small amount of each extract was reserved as reference extracts and for bioactivity screening. The extraction of the aerial parts and subterranean parts of *C. odorata* is shown in Scheme 1 and 2, respectively.



Scheme 1. Extraction scheme of the aerial parts of *C. odorata*



Scheme 2. Extraction scheme of the subterranean parts of *C. odorata*

Isolation of Chemical Constituents from *Chormolaena odorata*

When the extract EU9C was subjected to bioactivity screening tests, it exhibited anti-HSV-1 activity (IC_{50} 1.74 μg/ml), antituberculosis activity (MIC 100 μg/ml) and antibacterial activity (against *E. coli* and *S. aureus*). After partitioning the extract, EU9C, with 90%methanol and hexane, the results showed that active components were distributed mainly in the 90%methanol extract. According to the results, the fraction EU9M was selected first to isolate the bioactive compounds.

The methanol extract, EU9M (12 g), was dissolved in a small volume of DCM and mixed with kieselguhr (16 g). The mixture was dried and then fractionated by quick column chromatography using a sintered glass filter column (Buchner filter, Pyrex no.4) of silica gel, with a height of 2.0 inches and diameter of 4.0 inches. Groups of fractions were obtained by using the eluent (200 ml each) in the order as shown below.

Subfraction no.	Eluent
1-3	hexane
4-13	hexane : EtOAc (3 : 1)
14-22	hexane : EtOAc (1 : 1)
23-24	hexane : EtOAc (1 : 3)
25-37	EtOAc
38-42	2.5% methanol in EtOAc
43-47	5% methanol in EtOAc
48-55	10% methanol in EtOAc
56-60	20% methanol in EtOAc
61-63	30% methanol in EtOAc
64-69	50% methanol in EtOAc

The eluates were evaporated and combined according to their TLC analysis patterns giving nine fractions, EU9M001-EU9M009. All fractions were tested for anti-HSV-1 activity and the results (IC_{50} values) are shown in Table 3.1.

Table 3.1 The anti-HSV-1 activity results of fraction EU9M001-EU9M009

Fraction	Subfraction no.	Weight	Anti-HSV-1 activity
EU9M001	1-4	66.2 mg	not test
EU9M002	5-13	1.62 g	inactive
EU9M003	14-16	2.89 g	8.45 μg/ml
EU9M004	17-23	2.45 g	3.82 μg/ml
EU9M005	24-29	1.96 g	2.28 μg/ml
EU9M006	30-42	1.48 g	2.00 μg/ml
EU9M007	43-55	1.34 g	8.40 μg/ml
EU9M008	56-60	0.87 g	17.19 μg/ml
EU9M009	61-69	1.51 g	27.18 μg/ml

While the bioassay was undertaken and before the results were known, the fraction EU9M002 was randomly selected for fractionation of its components. Ten compounds were isolated from fraction EU9M002 including EU9M021K, 028K, 029K, 044K, 055, 058, 063, 067, 068 and 089.

1. Isolation of EU9M021K

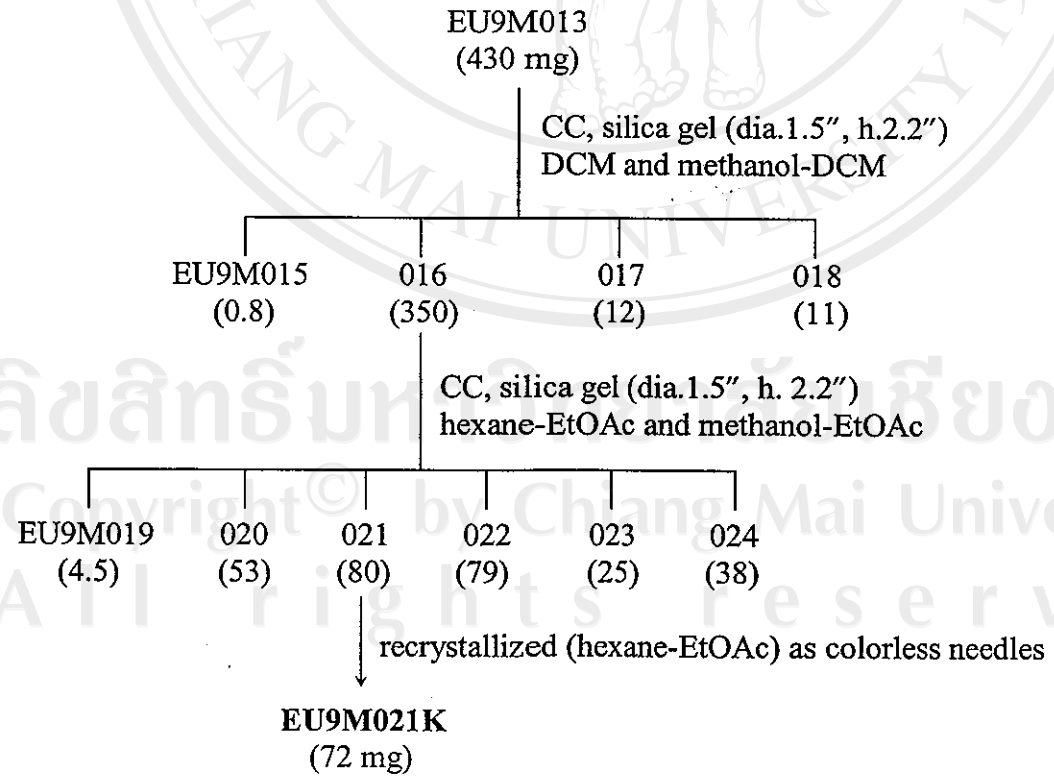
Fraction EU9M 002 was further fractionated using quick column chromatography of silica gel (diameter 2.0 inches, height 2.1 inches). Groups of fractions were obtained by using the eluents (200 ml each) in the order as shown below.

Subfraction no.	Eluent
1-4	Hexane : DCM (3 : 1)
5-28	Hexane : DCM (1 : 1)
29-30	Hexane : DCM (1 : 3)
31-34	DCM
35-40	2.5%methanol in DCM

The eluates were evaporated and combined according to their TLC patterns giving five fractions, EU9M010-EU9M014.

Fraction	Subfraction no.	Weight (mg)
EU9M010	1-3	72.5
EU9M011	4-8	687.6
EU9M012	9-14	127.6
EU9M013	15-17	445.4
EU9M014	18-20	65.5

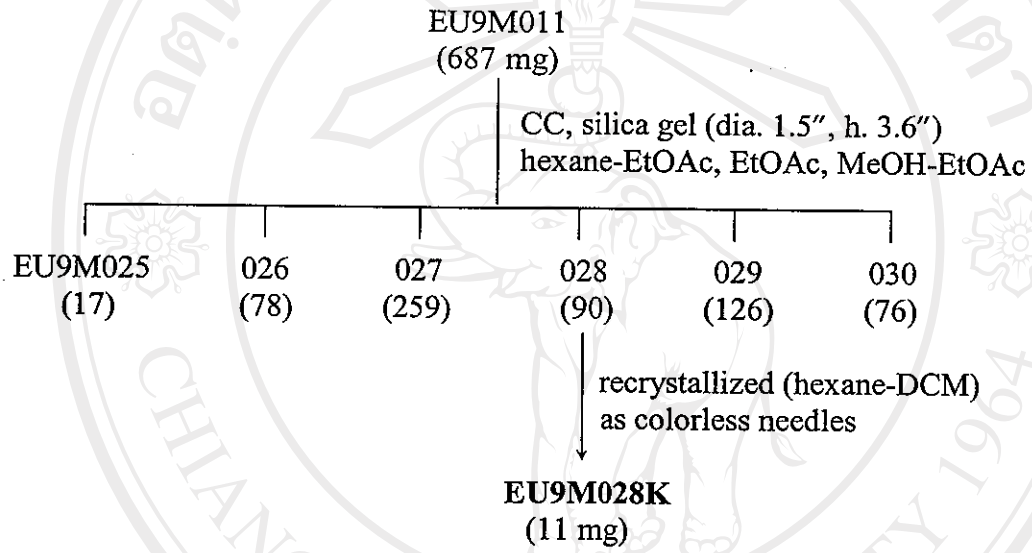
A portion of the fraction EU9M013 (430 mg) was further purified using short column chromatography on silica gel (diameter 1.5 inches, height 2.2 inches) and flash column chromatography on silica gel (diameter 1.5 inches, 3.6 inches) to obtain EU9M021K, m.p. 179-180^{o}C, as presented in Scheme 3. This compound exhibited a quenching spot under UV light of 254 nm and a yellow spot was observed after treatment of the chromatogram with anisaldehyde TS.



Scheme 3. Isolation scheme of compound EU9M021K from the 90%methanol extract.

2. Isolation of EU9M028K

The fraction EU9M011 (687 mg) was fractionated by using short column chromatography on silica gel (diameter 1.5 inches, height 3.6 inches) with hexane-EtOAc gradient and EtOAc as eluents. Each 25 ml fraction was collected and combined according to TLC patterns which showed two distinguishable spots under UV light 254 nm and after exposure to anisaldehyde TS. Two compounds, EU9M028K and EU9M029K were isolated from this fraction as shown in Scheme 4.



Scheme 4. Isolation scheme of compound EU9M028K from the 90%methanol extract.

3. Isolation of EU9M029K

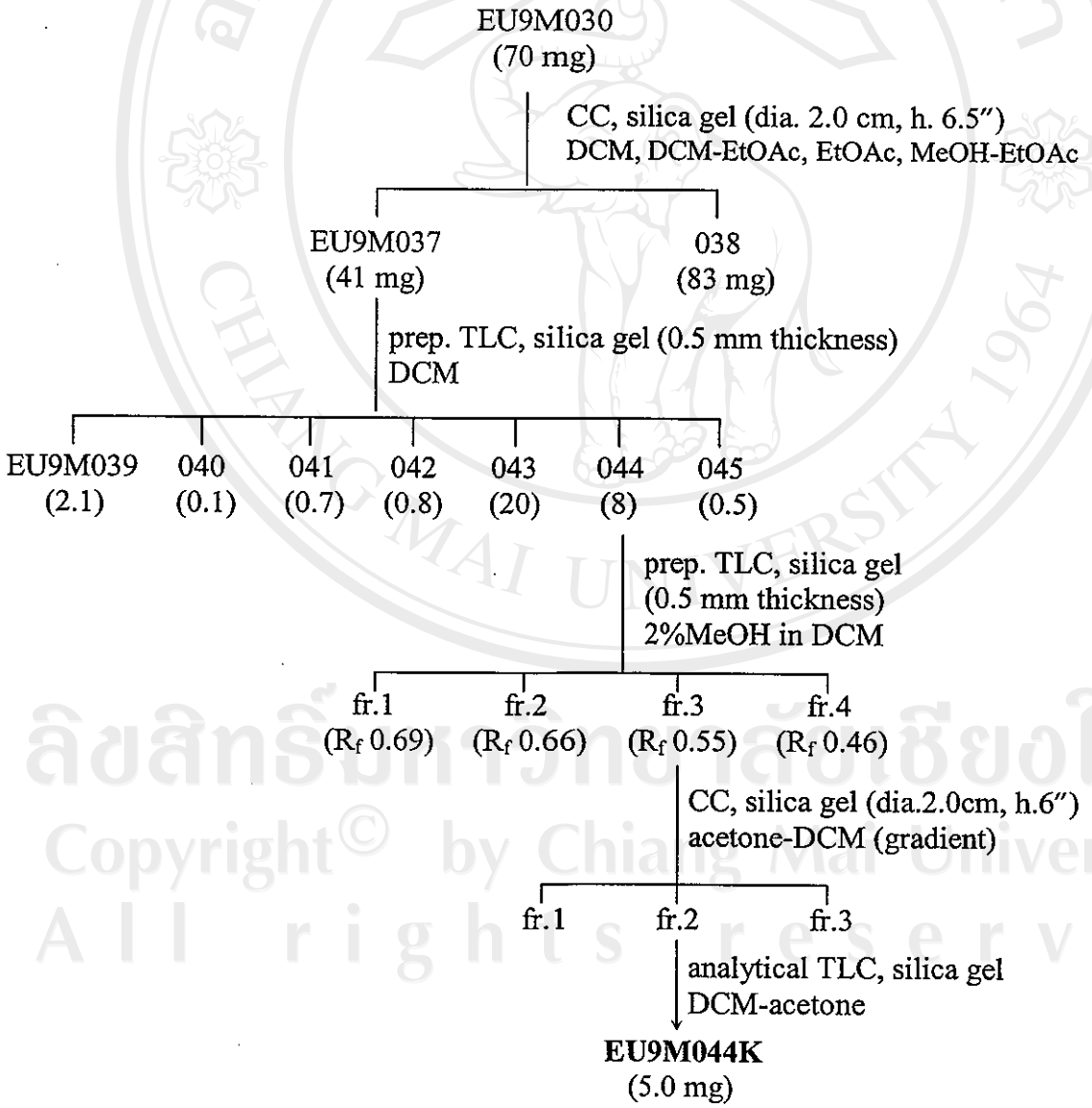
The isolation of EU9M029 is shown in Scheme 4 (above). This fraction was recrystallized in hexane-EtOAc (7:1) to obtain EU9M029K (27 mg) as orange needles, mp 140-141°C. The mother liquor was evaporated and fractionated using flash column chromatography on silica gel to obtain a mixture of steroids (EU9M051).

4. Isolation of EU9M044K

The fraction EU9M030 (70 mg) was fractionated using flash column chromatography on silica gel (diameter 2.0 cm, height 6.5 inches). Fractions were obtained by using the eluent in the order as shown below.

Subfraction no.	Eluent
1-24 (15 ml each)	DCM
25-29 (15 ml each)	DCM : EtOAc (7 : 1)
30 (200 ml)	EtOAc
31-32 (200 ml each)	10%methanol in EtOAc

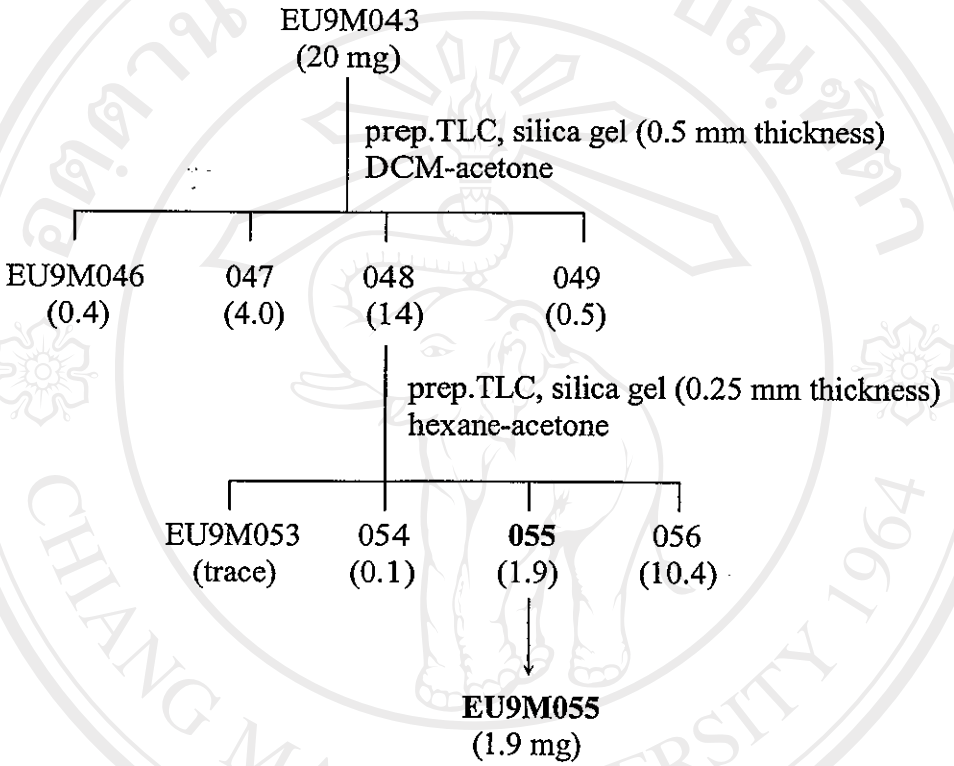
These subfractions were combined to obtain 2 fractions that were EU9M037 and EU9M038. The fraction EU9M037 was further isolated using column chromatography, analytical and preparative thin-layer chromatography to give EU9M044 as shown in Scheme 5.



Scheme 5. Isolation scheme of compound EU9M044K from the 90%methanol extract.

5. Isolation of EU9M055

The fraction EU9M043 (20 mg) was purified using preparative TLC on silica gel to obtain 4 fractions, EU9M046-049. Fraction EU9M048 was further purified by preparative TLC on silica gel to yield EU9M055. The fractionation process of EU9M055 is shown in Scheme 6.



Scheme 6. Isolation scheme of compound EU9M055 from the 90%methanol extract.

6. Isolation of EU9M058

Compound EU9M058 (0.5 mg) was isolated from fraction EU9M047 (see Scheme 6) by preparative TLC (silica gel, 0.25 mm thickness) using hexane-acetone as mobile phase (see also Scheme 7).

7. Isolation of EU9M063

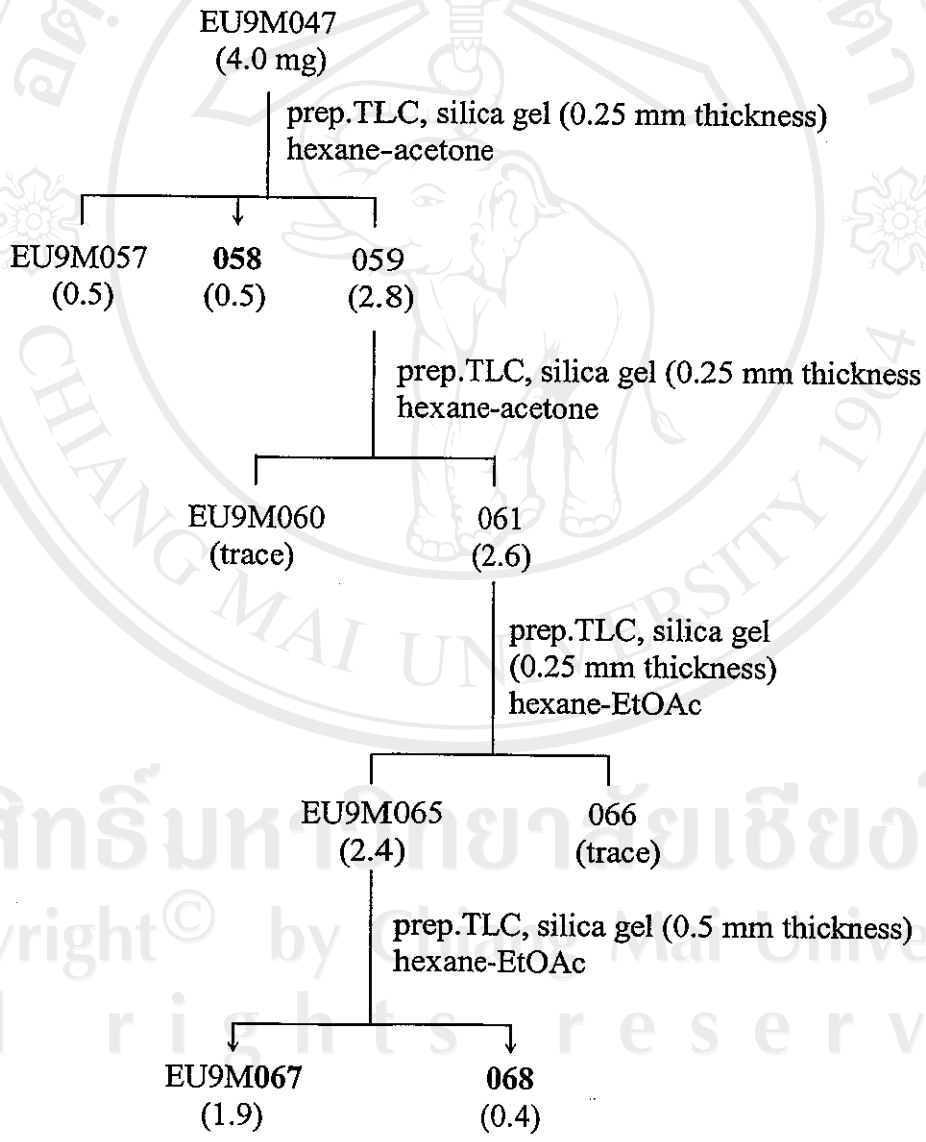
Compound EU9M063 (10 mg) was obtained from fraction EU9M056 (see Scheme 6) by preparative TLC (silica gel, 0.25 mm thickness) using hexane-acetone as mobile phase.

8. Isolation of EU9M067

Compound EU9M067 was afforded from fraction EU9M047 using preparative TLC as shown in Scheme 7.

9. Isolation of EU9M068

Compound EU9M068 was also isolated from fraction EU9M047 using preparative TLC as shown in Scheme 7.



Scheme 7. Isolation scheme of compounds EU9M058, 067 and 068 from the 90%methanol extract.

10. Isolation of EU9M089

From Scheme 3, fraction EU9M023 and 024 were co-fractionated using preparative TLC (silica gel, 1.0 mm thickness) with DCM as mobile phase to give compound EU9M089 (10 mg).

Fraction EU9M003 was also investigated before testing the biological activities. Fractionation of this fraction afforded 6 compounds including EU9M101, 104, 112, 135, 138 and 147. One of them, EU9M104 was identical with EU9M063.

11. Isolation of compound EU9M101

Fraction EU9M003 was isolated using column chromatography on silica gel (diameter 1.5 inches, height 6 inches). Groups of subfractions were obtained by using the eluent (50 ml each) in the order as shown below.

Subfraction no.	Eluent
1-12	Hexane : EtOAc (5 : 1)
13-19	Hexane : EtOAc (3 : 1)
20	Hexane : EtOAc (1 : 1)
21-24	EtOAc
25	5%methanol in EtOAc
26	10%methanol in EtOAc
27	15%methanol in EtOAc
28	20%methanol in EtOAc

The eluates were evaporated and combined according to their TLC patterns providing three fractions, EU9M100-EU9M102.

Fraction	Subfraction no.	Weight
EU9M100	1-19	2.35 g
EU9M101	20-24	82 mg
EU9M102	25-28	92 mg

The yellow solid of EU9M101 was obtained after the fraction was concentrated and repeatedly rinsed with cold methanol. Although this compound was eluted with ethyl acetate from the column, it did not dissolve in ethyl acetate but it dissolved very well in dimethylsulfoxide (see scheme 8).

12. Isolation of compound EU9M112K

Fraction EU9M100 (2.3 g) was further purified using quick column chromatography on silica gel (diameter 2.0 inches, height 3.5 inches). Groups of fractions were obtained by using the eluents (200 ml each) in the order as shown below.

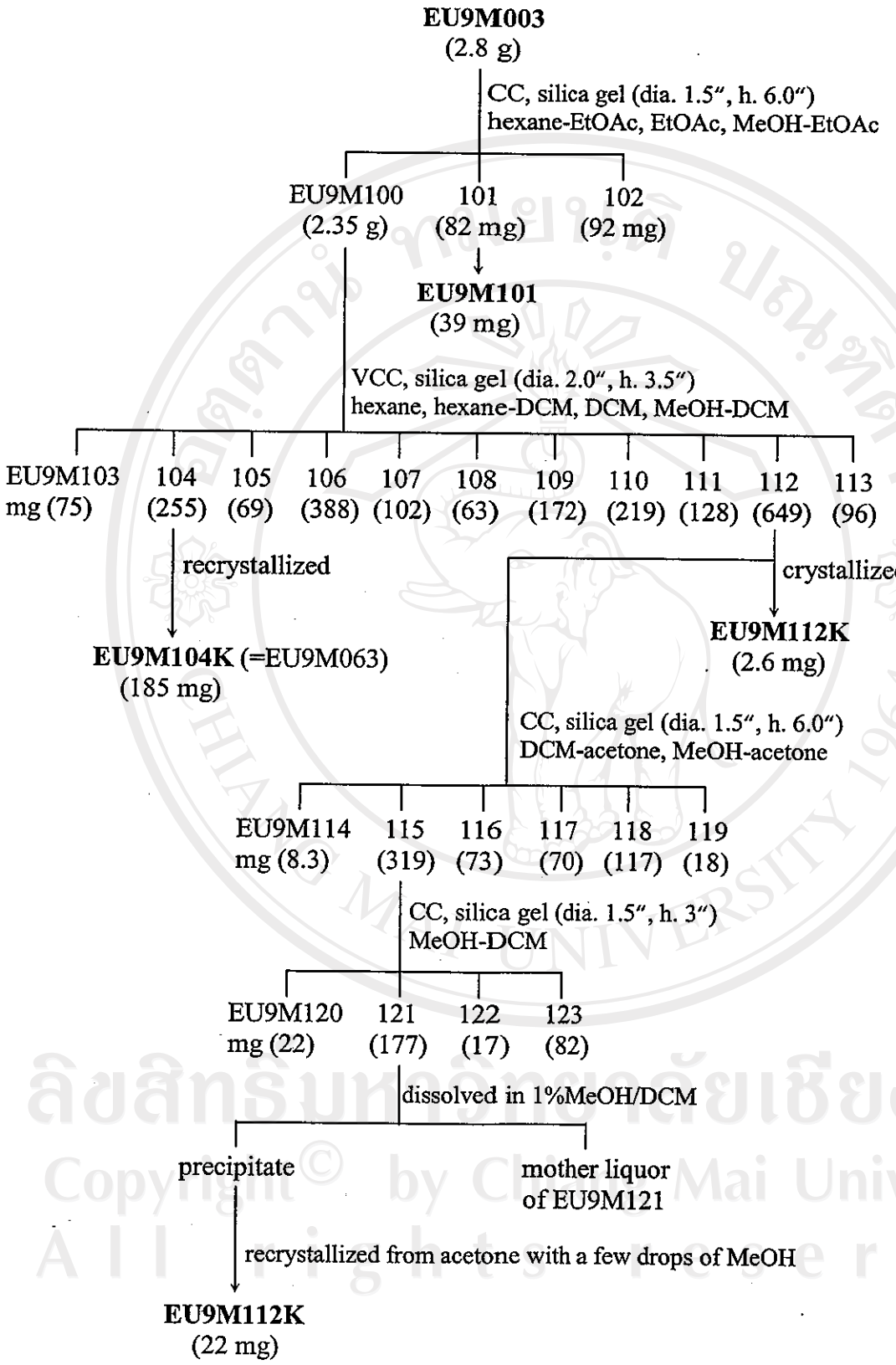
Subfraction no.	Eluent
1	Hexane
2-3	Hexane : DCM (9 : 1)
4-5	Hexane : DCM (8 : 2)
6-7	Hexane : DCM (7 : 3)
8-9	Hexane : DCM (2 : 1)
10-11	Hexane : DCM (3 : 2)
12-21	Hexane : DCM (1 : 1)
22-30	Hexane : DCM (3 : 2)
31-34	Hexane : DCM (1 : 1)
35-44	Hexane : DCM (90 : 110)
45-50	Hexane : DCM (80 : 120)
51-52	Hexane : DCM (70 : 130)
53-54	Hexane : DCM (60 : 140)
55-56	Hexane : DCM (50 : 150)
57-59	Hexane : DCM (40 : 160)
60-62	Hexane : DCM (10 : 190)
63-90	DCM
91-95	2.5%methanol in DCM
96	10%methanol in DCM

The eluates were evaporated and combined according to their TLC patterns providing eleven fractions, EU9M103-EU9M113, as shown below.

Fraction	Subfraction no.	Weight (mg)
EU9M103	1-18	75.1
EU9M104	19-25	255.5
EU9M105	26-36	69.2
EU9M106	37-47	388.2
EU9M107	48-55	102.5
EU9M108	56-60	62.9
EU9M109	61-66	172.6
EU9M110	67-78	219.4
EU9M111	79-91	127.9
EU9M112	92-95	649.2
EU9M113	96	33.2

After dissolving the fraction EU9M112 in a small amount of DCM, before loading on the column, crystals of the compound EU9M112K (2.6 mg) were obtained. The remaining fraction EU9M112 was isolated using flash column chromatography on silica gel (diameter 1.5 inches, height 6.0 inches) to give six fractions, EU9M114-EU9M119. The fraction EU9M115 (319 mg) was further purified using column chromatography on silica gel (diameter 1.5 inches, height 3.0 inches) to obtain 4 fractions, EU9M120-EU9M123. The fraction EU9M121 was treated with 1%MeOH in DCM and a yellow precipitate was obtained. The mother liquor was removed and the precipitate was further purified by crystallization in acetone with a few drops of methanol to yield fine yellow needles as shown in Scheme 8.

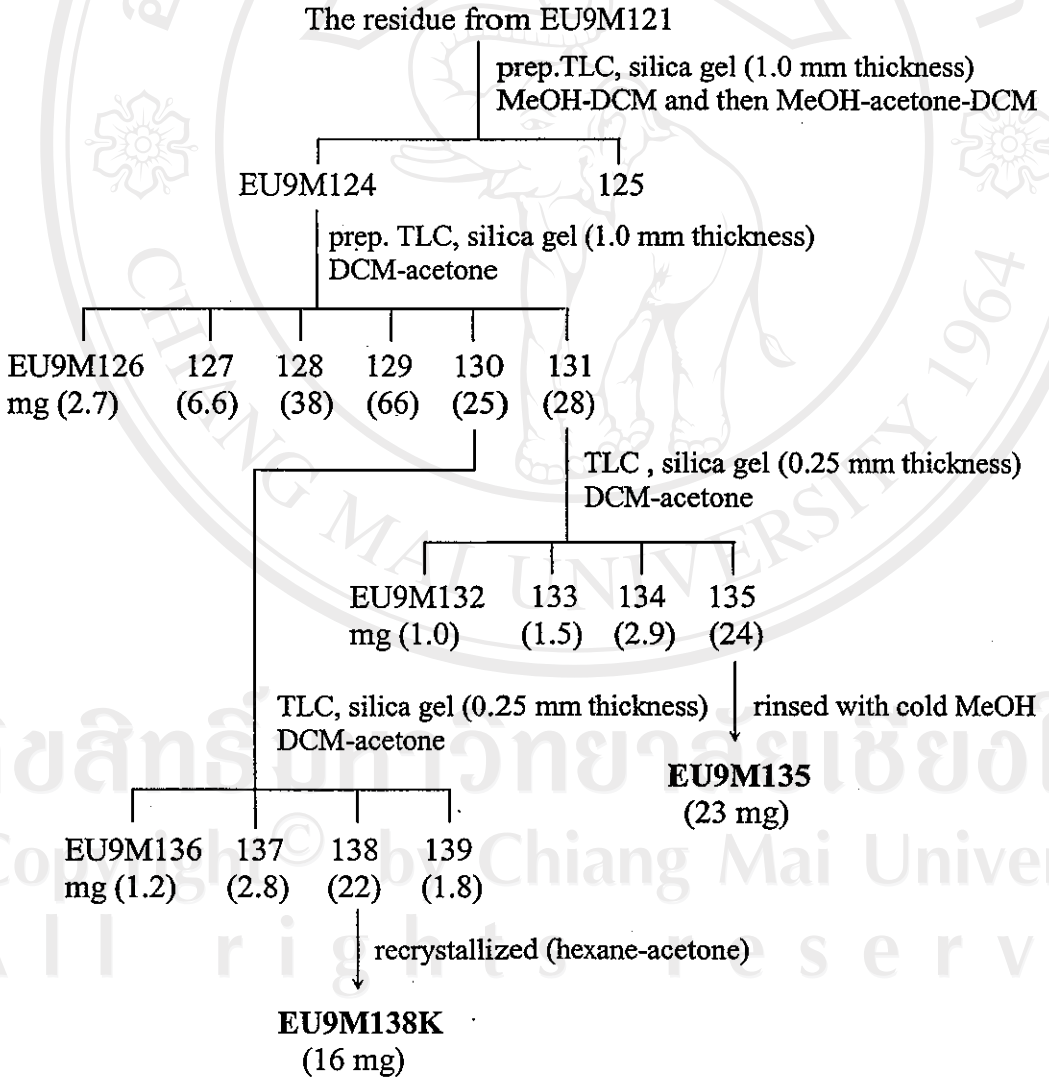
Fraction EU9M104 was further purified by recrystallization in hexane-acetone to give pale yellow crystalline material which were identical with EU9M063. The structure of a single crystal of this compound was also determined by x-ray crystallography.



Scheme 8. Isolation scheme of compounds EU9M101 and EU9M112K from the 90%methanol extract.

13. Isolation of compound EU9M135

The residue from the mother liquor of the fraction EU9M121 (177 mg) was purified using preparative TLC (silica gel, 1.0 mm thickness) to give 2 fractions, EU9M124 and EU9M125. The TLC pattern of fraction EU9M124 showed three distinguishable spots under UV light at 254 nm and after exposure to anisaldehyde TS. This fraction was then further purified using preparative TLC (silica gel, 0.25 mm thickness) to give six fractions, EU9M126-EU9M131. The fraction EU9M131 (28 mg) was further purified using TLC (silica gel, 0.25 mm thickness) to give compound EU9M135 as shown in Scheme 9.



Scheme 9. Isolation scheme of compounds EU9M135 and EU9M138 from the 90%methanol extract.

14. Isolation of compound EU9M138K

The fraction EU9M130 was purified using analytical silica gel TLC to give four fractions, EU9M136-EU9M139. The major fraction, EU9M138, was recrystallized from hexane-acetone to obtain EU9M138K as white needles (see Scheme 9).

15. Isolation of compound EU9M147K

The fraction EU9M128 was dissolved in acetone and then was purified by preparative TLC (silica gel, 0.25 mm thickness) using hexane-acetone as mobile phase to obtain EU9M145-EU9M147. The major fraction, EU9M147 (24 mg), was decolorized by rinsing with methanol and the residue was further purified using analytical silica gel TLC to give two fractions, one was EU9M112 (2 mg) and another was EU9M147K (20 mg).

Isolation of the Essential Oil from *Chormolaena odorata*

The fresh aerial parts (1.0 kg) including leaves, twigs, branches and stems were cut into small pieces and then hydrodistilled for 6 hours to afford a pale yellow oil. The obtained essential oil was dried over anhydrous sodium sulfate and, after filtration, stored under refrigeration until analyzed and tested. The essential oil was isolated in a yield of 0.2% (v/w on fresh weight basis; ca 2 ml).