CHAPTER 3

Experimental

3. 1 Source of plant materials

Plant samples used in this experiment including :

3.1.1 *Combretum deciduum* Coll. & Hemsl. was collected from Queen Sirikit Botanic Garden, Mae Rim, Chiang Mai, Thailand, in July 2010 (Figure 3.1).

3.1.2 *Combretum griffithii* Heur. & M.A. was collected from Queen Sirikit Botanic Garden, Mae Rim, Chiang Mai, Thailand, in July 2010 (Figure 3.2).

3.1.3 *Combretum latifolium* Bl. was collected from Wang Nuea, Lampang, Thailand, in December 2009 (Figure 3.3).

3.1.4 *Combretum quadrangulare* Kurz was collected from Doi Saket, Chiang Mai, Thailand, in June 2009 (Figure 3.4).

All plant materials were taxonomically identified by comparison with herbarium specimens from International Code of Botanical Nomenclature (ICBN) at the Department of Biology, Faculty of Sciences, Chiang Mai University, Chiang Mai, Thailand ; QBG No. 29269, QBG No. 97220, QBG No. 94582 and QBG No. 92695. And the voucher specimens were deposited at the Faculty of Pharmacy and Department of Biology, Faculty of Sciences, Chiang Mai University ; Nopsiri W. No. 6, Nopsiri W. No. 5, Nopsiri W. No. 4 and Nopsiri W. No. 7 as follow.

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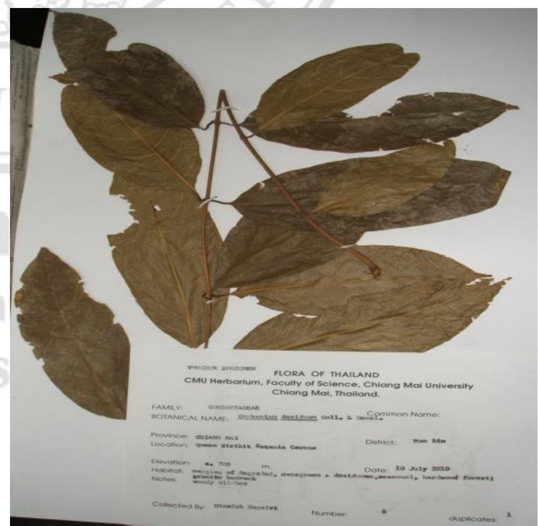
A

B



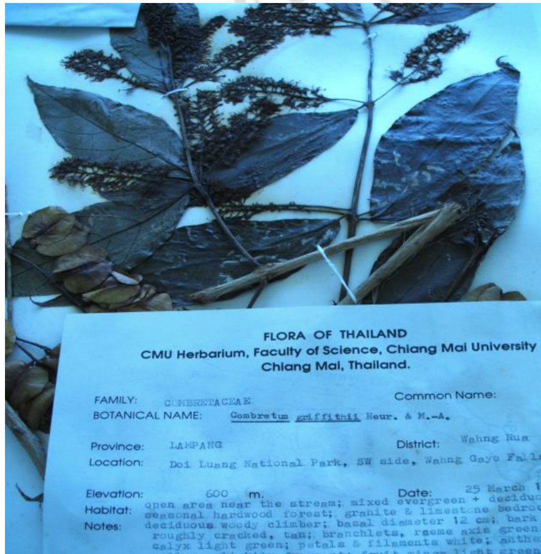
C

D

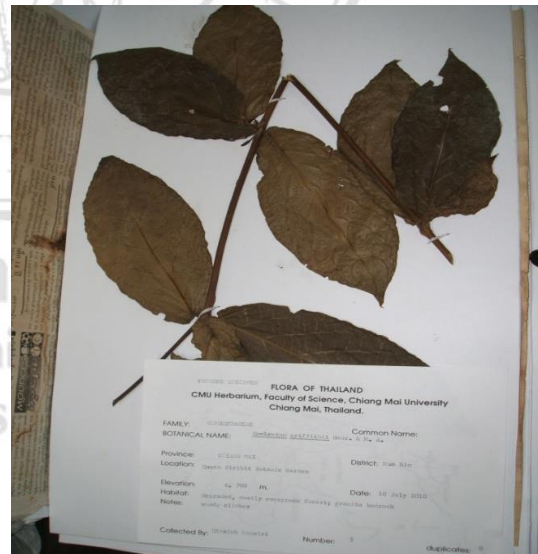
Figure 3.1 Leaves (A and B) and herbariums (C and D) of *Combretum deciduum* Coll. & Hemsl.

A

B



C



D

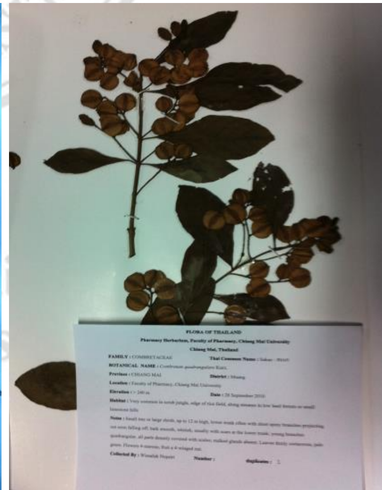
Figure 3.2 Leaves (A), flowers (B) and herbarium (C and D) of *Combretum griffithii* Heur. & M.A.

A B

C D E

Figure 3.3 Climbing tree (A), bouquets (B), leaves and herbarium (D and E) of *Combretum latifolium* Bl.

A



B C D

Figure 3.4 Tree (A), fruits (B) and herbarium (C and D) of *Combretum quadrangulare* Kurz

3.2 Ethnobotanical survey

The ethnobotanical surveys of some *Combretum* species were carried out in the northern Thailand. The traditional healers in Phrae province, northern Thailand were chosen as sources of information by interview with a questionnaire. The data that was sought about *Combretum* species includes : local name, part used, indication, method of preparation, dosage and duration of use.

3.2.1 Description of the study area

The ethnobotanical excursion was carried out in Rong Kwang district (Amphoe) in the northeastern part of Phrae province (Figure 3.5 [Website 1 ; 2]). Phrae is one of the northern provinces of Thailand, is situated 555 km from Bangkok, the capital city of Thailand. Neighboring provinces are (from north clockwise) Phayao, Nan, Uttaradit, Sukhothai and Lampang. Rong Kwang is situated 30 km from Phrae Province. The district is subdivided into 11 subdistricts (Tambon) : Rong Kwang, Rong Khem, Nam Lao, Ban Wiang, Thung Si, Mae Yang Tan, Mae Yang Peaw, Phai Thon, Huai Rong, Mae Sai and Mae Yang Rong, which are further subdivided into 93 villages (Muban). Total population are 50,459 people, 24,389 male population and 26,070 female population. There are Mong mountaineer and Tong Hluang tribe. There are 46 schools, 39 temples and 1 church. Occupation of most people have been farmers. Crop are rice, tobacco, corn, soybean, green beans and wax. The altitude is 205 m from the sea level. This region has temperature average 36°C (max) and 19°C (min), winter season (November-February), summer (March-May) and rainy season (June-October). The everage rainfall are 1,000-1,500 mm per year. Total area of Rong Kwang district is 642 km^2, crop area 218 km^2 and conserved forest area 424 km^2. This area is plentifully with natural resource, forest plants, animals and mineral (Manganese) [Website 3 ; 4 ; 5].

3.2.2 Ethnobotanical fieldwork

Eleven subdistricts in Rong Kwang district were visited for the ethnobotanical survey. These subdistricts were Rong Kwang, Rong Khem, Nam Lao, Ban Wiang, Thung Si, Mae Yang Tan, Mae Yang Peaw, Phai Thon, Huai Rong, Mae Sai and Mae Yang Rong. Sixty one traditional healer, 37 men and 24 women, were interviewed on their traditional medicinal uses of *Combretum* species. The structured

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interviews were performed with the aid of a questionnaire which consisted of general and more specific questions (Appendix A). *Combretum* species were shown to the healers, and they were asked about their species preferences and uses of these plants. The interviews were started with general questions, after which more specific questions were asked about the medicinal plants shown to the healers.

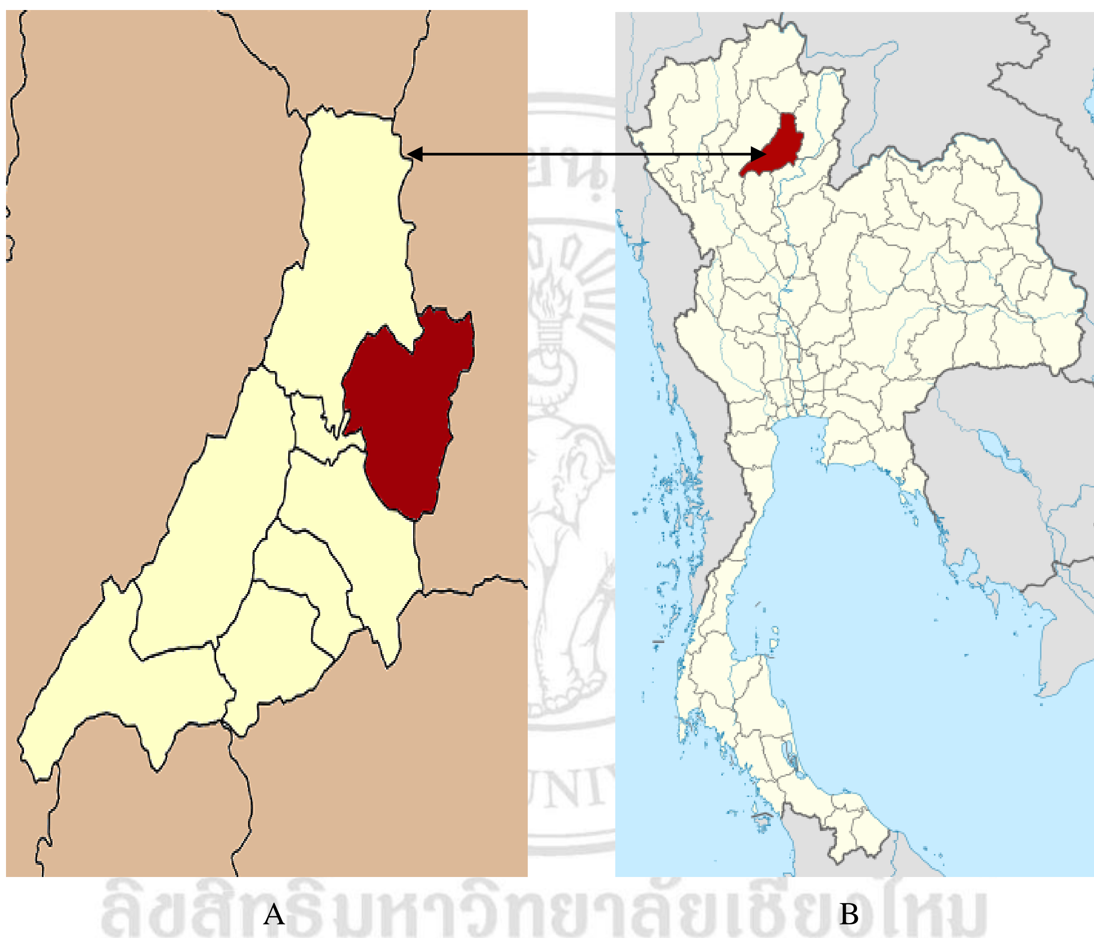

Figure 3.5 Map of Rong Kwang district (the red area), Phrae province (A), Thailand (B)

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3.3 General techniques

3.3.1 Analytical Thin-Layer Chromatography

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60 GF-254 (E. Merck) precoated on aluminium plate
Layer thickness	:	200 μm

Distance	:	4 cm
Temperature	:	Laboratory temperature (25-30°C)
Detection	:	1) Ultraviolet light at wavelength 254 and 365 nm 2) Spraying reagent Vanillin reagent (Vanillin reagent consisted of vanillin 15 g, concentrated sulfuric acid 2.5 mL and ethanol 250 mL). Stained TLC plates give specific color spots with this reagent after heating at 90-100 ºC for 2-5 min.

3.3.2 Preparative Thin-Layer Chromatography

Technique	:	One dimension, ascending
Adsorben	:	Silica gel 60 GF-254 (E. Merck) precoated on glass plate
Layer thickness	:	500 µm
Distance	:	15 cm
Temperature	:	Laboratory temperature (24-30 °C)
Detection	:	Ultraviolet light at wavelength 254 nm and 365 nm

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3.3.3 Column Chromatography

1) Quick Column Chromatography

Adsorbent	:	Silica gel 60 (No. 7730) particle size 0.005-0.040 mm (230-400 mesh ASTM) (E. Merck)
Packing method	:	Dry packing
Sample loading	:	The sample was dissolved in a small amount of eluent and then applied gently on top of the column.

Detection	:	Fractions were examined by TLC under UV light at the wave length 254 nm and 365 nm

2) Flash Column Chromatography

Adsorbent	:	Silica gel 60 (No. 7733) particle size 0.200-0.500 mm (200-400 mesh ASTM) (E. Merck)
Packing method	:	Wet packing
Sample loading	:	The sample was dissolved in a small amount of eluent and then applied gently on top of the column.
Detection	:	Fractions were examined by TLC under UV light at the wave length 254 nm and 365 nm

3.3.4 Crystallization technique

The compounds were crystallized from differentiate solvents. Each compound was dissolved in selected solvent until saturated and let standing at room temperature until amorphous powder or crystals were formed.

3.3.5 Spectroscopy

1) Infrared (IR) Absorption Spectra

IR spectra (KBr disc) were recorded on a FT-IR spectrometer (Tensor 27, Broker Optics, Department of Chemistry, Faculty of Science, Chiang Mai University). Spectra of solid sample were recorded as KBr discs.

2) Mass Spectra

Electron Impact (EIMS) were measured with a JEOL GC mate (Japan) or a FISONS VG TRIO 2000 mass spectrometer (Department of Chemistry, Faculty of Science, Chulalongkorn University).

3) Gas Chromatography-Mass Spectrometry (GC-MS)

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The GC-MS analysis was performed on Hawlett-Packard 7890A coupled with a Hawlett-Packard 5975C mass selective detector. HP-5MS fused silica capillary column, 30 m × 0.25 mm, 0.25 μm film thickness composed of 5% Phenylmethylsiloxane.

4) Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C-NMR) Spectra

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained with a Bruker Avance 400 NMR spectrometer (Faculty of Sciences, Chiang Mai University).

The solvent for NMR spectra was deuterated chloroform ($CDCl_3$). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

3.3.6 Solvents

Throughout this work, all organic solvents were analytical grade and commercial grade organic solvents were redistilled prior to use.

3.4 Extraction of volatile compounds

Fresh leaves of *Combretum* species were washed with distilled water, chopped into small pieces, and subjected to hydrodistillation in a Clevenger-type apparatus for 8 h. The volatile compounds were collected, dehydrated over anhydrous sodium sulfate, and kept at 4 °C for further analysis.

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3.5 Analysis of volatile compounds

The volatile compounds were analyzed on a Hewlett-Packard GC-7890A gas chromatography equipped with a HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), programming from 50 °C for 5 min to 200 °C at 5 °C/min, ending at 250 °C (10 min) : the total run time was 30 min; carrier gas He, constant flow rate of 0.5 mL/min ; injector temperature was 270 °C ; detector temperature was 280 °C. Samples were injected by splitting mode (1:25). The GC-MS analysis was performed on Hawlett-Packard GC-7890A coupled with a Hawlett-Packard 5975C mass selective detector were; electron impact ionization (EI) source

temperature, 230 °C ; interface temperature, 270 °C ; ionization energy, 70 eV ; mass range of 30-500 *m/z* ; quadrupole temperature, 150 °C.

The volatile componds were identified by comparing their retention indices (RI) relative to *n*-alkane index on HP-5 column and by comparison of mass spectra from libraries (Wiely7n.1 and NIST) with corresponding data of authentic compounds or published spectra [Zito, 2010].

3.6 Extraction

All the leaves of *Combretum* species were washed and dried in a hot air oven at 40 ºC for 2 days. Then the dried leaves were powdered before macerated in organic solvent. Each plant materials were extracted with *n*-hexane (1.5 L×3 days) at room temperature, followed by filtration. The filtrate was evaporated to dryness under reduced pressure to obtain *n*-hexane crude extract [I]. Similarly, the extraction was carried out using dichloromethane (1.5 L×3 days) and methanol (1.5 L×3 days) to give the corresponding dichloromethane [II] and methanolic extracts [III]. A summary of the extraction protocol that was used in this study is presented in Scheme 3.1.

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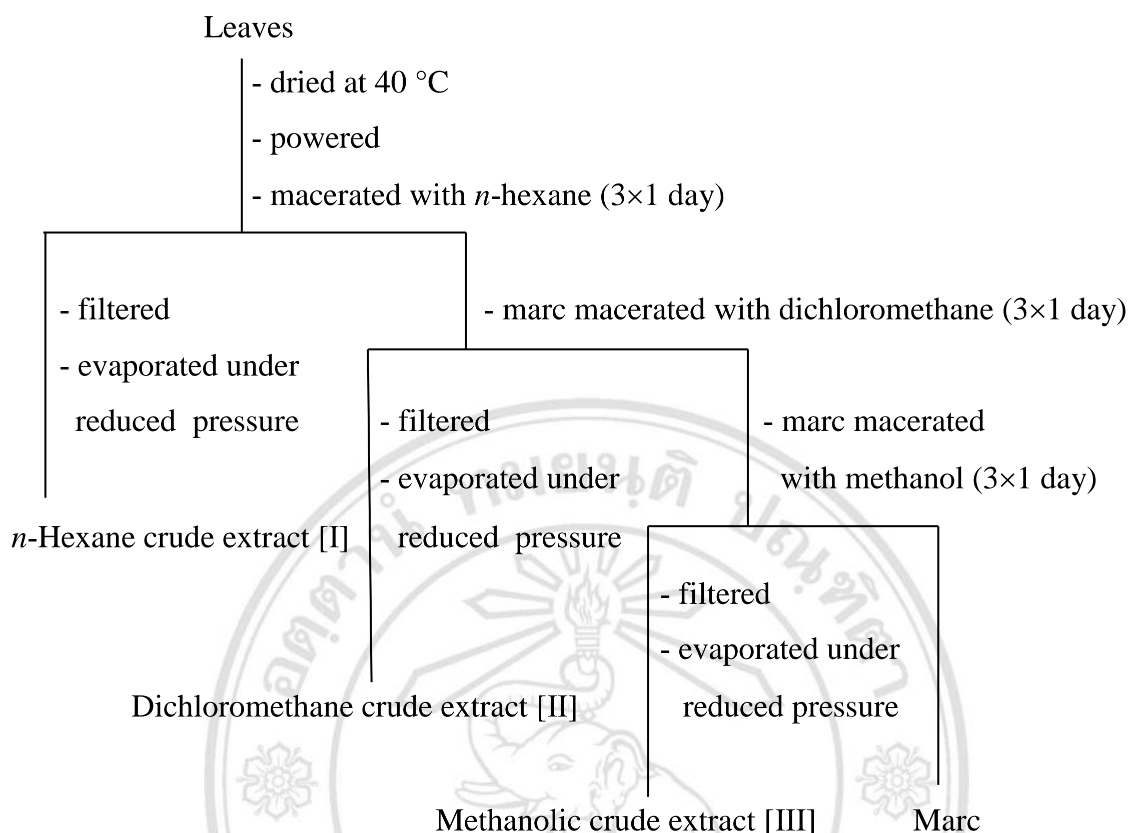

Scheme 3.1 Extraction scheme of all plant materials

3.6.1 Extraction of *Combretum deciduum* Coll. & Hemsl.

The dried powdered leaves of *Combretum deciduum* Coll. & Hemsl. (402.31 g) were macerated with *n*-hexane for one day three times (3×1.5 L), then the marc was macerated with dichloromethane (3×1.5 L). The process was repeated with methanol (3×1.5 L) in the previously described condition and filtered. Each filtrate was combined and evaporated to dryness at 50 °C under reduced pressure to yield dried *n*-hexane crude extract [I] (13.19 g, 3.28% based on dried weight of leaves), dichloromethane crude extract [II] (5.29 g, 1.32% based on dried weight of leaves) and methanolic crude extract [III] (106.97 g, 26.59% based on dried weight of leaves).

3.6.2 Extraction of *Combretum griffitii* Heur. & M.A.

The dried powdered leaves of *Combretum griffitii* Heur. & M.A. (285.04 g) were macerated with *n*-hexane for one day three times (3×1.5 L), then the marc was macerated with dichloromethane (3×1.5 L). The process was repeated with methanol (3×1.5 L) in the previously described condition and filtered. Each filtrate was combined

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and evaporated to dryness at 50 °C under reduced pressure to yield dried *n*-hexane crude extract [I] (18.35 g, 6.44% based on dried weight of leaves), dichloromethane crude extract [II] (12.89 g, 4.52% based on dried weight of leaves) and methanolic crude extract [III] (38.11 g, 13.37% based on dried weight of leaves).

3.6.3 Extraction of *Combretum latifolium* Bl.

The dried powdered leaves of *Combretum latifolium* Bl. (769.84 g) were macerated with *n*-hexane for one day three times (3×1.5 L), then the marc was macerated with dichloromethane (3×1.5 L). The process was repeated with methanol (3×1.5 L) in the previously described condition and filtered. Each filtrate was combined and evaporated to dryness at 50 °C under reduced pressure to yield dried *n*-hexane crude extract [I] (29.43 g, 3.82% based on dried weight of leaves), dichloromethane crude extract [II] (5.37 g, 0.70% based on dried weight of leaves) and methanolic crude extract [III] (134.95 g, 17.53% based on dried weight of leaves).

3.6.4 Extraction of *Combretum quadrangulare* Kurz

The dried powdered leaves of *Combretum quadrangulare* Kurz (840.00 g) were macerated with *n*-hexane for one day three times (3×1.5 L), then the marc was macerated with dichloromethane (3×1.5 L). The process was repeated with methanol (3×1.5 L) in the previously described condition and filtered. Each filtrate was combined and evaporated to dryness at 50 °C under reduced pressure to yield dried *n*-hexane crude extract [I] (18.02 g, 2.15% based on dried weight of leaves), dichloromethane crude extract [II] (90.12 g, 10.73% based on dried weight of leaves) and methanolic crude extract [III] (123.98 g, 14.76% based on dried weight of leaves).

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3.7 Isolation

3.7.1 Isolation of *Combretum griffitii* Heur. & M.A.

The crude methanolic extract, CG (9.49 g) was dissolved in a small amount of methanol and packed onto top of wet silica gel column (4.5×16 cm). The column was eluted with each 500 mL of *n*-hexane, *n*-hexane : ethyl acetate (9:1), *n*-hexane : ethyl acetate (4:1), *n*-hexane : ethyl acetate (7:3), *n*-hexane : ethyl acetate (3:2), *n*-hexane : ethyl acetate (1:1), *n*-hexane : ethyl acetate (2:3), *n*-hexane : ethyl acetate (3:7), *n*-

hexane : ethyl acetate (1:4), *n*-hexane : ethyl acetate (1:9), ethyl acetate, ethyl acetate : methanol (9:1), ethyl acetate : methanol (4:1), ethyl acetate : methanol (7:3), ethyl acetate : methanol (3:2), ethyl acetate : methanol (1:1), ethyl acetate : methanol (2:3), ethyl acetate : methanol (3:7), ethyl acetate : methanol (1:4), ethyl acetate : methanol (1:9) and then washed with methanol until no traces could be detected. Fractions of 125 ml were collected and compared by TLC. The eluting solvents were altered to give more polar solvent systems when the difference of patterns on TLC were observed. Those fractions of similar pattern were combined and evaporated to dryness under reduced pressure. By this procedure :-

1) Fractions 1-3 were combined and designated as fraction CG1 (0.0023 g).

2) Fractions 4-19 were combined and designated as fraction CG2 (0.0250 g).

3) Fractions 10-12 were combined and designated as fraction CG3 (0.1287 g).

4) Fractions 13-15 were combined and designated as fraction CG4 (0.1470 g).

5) Fractions 16-20 were combined and designated as fraction CG5 (0.3350 g).

6) Fractions 21-30 were combined and designated as fraction CG6 (0.1850 g).

7) Fractions 31-34 were combined and designated as fraction CG7 (0.0054 g).

8) Fractions 35-37 were combined and designated as fraction CG8 (0.2352 g).

9) Fractions 38-40 were combined and designated as fraction CG9 (0.0029 g).

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10) Fractions 41-44 were combined and designated as fraction CG10 (0.0023 g).

11) Fractions 45-67 were combined and designated as fraction CG11 (0.0018 g).

12) Fractions 68-88 were combined and designated as fraction CG12 (0.0012 g).

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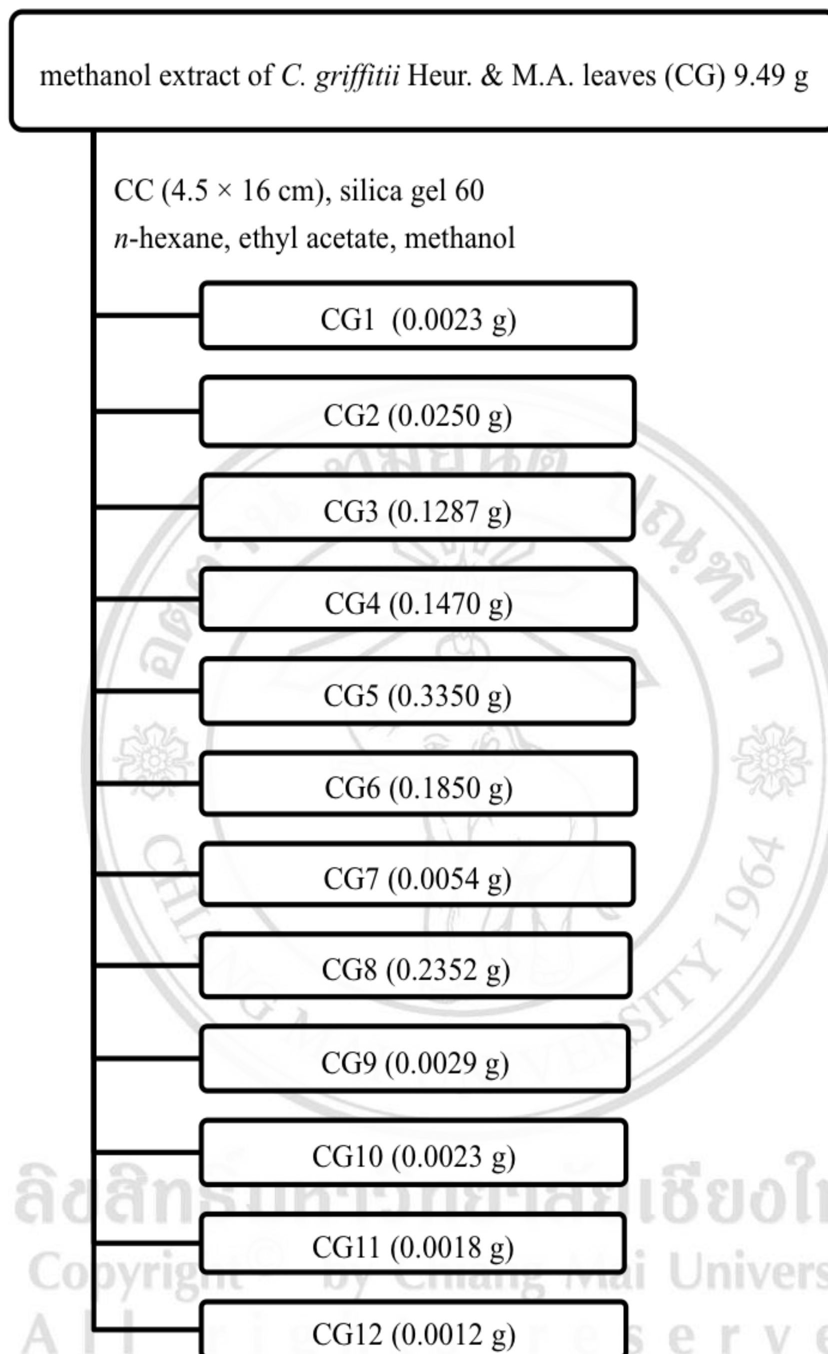

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Scheme 3.2 Isolation scheme of methanolic extract of *Combretum griffithii* Heur. & M.A. leaves (CG)

1) Isolation of fraction CG-3.2 from fraction CG3

Fraction CG3 (0.1287 g) was shown by TLC to contain at least two compounds. It was dissolved in small amount of *n*-hexane and packed onto the top of wet silica gel column (3×16 cm). The column was eluted with *n*-hexane, *n*-hexane : ethyl acetate (95:5) then washed with methanol until no traces of compounds could be detected. Fourty-three fractions, each of approximately 50 mL, were collected. The volumes of eluent were 300, 650, 500 and 500 mL, respectively. The fractions were examined by TLC and the liked fractions were combined to give the following portions :-

1.1) Fractions1-10 were combined and designated as fraction CG-3.1 (0.0001 g).

1.2) Fractions 11-23 were combined and the TLC chromatogram shown only one spot under UV light at 254 nm, R_f 0.25 [silica gel/*n*-hexane : ethyl acetate (95:5)]. These fractions were evaporated under reduced pressure to give 0.0685 g of compound CG-3.2 as colorless needles crystals (0.72% based on dried weight of crude plant extract). It was later identified as 3β-Taraxerol.

1.3) Fractions 24-33 were combined and designated as fraction CG-3.3 (0.0008 g).

1.4) Fractions 34-43 were combined and designated as fraction CG-3.4 (0.3662 g).

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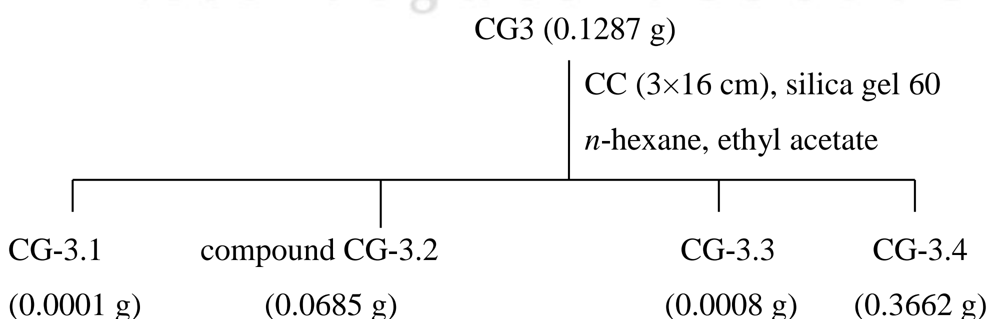

Scheme 3.3 Isolation scheme of compound CG-3.2

3.7.2 Isolation of *Combretum latifolium* Bl.

The crude methanolic extract (1 g) was dissolved in the small amount of solvent and applies in the beginning line of the preparative TLC plate that was coated with polar solid stationary phase (silica gel 60), starting and ending at least 1 cm from either side. The plate was developed with each 100 mL of chloroform : methanol (9:1), and then 11 bands visualized and marked under ultraviolet light (254 and 365 nm). The bands were scraped off from the glass plate to remove the components. Each band was eluted by acetone and then filtered using a sintered glass funnel. By this procedure :-

1) Band 1 was designated as compound CLP1 (0.0137 g).

2) Band 2, the TLC chromatogram shown only one spot under UV light at 254 nm, R_f 0.70 [silica gel/chloroform : methanol (9:1)]. These compound was evaporated under reduced pressure to give 0.0362 g of compound CLP2 as white powder (3.62% based on dried weight of crude plant extract). It was later identified as lipid (long chain fatty acid) compound.

3) Band 3 was designated as compound CLP3 (0.0145 g).

4) Band 4, the TLC chromatogram shown only one spot under UV light at 254 nm, R_f 0.67 [silica gel/chloroform : methanol (9:1)]. These compound was evaporated under reduced pressure to give 0.0361 g of compound CLP2 as white powder (3.62% based on dried weight of crude plant extract). It was later identified as lipid (long chain fatty acid) compound.

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5) Band 5 was designated as compound CLP5 (0.0925 g).

6) Band 6 was designated as compound CLP6 (0.1006 g).

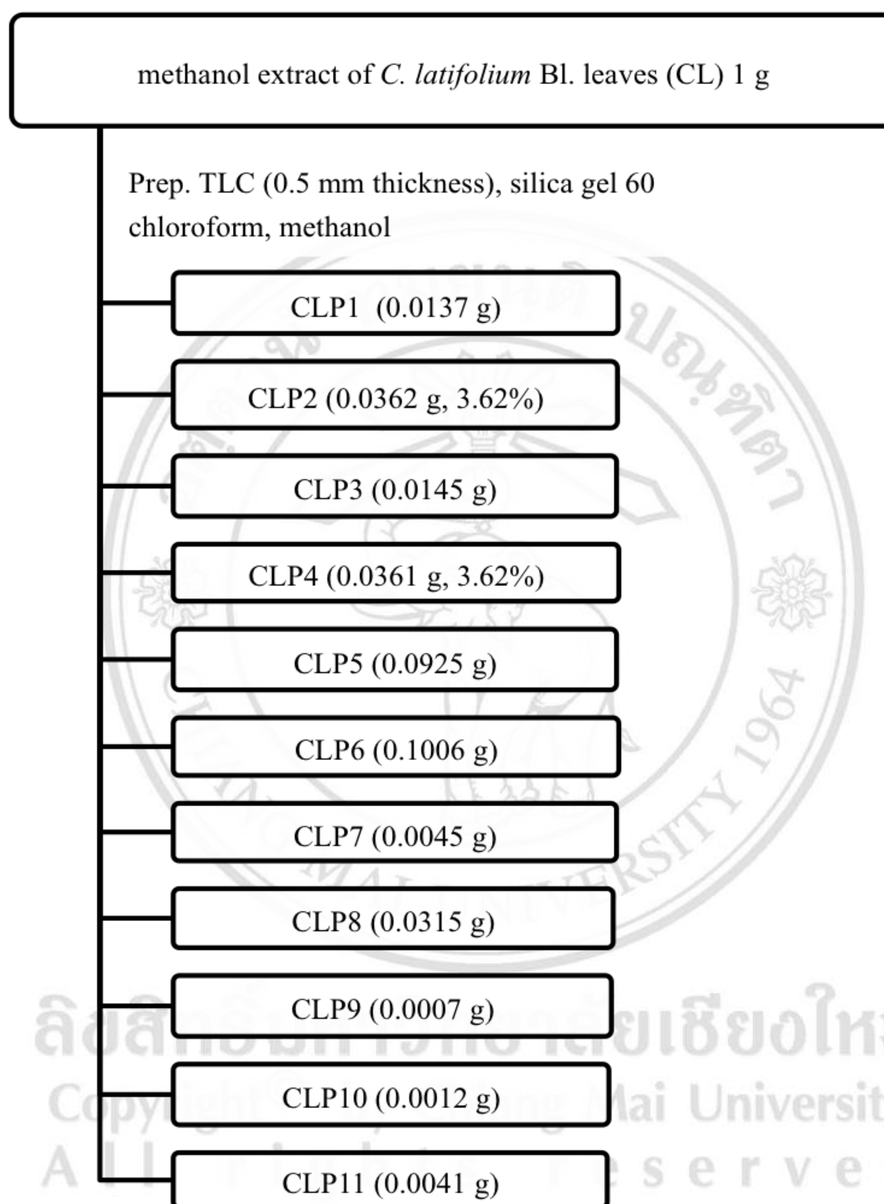
7) Band 7 was designated as compound CLP7 (0.0045 g).

8) Band 8 was designated as compound CLP8 (0.0315 g).

9) Band 9 was designated as compound CLP9 (0.0007 g).

10) Band 10 was designated as compound CLP10 (0.0012 g).

11) Band 11 was designated as compound CLP11 (0.0040 g).



Scheme 3.4 Isolation scheme of methanolic extract of *Combretum latifolium* Bl. leaves (CL)

3.7.3 Isolation of *Combretum quadrangulare* Kurz

The crude methanolic extract (10.41 g) was dissolved in a small amount of methanol and packed onto top of wet silica gel column (4.5×16 cm). The column was eluted with each 500 mL of *n*-hexane, *n*-hexane : ethyl acetate (9:1), hexane : ethyl acetate (4:1), *n*-hexane : ethyl acetate (7:3), *n*-hexane : ethyl acetate (3:2), *n*-hexane : ethyl acetate (1:1), *n*-hexane : ethyl acetate (2:3), *n*-hexane : ethyl acetate (3:7), *n*-hexane : ethyl acetate (1:4), *n*-hexane : ethyl acetate (1:9), ethyl acetate, ethyl acetate : methanol (9:1), ethyl acetate : methanol (4:1), ethyl acetate : methanol (7:3), ethyl acetate : methanol (3:2), ethyl acetate : methanol (1:1), ethyl acetate : methanol (2:3), ethyl acetate : methanol (3:7), ethyl acetate : methanol (1:4), ethyl acetate : methanol (1:9) and then washed with methanol until no traces could be detected. Fractions of 250 ml were collected and compared by TLC. The eluting solvents were altered to give more polar solvent systems when the difference of patterns on TLC were observed. Those fractions of similar pattern were combined and evaporated to dryness under reduced pressure. By this procedure :-

1) Fractions 1-3 were combined and designated as fraction CQ1 (0.0012 g).

2) Fractions 4-7 were combined and designated as fraction CQ2 (0.0350 g).

3) Fractions 8-15 were combined and designated as fraction CQ3 (0.2386 g).

4) Fractions 16-25 were combined and designated as fraction CQ4 (0.3258 g).

5) Fractions 26-30 were combined and designated as fraction CQ5 (0.2262 g).

6) Fractions 31-35 were combined and designated as fraction CQ6 (0.3782 g).

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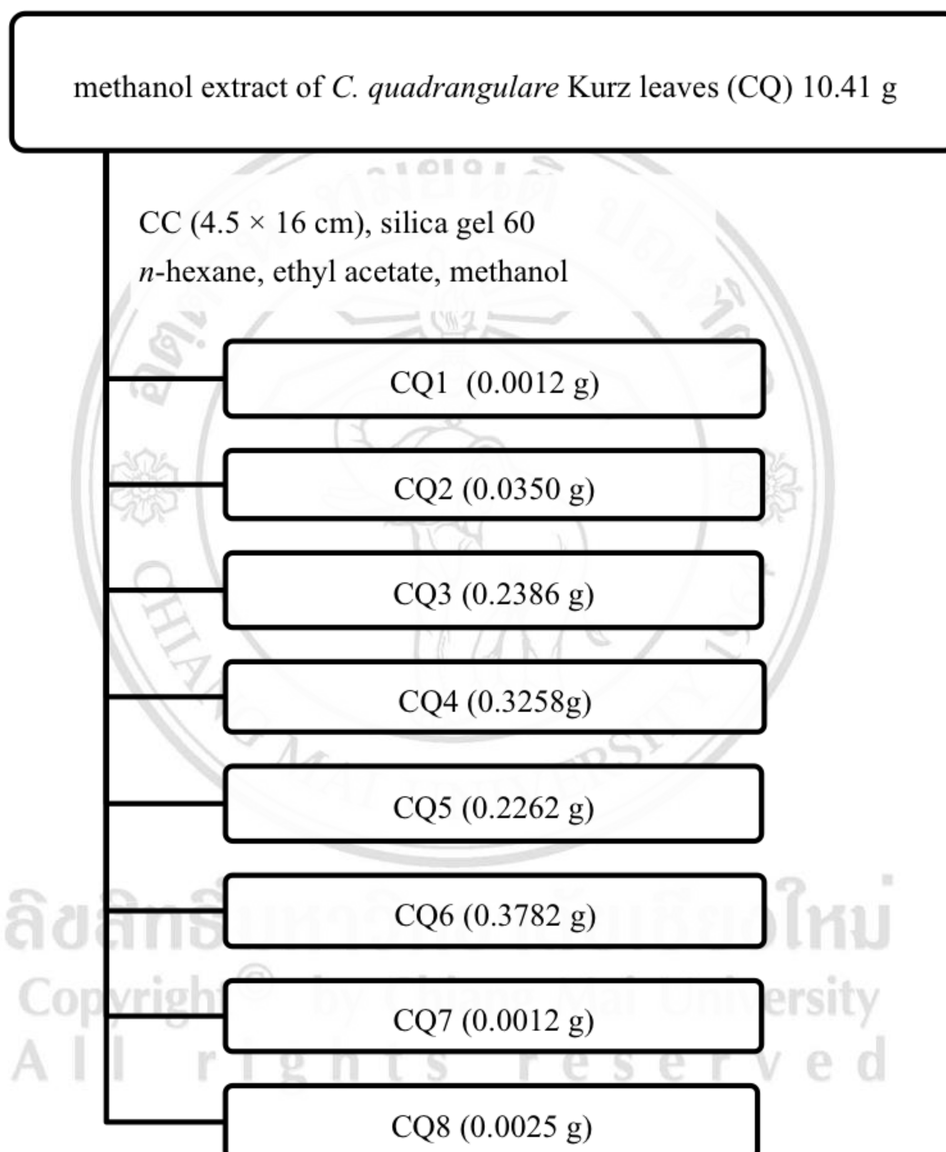
7) Fractions 36-40 were combined and designated as fraction CQ7 (0.0012 g).

8) Fractions 41-54 were combined and designated as fraction CQ8 (0.0025 g).



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Scheme 3.5 Isolation scheme of methanolic extract of *Combretum quadrangulare* Kurz leaves (CQ)

1) Isolation of fraction CQ-3.2 from fraction CQ3

Fraction CQ3 (0.2386 g) was shown by TLC to contain at least two compounds. It was dissolved in small amount of *n*-hexane and packed onto the top of wet silica gel column (3×16 cm). The column was eluted with *n*-hexane, *n*-hexane : ethyl acetate (9:1) then washed with methanol until no traces of compounds could be detected. Fifty fractions, each of approximately 50 mL, were collected. The volumes of eluent were 950, 500, 950 and 600 mL, respectively. The fractions were examined by TLC and the liked fractions were combined to give the following portions :-

1.1) Fractions 1-19 were combined and designated as fraction CQ-3.1 (0.0003 g).

1.2) Fractions 20-29 were combined and the TLC chromatogram shown only one spot under UV light at 254 nm, R_f 0.45 [silica gel/*n*-hexane : ethyl acetate (9:1)]. These fractions were evaporated under reduced pressure to give 0.0043 g of compound CQ-3.2 as white crystals (0.04% based on dried weight of crude plant extract). It was later identified as Lupeol.

1.3) Fractions 30-38 were combined and designated as fraction CQ-3.3 (0.0028 g).

1.4) Fractions 39-50 were combined and designated as fraction CQ-3.4 (0.0003 g).

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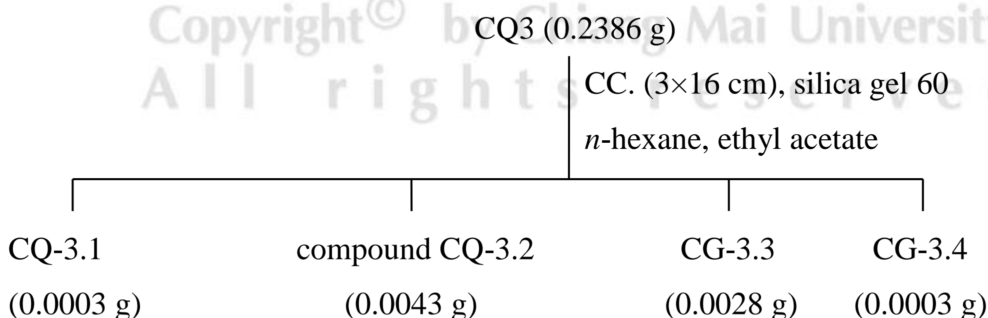

Scheme 3.6 Isolation scheme of compound CQ-3.2

2) Isolation of compounds CQ-4.2 from fraction CQ4

Fraction CQ4 (0.3258 g) was shown by TLC to contain at least two compounds. It was dissolved in small amount of *n*-hexane and packed onto the top of wet silica gel column (3×16 cm). The column was eluted with *n*-hexane, *n*-hexane : ethyl acetate (9:1) then washed with methanol until no traces of compounds could be detected. Fifty fractions, each of approximately 50 mL, were collected. The volumes of eluent were 300, 2500 and 500 mL, respectively. The fractions were examined by TLC and the liked fractions were combined to give the following portions :-

2.1) Fractions 1-9 were combined and designated as fraction CG-4.1 (0.0007 g).

2.2) Fractions 10-16 were combined and the TLC chromatogram shown only one spot under UV light at 254 nm, R_f 0.42 [silica gel/*n*-hexane : ethyl acetate (9:1)]. These fractions were evaporated under reduced pressure to give 0.0065 g of compound CQ-4.2 as colorless needles crystals (0.06% based on dried weight of crude plant extract). It was later identified as 3*β*-Taraxerol.

2.3) Fractions 17-22 were combined and designated as fraction CQ-4.3 (0.0043 g).

2.4) Fractions 23-34 were combined and designated as fraction CQ-4.4 (0.0123 g).

2.5) Fractions 35-50 were combined and designated as fraction CQ-4.5 (0.0013 g).

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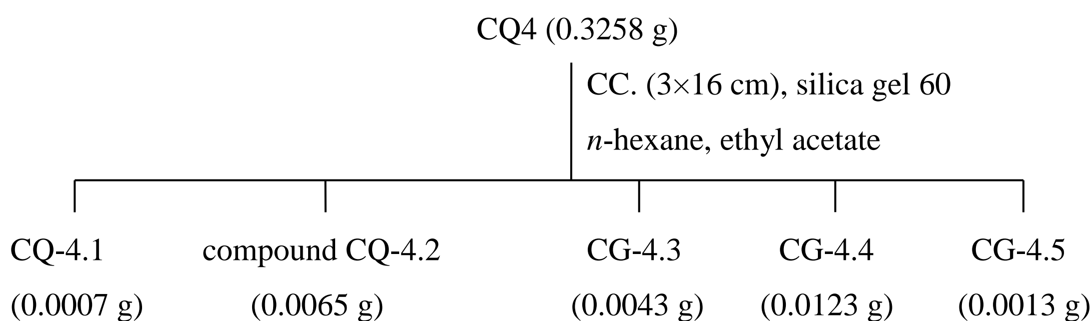

Scheme 3.7 Isolation scheme of compound CQ-4.2

3.8 Biological activities determination

All extracts and the volatile oils were determined for their biological activities. The most biological active fractions of each test were selected for further fractionation by chromatographic techniques including normal phase flash column chromatography, normal phase column chromatography and preparative TLC. The isolated compounds were also investigated for their biological activities.

3.8.1 Antibacterial activity tests

The crude plant extracts and isolated compound obtained from the above procedure will be tested for antibacterial activities.

Three bacterial strains, representing human bacterial pathogens were used for the antibacterial activity test. One gram-positive bacterium was *Staphylococcus aureus* (ATCC 25923). The two gram-negative bacteria were *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Furthermore, the following bacteria were used : *Staphylococcus aureus* ATCC 25923, *S. aureus* MRSA (methicillin resistant), *S. aureus* MSSA sp 127 (methicillin sensitive, clinical isolate from human sputum), *S. aureus* MSSA 507 (clinical isolate from human), *S. aureus* MSSA bal 14 (clinical isolate from human bronchoalveolar lavage), *S. aureus* MSSA sp 116 (clinical isolate from human sputum), *S. aureus* MSSA sp 152 (clinical isolate from human sputum), *Escherichia coli* ATCC 25922, *E. coli* ESBL (-) (extended-spectrum *β*-lactamase negative), *E. coli* ESBL (+) (extended-spectrum *β*-lactamase positive), *E. coli* ESBL (+) u 296 (clinical isolate from human urine), *E. coli* ESBL (-) sp 122 (clinical isolate from human sputum), *E. coli* ESBL (+) u 301 (clinical isolate from human urine), *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* MDS (multidrug-

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sensitive), *P. aeruginosa* MDR (multidrug-resistant), *P. aeruginosa* MDR u 281 (clinical isolate from human urine), *P. aeruginosa* MDS sp 149 (clinical isolate from human sputum), *Klebsiella pneumoniae* ESBL (-), *K. pneumonia* ESBL (+), *Enterobacter cloacae*, *Serratia marcescens*, *Acinetobacter baumannii* MDS and *A. baumannii* MDR.

1) Agar dilution method

The agar dilution method is used to determine the minimum inhibitory concentration (MIC) of extract for antibacterial activity against gram-positive and gram-negative bacteria [Ferraro, 2000].

1.1) Preparing agar dilution plates

1.1.1) One g of *n*-hexane, dichloromethane and metanolic crude extract of *Combretum* species leaves were resuspended with *n*-hexane, dichloromethane and metanol, respectively to 10 mL (concentration of each crude extract = 100,000 μg/mL). Each of the redissolved extract is two-fold serially diluted with *n*-hexane, dichloromethane and methanol, respectively to 8-well plates.

1.1.2) Mixed 2 mL of each concentration solution from 1.1.1) with 18 mL of autoclaved Mueller-Hinton agar.

1.1.3) The mixtures of 1.1.2) were poured quickly after mixing into sterile Petri dishes (9 cm). The concentrations were 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156.25, 78.12 μg/mL, respectively.

1.1.4) Twenty mL of autoclaved Mueller-Hinton agar were used as positive control and the mixture of 18 mL of autoclaved Mueller-Hinton agar with 2 mL of *n*-hexane, dichloromethane and methanol were used as solvent control.

1.2) Preparing the inoculums

A standardized inoculum for the agar dilution method was prepared by either growing microorganisms to show the turbidity of the 0.5 McFarland standard, by suspended pathogenic bacteria in Trypti-case soy broth.

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1.3) Inoculating agar dilution plates

1.3.1) Each inoculum was applied to the agar surface by Multiple Inoculator.

1.3.2) The pathogenic bacteria were inoculated.

1.3.3) A control plate was inoculated first, then starting with the lowest concentration of samples tested. A second control plate was inoculated last to ensure that there was no contamination or significant antimicrobial carry-over during the inoculation.

1.4) Incubating agar dilution plates

The inoculated plates are allowed to stand at room temperature until the moisture in the inoculum spots had been absorbed into the agar, until the spots are dry. The plates were inverted and incubated at 37 °C for 18-24 h.

1.5) Determinating agar dilution end points

The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth, disregarding a single colony or a faint haze caused by the inoculum.

2) Agar disc diffusion method

The agar disc diffusion assay was used at last for pure compound and volatile oils [Washington, 1981].

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2.1) Preparing agar disc diffusion plates

Petri dishes with 20 mL of Mueller-Hinton agar were prepared. Samples were dissolved in methanol then 5 μL of test concentration (1 mg/mL) added to discs and the same volume (5 μL) of solvents was used as a control.

2.2) Preparing the inoculums

Microbial cell suspensions were used in a similar way as the dilution method.

2.3) Inoculating agar disc diffusion plates

The innoculi were spread on the agar surface. Discs with 6 mm diameter were put on the agar plate and 5 μL of samples were loaded into the discs.

2.4) Incubating agar disc diffusion plates

The inoculated plates were run in a similar way as the dilution method.

2.5) Determinating agar disc diffusion end points

The clear zone diameters were measured after incubation. The measurements were done basically from the edge of the zone to the opposite edge of the zone.

3) Screening of antibacterial activity of crude extracts by agar dilution method

Crude extracts were screened for antibacterial activity against *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, and determined by the agar dilution method [Ferraro, 2000].

4) Determination of MIC (Minimum Inhibitory Concentration) of crude extracts against pathogenic bacteria by agar dilution method

MIC of crude extracts against pathogenic bacteria were determined by the agar dilution method [Ferraro, 2000].

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5) Determination of clear zone diameter of isolated compound of *Combretum* species against pathogenic bacteria by agar disc diffusion method

The isolated compounds and volatile oils were tested for antibacterial activity against pathogenic bacteria by the agar disc diffusion method [Washington, 1981].

3.8.2 Antifungal activity tests

The crude plant extracts obtained from the above procedure will be tested for antifungal activity.

Three fungi strains, representing human fungi pathogens were used for the antifungal activity test. The three fungi were *Aspergillus flavus*, *Candida albicans* and *Trichophyton mentagrophytes*.

1) Agar well diffusion method

The agar well diffusion assay was used for crude plant extracts [Adeniyi, 1996].

1.1) Preparing agar well diffusion plates

Petri dishes with 20 mL of Mueller-Hinton agar were prepared. Samples were dissolved in methanol then 200 µL of test concentration (25 mg/mL) added to well and the same volume (200 µL) of solvents was used as a control.

1.2) Preparing the inoculums

Microbial cell suspensions were used in a similar way as the dilution method.

1.3) Inoculating agar well diffusion plates

The innoculi were spread on the agar surface. In each of the plates, wells with 9 mm diameter were cut out using sterile cork borer on the agar and 200 µL of samples were loaded into the wells.

1.4) Incubating agar well diffusion plates

The inoculated plates were run in a similar way as the dilution method.

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1.5) Determinating agar well diffusion end points

The clear zone diameters were measured after incubation. The measurements were done basically from the edge of the zone to the opposite edge of the zone.

2) Screening of antifungal activity of crude extracts by agar well diffusion method

Crude extracts were screened for antifungal activity against *Aspergillus flavus*, *Candida albicans* and *Trichophyton mentagrophytes*, and determined by the agar well diffusion method [Adeniyi, 1996].

3) Determination of clear zone diameter of crude extracts of *Combretum* species against pathogenic fungi by agar well diffusion method

The isolated compound was tested for antifungal activity against pathogenic fungi by the agar well diffusion method [Adeniyi, 1996].

3.8.3 Anti-*Mycobacterium tuberculosis* Activity

Green fluorescent protein (GFP) expressing *Mycobacterium tuberculosis* strain H37Ra was established by Changsen, *et al.* [2003]. H37Ra *gfp* is cultivated on 7H10 agar containing 30 µg/mL of Kanamycin at 37 ºC for 4 weeks or until observed. Starter clutters were prepared by fully looping 2-3 single colony into 7H9 broth supplemented with 0.2% v/v glycerol, 0.1% w/v of casitone, 0.05% v/v Tween 80, 10% v/v Middlebrook OADC enrichment solution (BD Biosciences) and 30 µg/mL of Kanamycin. The mixture was then incubated at 37 ºC in 200 rpm shaker incubator until the optical density (OD) at 550 nm was found to be between 0.5 and 1. For batch cultivation, the starter cultures were transferred at the rate of 1/10 volume to the 7H9 broth and incubated at 37 ºC in 200 rpm shaker incubator until the OD550 nm was approximately 0.5 to 1. The cells were pelleted, washed and suspended in PBS buffer, then sonicated 8 times for 15 sec each. The sonicated samples were then aliquoted and frozen at -80 ºC for up to 2 to 3 months prior to use. Titer stocks were determined by colony forming unit (cfu) assay and the seeding density for anti-TB assay was optimized by serial dilutions. The dilution that grew at logarithmic phase on day 7 would be used as an optimal bacteria seeding density. For assay in 384-well format, the seeding was approximately 2 × 104 to 1 × 105 cfu/mL/well. The assay was performed in duplicate; each well containing 5 µL of test samples serially diluted in 5% dimethylsulfoxide, followed by 45 µL of cell suspension prepared as described above. Plates were incubated at 37 ºC for 7 days and the fluorescence signals were measured using SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom-

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reading mode at the excitation and emission wavelengths of 485 nm and 535 nm, respectively. Fluorescence signals on day zero were used as background, which was used to subtract the signals on day 7. The percentage of growth inhibition was calculated from the mean of fluorescence unit of cells treated with sample (FUt) and untreated cells (FUc), as the following equation:

$$\% \text{ cytotoxicity} = [1 - (FUT / FUC)] \times 100$$

The lowest drug concentration that inhibited cell growth by 90% was reported as the Minimum Inhibitory Concentration (MIC). Rifampicin, streptomycin, isoniazid, ofloxacin and ethambutol were used as positive controls, and 0.5% DMSO was used as negative control.

3.8.4 Antioxidant activity tests

The antioxidant activity of the crude plant extracts obtained from the above procedure were determined by ABTS, DPPH and total phenolic method.

1) ABTS method

The antioxidant activity of the crude plant extracts were investigated using the ABTS radical cation ($ABTS^{•+}$) scavenging assay which was conducted according to the method of Roberta *et al*. [1999], compared with Trolox standards (concentration rang 0.5-2.5 mM). For the ABTS assay, 20 µL of crude plant extracts (0.1 mg/mL) were mixed with 2.0 ml of diluted ABTS solution ($A_{734nm} = 0.700 \pm 0.020$) 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 $ABTS^{•+}$ in percent (I%) was calculated by the following equation :

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

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 was calculated and plotted as a function of concentration of antioxidants and of Trolox and vitamin C for the standard reference data.

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2) DPPH method

A stock solution (5.0×10^{-4} mol/L) of DPPH was prepared by dissolving the appropriate amount (10.0 mg) in 50 mL ethanol. This solution was kept at 4 °C and protected from light, and it was stable during a week. The DPPH working solution containing 1.0×10^{-4} mol/L 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 [Thongchai, 2009].

The test sample (20 µL) was added to 180 µL of 10 µL DPPH solution in a 96-well microtiter plate. The reaction mixture was incubated at 37 °C for 30 min, and then the absorbance of each well was measured at 540 nm. The DPPH 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 crude plant extracts, a graph showing concentration versus % DPPH reduction was plotted. The IC_{50} was calculated from the calibration curve and activity was expressed as the percentage DPPH scavenging relative to the control using the following equation :

$$\text{DPPH scavenging activity (\%)} = [\,(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

3.8.5 Anticancer activity tests

The anticancer activity of the crude plant extracts were performed by using three cancerous human-cell lines are KB cell line (Epidermoid carcinoma of oral cavity, ATCC CCL-17), MCF-7 cell line (Breast adenocarcinoma, ATCC HTB-22) and NCI-H187 cell line (Small cell lung carcinoma, ATCC CRL-5804) and determined by resazurinmicro plate assay (REMA) following a modified method of the use of a fluorescent dye for mammalian cell cytotoxicity according to O'Brien *et al*. [2000]. Ellipticine, doxorubicin and tamoxifen were used as positive controls. 0.5% DMSO and sterile water were used as negative controls. In brief, cells at a logarithmic growth phase were harvested and diluted to 2.2×10^4 cells/mL for KB and 3.3×10^4 cells/mL for MCF-7 and NCI-H187 in fresh medium. Successively, 5 µL of test sample diluted in 5% DMSO, and 45 µL of cell suspension are added to 384-well plates, incubated at 37

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°C 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 is added to each well, and the plates are then incubated at 37 °C for 4 h. Fluorescence signal is measured using SpectraMaxM5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. Percent inhibition of cell growth is calculated by the following equation:

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

Whereas FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively. Dose response curves are plotted from 6 concentrations of 3-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).

1) Cytotoxicity assay

1.1) Sample preparation

The dried extracts of four *Combretum* spicies were weighed and dissolved in dimethylsulfoxide (DMSO) to make a stock concentration of 100 mg/mL. The samples were then serial diluted in the culture medium of cells at a ratio of 1:2 giving 8 concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 µg/mL [Plumb, 1989].

1.2) Cell culture

The target cells were Vero (African green monkey kidney; ATCC Cat. No. CCL-81).

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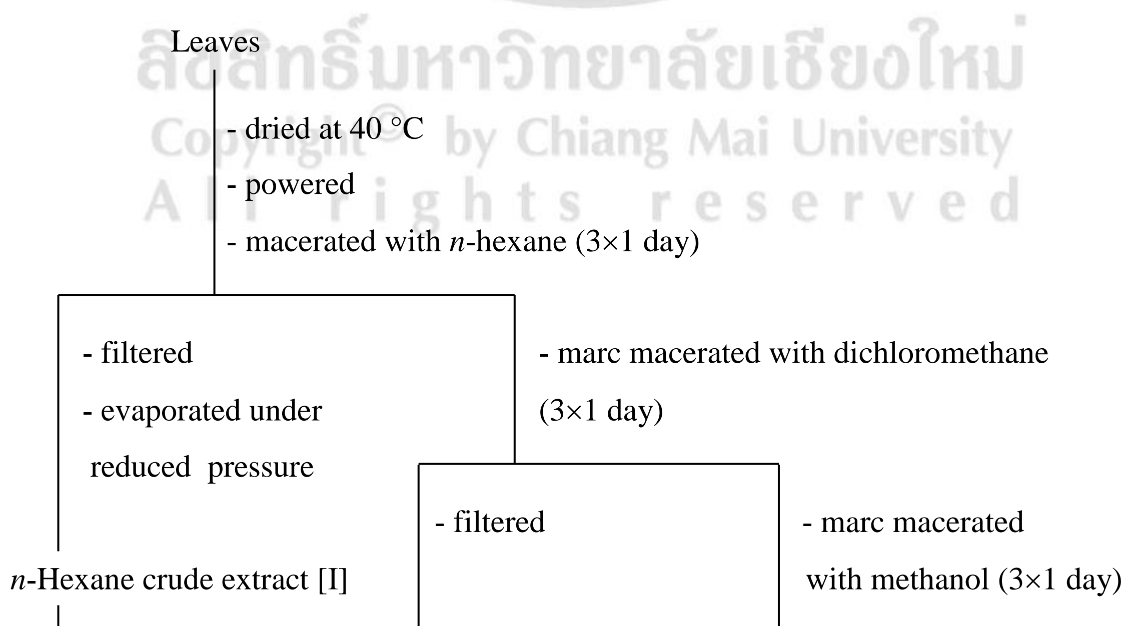
The cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 0.1 mM non essential amino acid, 100 unit/ml penicillin and 100 µg/mL streptomycin. The cells were incubated at 37 °C in a fully humidified, 5% CO_2 : air atmosphere [Plumb, 1989].

2) MTT cytotoxicity test

This assay was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and

ISO10993-5). The MTT assay [Plumb, 1989] is a tetrazolium-dye based colorimetric microtitration assay. Metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It is assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability. The cells were seeded in a 96-well plate at a density of 3,000 cells/well, and incubated for 48 h. The sample at various concentrations were added to the cells and incubated for 24 h. The test samples were removed from the cell cultures and the cells were reincubated for a further 24 h in fresh medium and then tested with MTT assay. Briefly, 50 µl of MTT in PBS at 5 mg/mL was added to the medium in each well and the cells were incubated for 4 h. Medium and MTT were then aspirated from the wells, and formazan solubilized with 200 µL of DMSO and 25 µL of Sorensen's Glycine buffer, pH 10.5. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each toxin sample. A dose-response curve was derived from 8 concentrations in the test range using 4 wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compared to controls.

Leaves were collected, extracted and determined by summary procedure as following:



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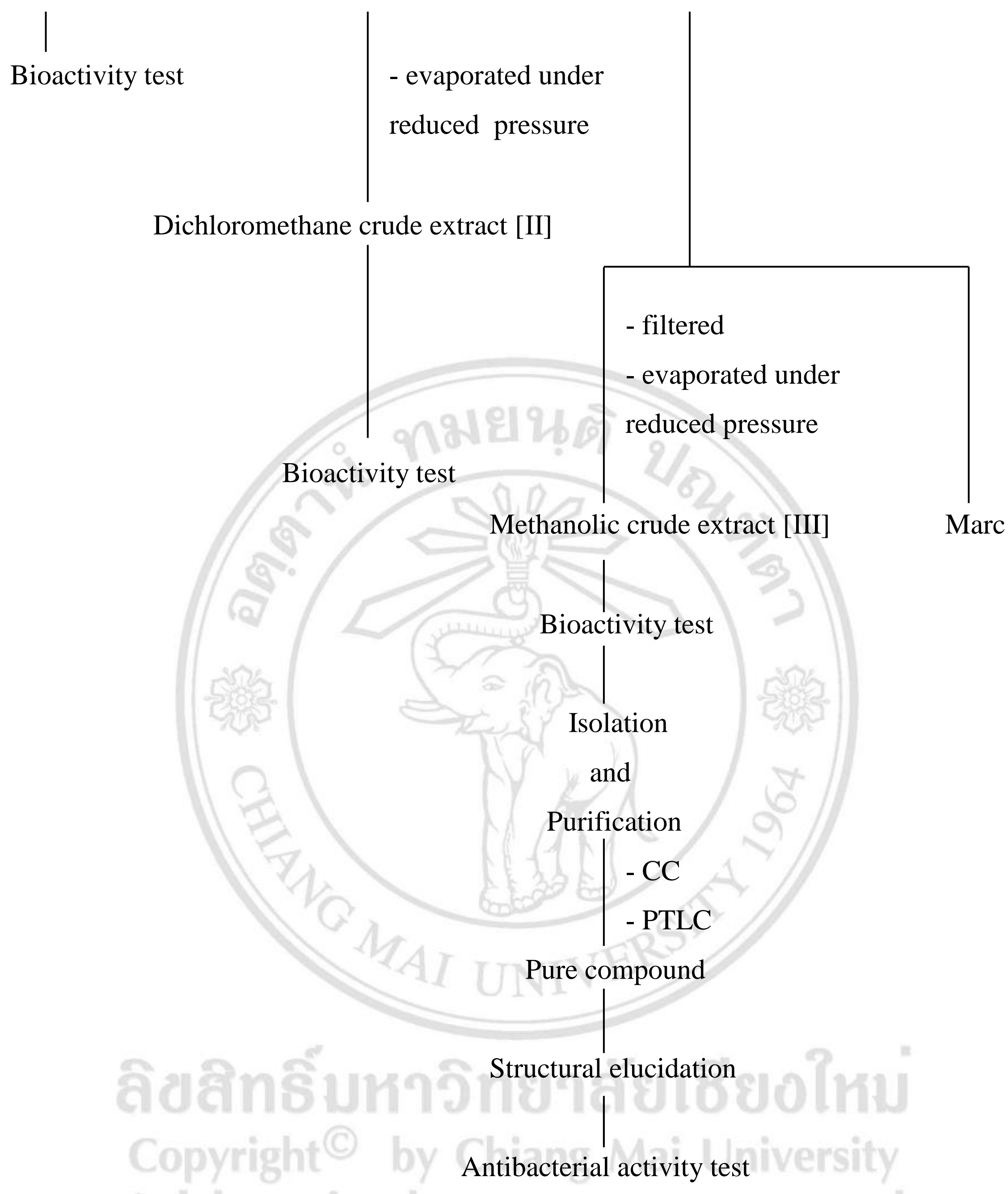

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Scheme 3.8 The extraction, isolation, purification and bioactivity test