CHAPTER 2

RESEARCH METHODOLOGY

2.1 Instruments

^{1}H and ^{13}C-Nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectra were recorded on a Fourier Transform NMR spectrometer (FTNMR), Varian UNITY INOVA 500 MHz using either operating solvent or tetramethylsilane (TMS) as an internal standard. Spectra were recorded as chemical shift parameter (δ) value in ppm scale (J in Hz). EI-MS data were recorded by MAT 95 XL Mass Spectrometer which runs low resolution technique with direct insert probe (DIP probe). Infrared spectra were recorded using FTS FT-IR spectrophotometer and major bands (□) were recorded in wave number (cm^{-1}). Ultraviolet (UV) absorption spectra were recorded using a SPECORD S 100 (Analytikjena). Optical rotations were obtained from a JASCO P-1020 polarimeter. The absorbance (OD) for free radical scavenging activity was measured at 520 nm and for lipid peroxidation formation was measured at 532 nm and the absorbance of each well in anti HIV-1 integrase assay was measured at 405 nm, using a power Wave X plate reader (Bio-TEK Instruments, Inc.). Silica gel 60 (Merck, 0.063-0.200 mm) was used for vacuum liquid chromatography (VLC). Silica gel 60 (Merck, 0.040-0.060 mm) and Sephadex ™ LH-20 were used for column chromatography (CC). The High performance liquid chromatographic (HPLC) apparatus was a Shimadzu SCL-10*Avp* equipped with ultraviolet visible (UV-vis) detector (SPD-10 *Avp*) and automatic injector (SIL-10 AD*vp*) was made to evaluate astilbin content. Chromatographic separation was carried out at room temperature using a μBondapak™ C18 analytical column (300 mm x 3.9 mm I.D., 10 μm).

2.2 Plant Materials

Smilax corbularia Kunth was collected from Petchaboon province. Authentications of plant materials were carried out at the herbarium of the Department of Forestry Bangkok, Thailand, where the herbarium vouchers have been kept to specify plant and species identified. The other of this plant has been kept as specimen in the herbarium of Southern Center of Thai Medicinal plant at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand (SKP 179001003).

2.3 Preparation of plant extracts

Rhizomes of this plant were washed with water to remove the remaining sand and to reduce the microbial load. The cleaned plant materials were cut into small pieces and dried at 50 ^{o}C, powdered and extracted in a similar way to that practiced by Thai traditional doctors such as ethanolic extraction.

2.3.1 Ethanolic extract

The dried ground plant materials (332.5 g) were macerated with 95 % ethanol for 3 days, filtered and concentrated to dryness under pressure. The marc was macerated 2 times and dried by evaporator. All extracts were combined and calculated for the percentage of yield.

Figure 2-1 Rhizomes of *Smilax corbularia* Kunth

2.4 Assays for antioxidant activity

The antioxidant activity of the extract was evaluated by DPPH radical scavenging assay, originally described by Blois (1958) and lipid peroxidation of liposome assay, originally described by Uchiyama and Mihara (1978).

2.4.1 DPPH radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a unstable radical because of the paramagnetism conferred by its odd electron (delocalization of the spare electron over the molecule as a whole). The solution (in absolute ethanol) appears as a deep violet color and shows a strong absorption band at 520 nm. The DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule, in which the absorption vanishes and the resulting decolorization is stomachiometric with the number of electrons taken up; the solution has pale violet color (Blois, 1958). A DPPH solution having a concentration of $6x10^{-5}$ M was used in the present study since at this low concentration the color is not too dense and the Lambert-Beer law is obeyed. If the tested substance is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has an antioxidant effect by the mechanism of free radical scavenging activity. The following assay procedure was modified from those described by Yamasaki *et al.,* (1994).

$$DPPH^{\cdot} + AH \longrightarrow DPPH\text{-}H + A^{\cdot}$$

Samples for testing were dissolved in either alcohol (e.g. absolute ethanol and methanol) or distilled water to obtain the highest concentration of 200 μg/ml. Each sample was further diluted for at least 5 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of sample solution (500 μl) was mixed with an equal volume of $6x10^{-5}$ M DPPH (in absolute ethanol) and allowed to stand at room temperature for 30 min. The absorbance (A) was then measured at 520 nm. BHT (butylated hydroxytoluene), a well known synthetic

antioxidant, was tested in the same system as a positive standard. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The result was expressed as percentage inhibition in the formulae below:-

$$\%\text{inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50 %) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations.

2.4.2 Lipid peroxidation of liposome assay

The principal components of the assay, apart from a standardized liposome source (such as a brain extract), are ascorbic acid and an iron source, such as $FeCl_3$. Ascorbic acid plays a key role in the initiation of the lipid peroxidation reaction. To get to the point, ascorbic acid, having pro-oxidant properties in the presence of transition metal such as iron (Fe), will reduce Fe (III) to Fe (II):

$$Fe^{3+} + \text{ascorbic acid} \longrightarrow Fe^{2+} + \text{ascorbate}^{\bullet}$$

The latter will react with atmospheric oxygen giving rise to the formation of superoxide:

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\bullet -}$$

The superoxide can then dismutate to form hydrogen peroxide:

$$2\ O_2^{\bullet -} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide can in turn react with Fe^{2+} to form hydroxyl radical:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH + OH^{\bullet -}$$

These reactions underlie the lipid peroxidation of liposomes assay and are utilized to test for antioxidant behavior. The genesis of hydroxyl radicals induces damage to liposomes, whereas addition of an antioxidant will scavenge free radicals and thus prevent this damage. The extent of the damage to liposomes and hence the antioxidant efficacy of any compound can be monitored and quantified with the aid of the thiobarbituric acid (TBA) test (Uchiyama and Mihara, 1978; Halliwell and Gutteridge, 1999).

The TBA test is one of the most frequently used tests for measuring the peroxidation of fatty acids, membranes, and food products. It is based on the fact that peroxidation of most membrane systems leads to formation of small amounts of free malonaldehyde (MDA). In the next step, MDA can react with thiobarbituric acid and generate a pink colored product (Figure 2-2).

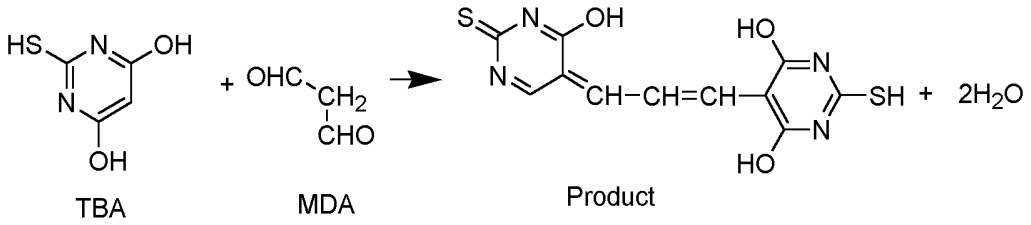


Figure 2-2: The reaction between thiobarbituric acid (TBA) and malonaldehyde (MDA) to produce the pink coloured product.

The product can be measured spectrophotometrically at 532 nm. The yield of the coloured product is proportional to the MDA formed, and thus to the extent of lipid peroxidation. Lipid peroxidation can thus be quantified, as can the protective effect of any compound that acts the occuring form of product. (Halliwell and Gutteridge, 1999)

In acidic solution, the product absorbs light at 532 nm and it is readily extractable into organic solvent such as butan-1-ol. The incorporation of any antioxidant compounds in the lipid peroxidation assay reaction mixture will lead to a reduction of the extent of peroxidation. This will ultimately lead to a lower yield of the chromogenic product, indicated

by a lower absorbance reading. This is essentially the principle of the TBA test, which works well when applied to defined membrane systems, such as liposomes or microsome which are biomembranes and are composed of phospholipid bilayers with protein. They are one of the major targets of ROS, the attack that affects membrane functions by inducting continuous lipid peroxidation (Halliwell and Gutteridge, 1999).

The extract test reaction mixture consisted of 0.2 ml of liposomes which were prepared from a bovine brain extract suspension in phosphate buffered saline (PBS) (5 mg/ml), 0.1 ml $FeCl_3$, 0.1 ml ascorbic acid (1 mM), 0.5 ml PBS and 0.1 ml of ethanolic or water extract to be assessed. Propyl gallate ($1x10^{-4}$ M) is positive control. All test tubes were incubated at 37 ^{o}C for 20 minutes. The lipid peroxidation of liposomes should occur within that incubation period, unless the test substance exerted a protective antioxidant effect.

The extract was prepared as a 10 % stock solution and dilutions were prepared to 5.0, 1, 0.5, 0.1, 0.05, 0.01 and 0.05 mg/ml. For a control, the extract was omitted and solvent (ethanol or water) was added instead to form the so-called full reaction mixture (FRM). The absorbance of liposome suspension alone was assayed after incubation also, in case it self-peroxidised during the incubation period. The extract alone was also assayed, in order for its absorbance reading to be taken in consideration as at a later stage. The assay was approved by a positive test performed by adding 0.1 ml propylgallate ($1x10^{-4}$ M), as known antioxidant that should prevent lipid peroxidation, in tubes of the reaction mixture. Four replicates were carried out for each mixture.

The TBA test was performed after the 20 minute incubation by adding 0.1 ml of 2 % butylated hydroxytoluene (BHT) in ethanol (another antioxidant to prevent lipid peroxidation during the TBA test itself) followed by 0.5 ml of 1 %w/v thiobarbituric acid (TBA) in 50 mM NaOH and 0.5 ml of 25 % HCl. The full reaction mixture was completed after adding the TBA reagents, heated to 85 ^{o}C for 30 minutes and then allowed to cool completely for at least 0.5-1 hour. At the end of this incubation period at 85 ^{o}C, thiobarbituric acid should have formed a coloured adduct with malonaldehyde.

As the lipid suspension would be turbid and unsuitable for spectroscopic analysis, the chromogen was extracted into 2.5 ml butanol in each tube. The mixture was vortexed to ensure complete extraction of the chromogen and then centrifuged at 3500 rpm for 20 minutes

at room temperature in order to separate the two layers. The upper layer, which contain the chromogen, was pipetted into a cuvette for each sample. Absorbance was read at 532 nm for all replicates of all reactions and mean absorbance were calculated (Uchiyama and Mihira, 1978).

The % inhibition of lipid peroxidation was assessed by comparing the absorbance of the full reaction mixture with that of the extract test reaction mixtures where the substance to be assessed was included. For calculation was followed**:**

% inhibition= 100 x [(FRM-B)-(ET-B-EA)]/(FRM-B)

FRM = Absorbance of the full reaction mixture (liposome and iron source plus solvent without the test substance)

B = Absorbance of the blank mixture (lyposome only)

ET= Absorbance of the extract test mixture (full reaction mixture plus test substance)

EA= Absorbance due to the extract alone

The EC_{50} value (effective concentration of sample required to scavenge radical by 50 %) was obtained by linear regression analysis of the dose response curve plotting % inhibition against concentration from prism program. The experiments for each extracts were calculated for mean and standard error of mean (SEM) values.

2.5 Assay for HIV-1 IN Inhibitory activity

2.5.1 Enzyme

HIV-1 IN protein was kindly provided by Dr. Robert Craigie (the National Institute of Health, Bethesda, Maryland, USA), and stored at -80 ^{o}C before use.

2.5.2 Oligonucleotide substrates

Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIAGEN Operon, USA and stored at -25 ^{o}C before use. The sequence of biotinylated LTR donor DNA and its unlabelled complement were 5′-biotin-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′ (LTR-D1) and 3′-GAAAATCAGTCACACCTTTTAGAGATCGTCA-5′ (LTR-D2) respectively; while those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3′-labelled complement were 5′-TGACCAAGGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAGTGA-5′ (TS-2), respectively.

2.5.3 Multiplate Integration Assay (MIA) Procedure

2.5.3.1 Annealing of the Substrate DNA

Firstly, LTR-D1 and LTR-D2, TS-1 and TS-2 were mixed separately and then the former solution was diluted to a concentration of 2 pmol/ml, while the later one was made to 5 pmol/ml using a buffer solution containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 100 mM KCl. Both solutions were then heated at 85 ^{o}C for 15 min in an incubator. After heating, each solution is gradually cooled to room temperature and was stored at -20 ^{o}C until use.

2.5.3.2 Pretreatment of the multiplate

A 96-well plate was coated with 50 μl of a steptavidin solution containing 40 μg/ml steptavidin, 90 mM Na_2CO_3 and 10 mM KCl. After discarding steptavidin coating solution, the coated plate was washed with 300 μl of sterile water twice and phosphate buffer saline (PBS, 300 ml) twice. The blocking buffer (300 μl) containing 1 % skin milk in PBS was added into each well and the plate was kept at room temperature for 30 min. After discarding the

blocking buffer, each well was washed with PBS solution (300 μl) four times and then the PBS solution was completely removed. A biotinylated LTR donor DNA (50 μl) solution containing 10 mM Tris-HCl (pH 8.0), 1 mM NaCl and 40 fmol/ml of LTR donor DNA was added in to each well and the plate was shaken well, centrifugated and kept gently at room temperature for 60 min. After discarding the LTR donor solution, the microplate was washed with PBS solution four times and then each well was filled with 300 μl of PBS solution. Just before the integration reaction, the PBS solution of each well was discarded and rinsed with 300 μl of distilled water four times, and then the distilled water was removed completely.

2.5.3.3 Integration reaction

The integration reaction was evaluated according to the method previously described (Tewtrakul *et al.,* 2001). A mixer (45 μl) composed of 12 μl of integrase buffer [containing 150 mM 3-(N-morpholino) propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25 % glycerol and 500 μg/ml bovine serum albumin], 1 ml of 5 pmol/ml digoxigenin-labelled target DNA and 32 μl of sterilized water were added into each well of a 96-well plate. Subsequently, 6 ml of sample solution and 9 μl of 1/5 dilution of integrase enzyme was added to the plate and incubate at 37 ^{o}C for 80 min. After wells were washed with PBS four times, 100 ml of 500 mU/ml alkaline phosphatase (AP) labeled anti-digoxigenin antibody were added and incubate at 37 ^{o}C for 1 hr. The plate was washed again with washing buffer containing 0.05 % Tween 20 in PBS four times and with PBS four times. Then, AP buffer (150 μl) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM p-nitrophenyl phosphate was added to each well and incubated at 37 ^{o}C for 1 hr.

Finally, the plate was measured using a microplate reader at a wavelength of 405 nm. A control composed of a reaction mixture, 50 % DMSO and an integrase enzyme, while a blank is buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM

ethylenediaminetetraacetate disodium salt (EDTA. 2Na), 0.1 % Nonidet-P 40 (NP-40), 20 % glycerol, 1 mM DTT and 4 M urea without the integrase enzyme. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control.

% Inhibition against HIV-1 IN = [(OD control - OD sample)/ OD control] x100

OD= absorbance detected from each well

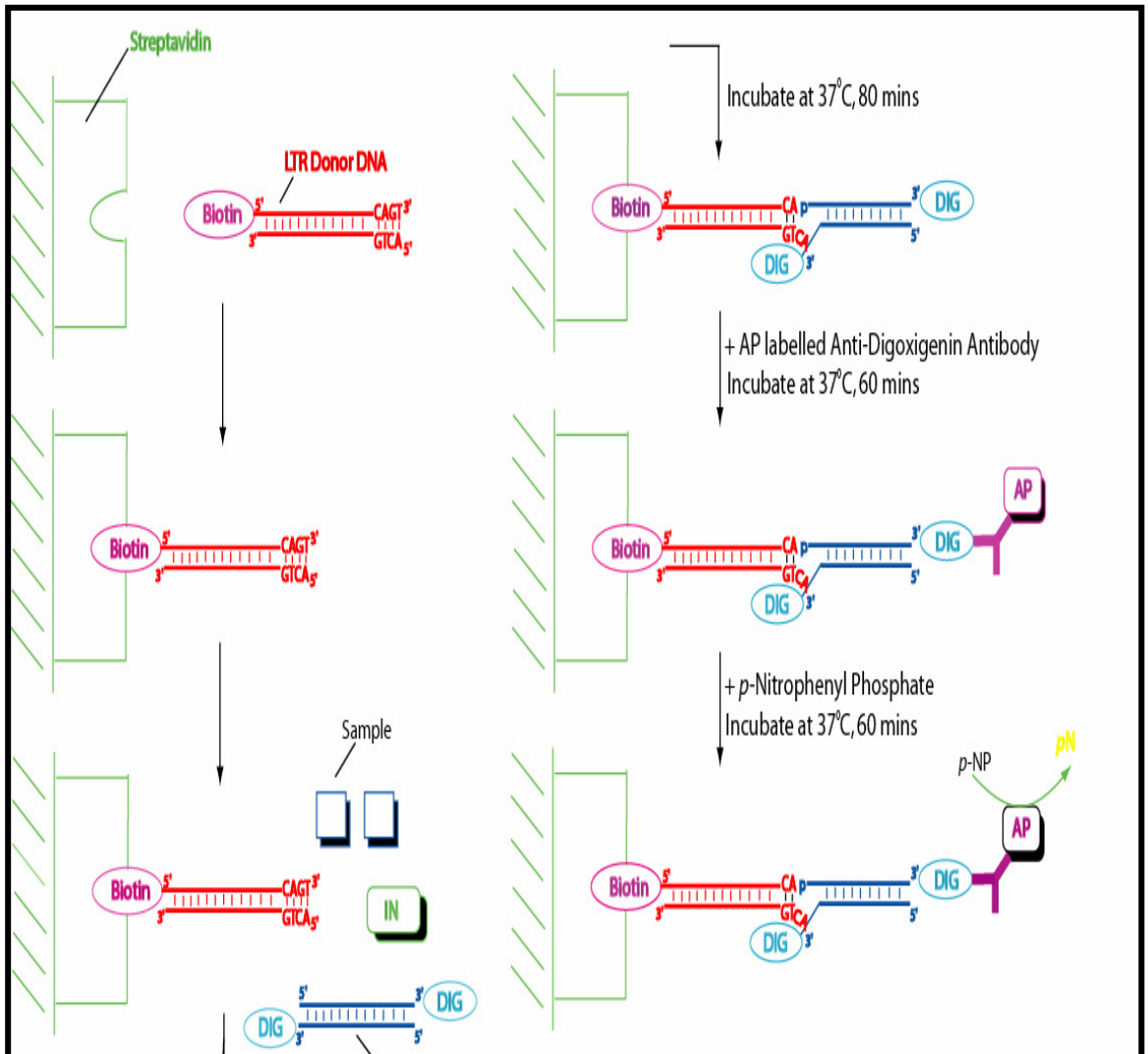


Figure 2-3 Diagram of the multiplate integration assay using the 96-well plate

2.6 Bioassay-guided fractionation

The gradient polarity of solvents (1000 ml for each solvent) for 6 fractions were hexane, hexane: $CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$: MeOH (1:1) and MeOH. Each fraction was concentrated by evaporator. The fraction of $CHCl_3$: MeOH (1:1) showed supernatant and precipitate. All fractions were tested for anti HIV-1 integrase activity.

The previous report found that the ethanolic extract of *Smilax corbularia* exhibited the highest activity against HIV-1 IN (IC_{50}=1.9 μg/ml) (Tewtrakul *et al.,* 2006).

Therefore the ethanolic extract of its rhizome was separated by vacuum liquid chromatography (VLC). Silica gel 60 (300 grams) was packed in filtering funnel. The ethanol extract (40 grams) was dissolved in methanol and added with silica gel 60 (60 grams). Then, the mixture was dried and ground with ceramic grinder. The ground silica mixture was loaded at the top of filtering funnel as show in figure 2-4. The elution solvents were hexane (2000 ml), hexane:chloroform (1:1) (2000 ml), chloroform (2000 ml), chloroform:methanol (1:1) (5000 ml) and methanol (7000 ml). Each fraction was concentrated by evaporator. The yields of fractions eluted by hexane, hexane: $CHCl_3$(1:1), $CHCl_3$, $CHCl_3$: MeOH (S), $CHCl_3$: MeOH (P) and MeOH were 0.0201, 0.2721, 0.1767, 28.0, 2.0 and 9.3 g, respectively. All six fractions were tested for free radical scavenging activity (section 3.1.1), lipid peroxidation of liposome assay (section 3.1.2) and anti HIV-1 integrase activity (section 3.1.3).

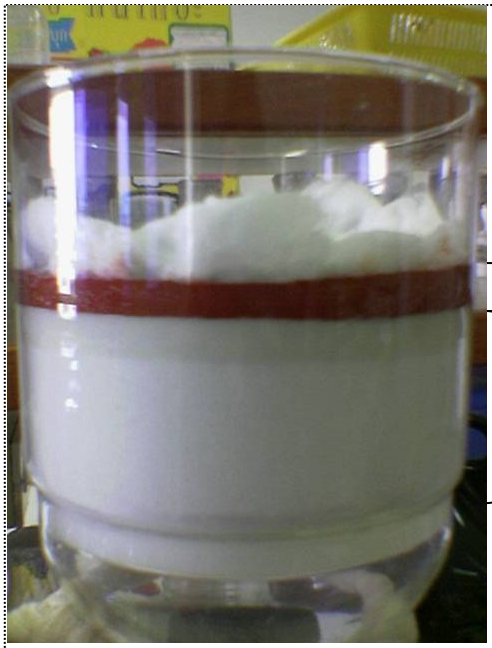


Figure 2-4 VLC column packing

2.7 Isolation of chemical constituents from *Smilax corbularia* Kunth

2.7.1 Isolation method for astilbin and engeletin

An aliquot (5 g) of $CHCl_3$: MeOH (S) was separated under a silica gel column using chloroform: methanol (7:3) as eluent, and finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure as follows:

The flow chart for compound separation was showed in figure 2-5. Ten fractions were obtained including A, B, C, D, E, F, G, H, I and J, giving yields as 67.2, 145.9, 52.4, 1.304, 998.6, 863, 501.4, 308.3, 501.3 and 104.2 mg respectively. Fraction E was crystallized by MeOH (721.2 mg) giving a code as E1 and supernatant (277 mg) as E2.

An aliquot of E1 (710 mg) was further separated under a silica gel column using chloroform:methanol (8.5:1.5) as eluent, finally the column was washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure.

A pure compound (2.7 mg) obtained from fraction X1 was given a code as SC5. It was yellow brown crystal. Its structure was elucidated using ^{1}H, ^{13}C-NMR and MS, and identified as engeletin as destribed in page 102 or section 3.2.1.5.

A pure compound (49 mg) obtained from fraction X3 was given a code as SC4. It was white crystal. The structure analysed using ^{1}H, ^{13}C-NMR and MS was identified as astilbin as described in structure elucidation part in page 95 or section 3.2.1.4.

2.7.2 Isolation method of quercetin, β-sitosterol-3-*O*-β-D glucopyranoside and β-sitosterol

An aliquot (5 g) of $CHCl_3$: MeOH (S) was chromatographed over a silica gel column using chloroform:methanol (8.5:1.5) as eluent, and finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure as follows:

The flow chart for compound separation was showed in figure 2-6. Ten fractions were obtained including A, B, C, D, E, F, G, H, I and J, giving yields as 1.5, 31.9, 22.0, 24.5, 42.7, 45.5, 91.9, 1276.3, 633.9 and 1365.1 mg, respectively.

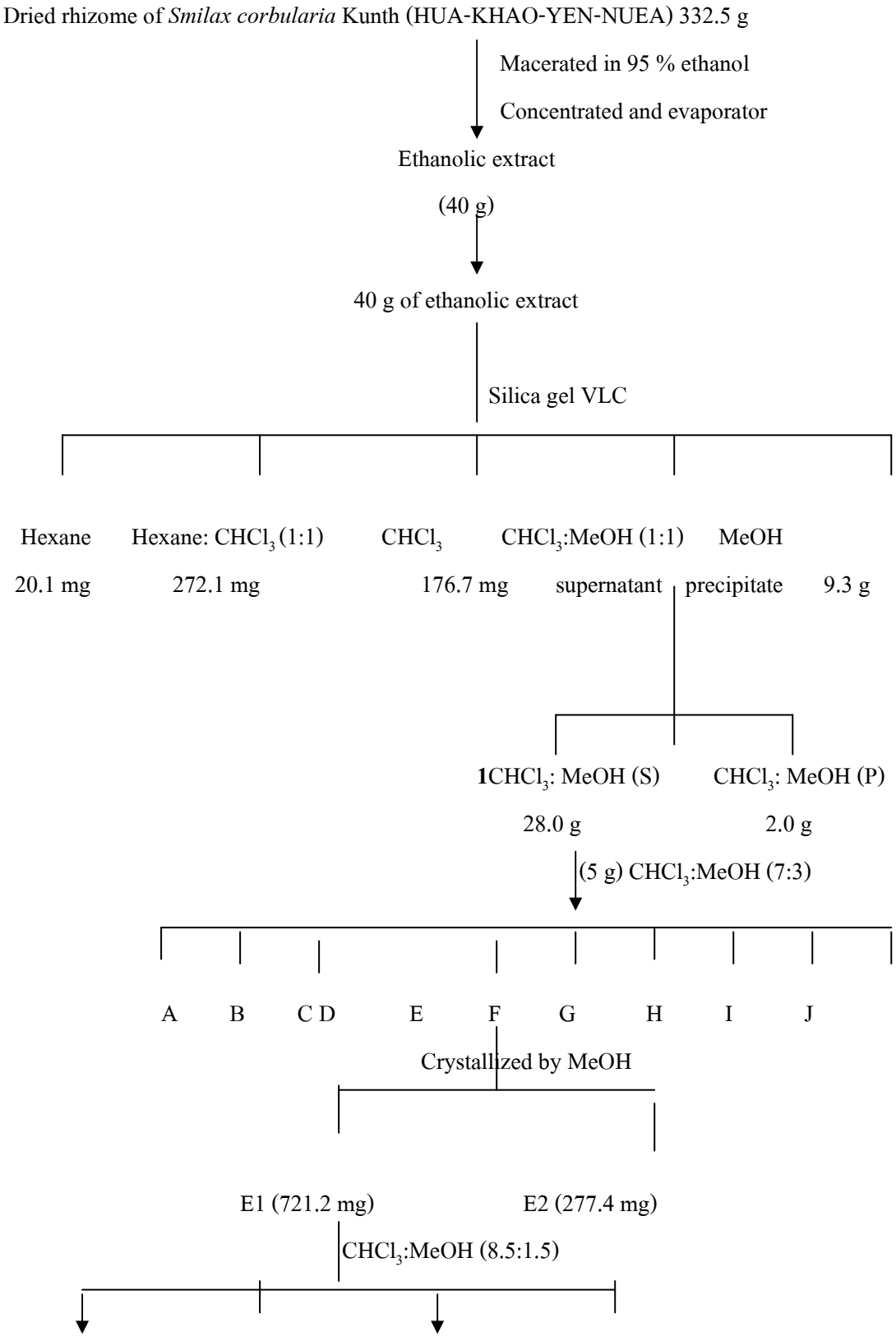
A pure compound (SC1) from fraction A was white solids (1.5 mg). It was identified by ^{1}H-NMR as β-sitosterol.

Fraction E was obtained as white yellow crystals (42.7 mg). The total white yellow crystals were washed with chloroform to obtain SC2 (a pure compound), as white crystals (3.0 mg). It was identified by ^{1}H-NMR as β-sitosterol-3-*O*-β-D-glucopyranoside.

An aliquot (44.0 mg) of F was chromatographed over a silica gel column using chloroform:methanol (9:1) as eluent finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure as follows:

The flow chart of separated compounds showed in figure 2-6. They got 3 fractions from F1, F2 and F3, giving yield as 16.0, 12.6 and 6.2 mg, respectively.

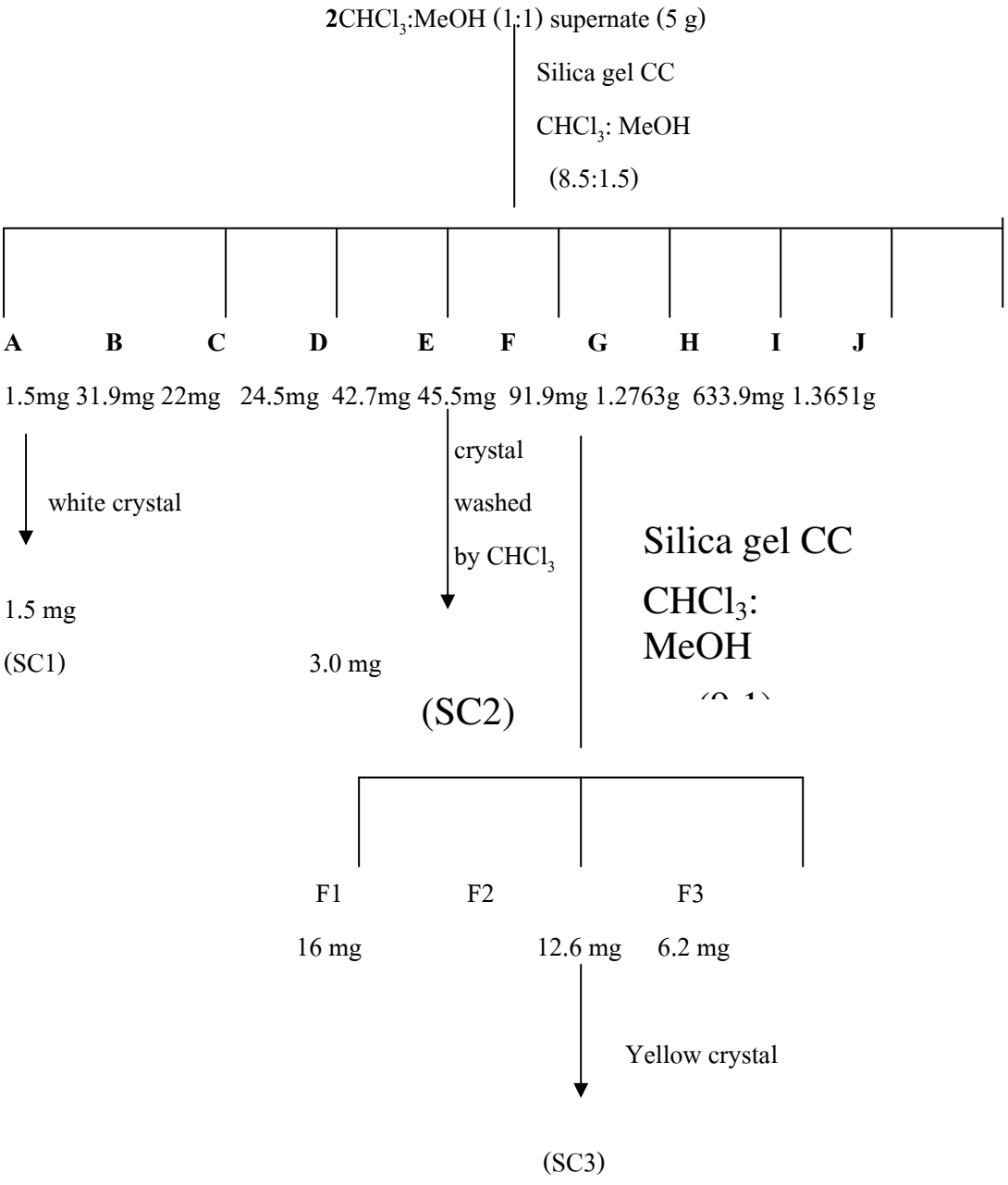
A pure compound (SC3) from fraction F2 was yellow crystals (12.6 mg). It was identified by ^{1}H-NMR and compared with authentic sample as quercetin.


Dried rhizome of *Smilax corbularia* Kunth (HUA-KHAO-YEN-NUEA) 332.5 g
Macerated in 95 % ethanol
Concentrated and evaporator
Ethanolic extract
(40 g)
40 g of ethanolic extract
Silica gel VLC
Hexane
20.1 mg
Hexane: $CHCl_3$ (1:1)
272.1 mg
$CHCl_3$
176.7 mg
$CHCl_3$:MeOH (1:1)
supernatant
MeOH
precipitate
9.3 g
1$CHCl_3$: MeOH (S)
28.0 g
$CHCl_3$: MeOH (P)
2.0 g
(5 g) $CHCl_3$:MeOH (7:3)
A B C D E F G H I J
Crystallized by MeOH
E1 (721.2 mg)
E2 (277.4 mg)
$CHCl_3$:MeOH (8.5:1.5)

*X1 (2.7 mg) X2 (66.8 mg) *X3 (49.0 mg) X4 (42.9 mg)

engeletin (SC5) astilbin (SC4)

Figure 2-5 Flow chart of separated compounds from E1 of the ethanolic extract of *Smilax corbularia* Kunth



SC1 = β-sitosterol

SC2 = β-sitosterol-3-*O*-β-D-glucopyranoside

SC3 = quercetin

Figure 2-6 Flow chart of separated compounds from $CHCl_3$:MeOH (1:1) supernate of *Smilax corbularia* Kunth

2.8 Quantitative determination of astilbin and engeletin using HPLC

2.8.1 Reagents

Methanol and acetic acid were chromatographic grade (Labscan, Bangkok, Thailand). Water was purified by a Milli-Q academic water purification system (Milford, MA, US).

Stock solution of astilbin and engeletin; 1.0 mg/ml, was preparaed by dissolving weighted quantities of standard compounds into methanol and was diluted to the desired concentration by using the stock solutions. Series of standard solutions were prepared with the concentrations 1.0-5.0 μg/ml.

2.8.2 Chromatographic conditions

The mobile phase consisted of methanol (A)- water (B)- acetic acid (C) with isocratic elution as follows: 40%A-59.7%B-0.3%C. The flow rate was 1.0 ml/min. The sample volume injected was 20 μl. UV spectra were set at 291 nm for both astilbin and engeletin.

2.8.3 Preparation of plant samples

The sample were prepared by accurately weighing 20.0 mg of each fraction was separated by vacuum liquid chromatography (VLC), adjustively to 10 ml with methanol into a

volumetric flask and the solutions were then sonicated for 15 min. The solutions were diluted with methanol for the ethanol extract to give final concentration in range of 50-200 μg/ml and filtered through a 0.45 μm filter membrane before analysis. Twenty microliters of the sample were directly injected into the HPLC column and separated under above chromatographic conditions.