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). The absorbance (OD) for free radical scavenging activity was measured at 520 nm and for lipid peroxidation formation was measured at 532 nm, the absorbance for the content of total phenolic compounds was measured at 765 nm and the absorbance of each well in cytotoxic activity assay was measured at 492 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-10A*vp* equipped with ultraviolet visible (UV-vis) detector (SPD-10A*vp*) and automatic injector (SIL-10AD*vp*) was made to evaluate gallic acid content. Chromatographic separation was carried out at room temperature using a μBondapak™ C18 analytical column (300 mm×3.9 mm I.D., 10 μm).

2.2 Plant Materials

Fruits of each plant were collected from several regions of Thailand during October - December, 2004 as shown in Table 1. Figures 2-1 to 2-3 indicates typical fruits of *P. emblica*, *T. chebula* and *T. bellerica*, respectively. Authentication 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. Others of these plants have been kept as specimens in the herbarium of Southern Center of Thai Medicinal Plant at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

2.3 Preparation of plant extracts

Fruits of these plants 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 °C, powdered and extracted in a similar way to that practiced by Thai traditional doctors, e.g. water extraction and ethanolic extraction. The formula drug "Triphala" was also extracted in the same procedure.

2.3.1 Water extracts

For each plant, the dried ground fruit materials (300 g) were boiled at 60°C for 30 minutes in distilled water and filtered by filter cloth. Then the filtrate was concentrated and dried by lyophilizer.

2.3.2 Ethanolic extracts

For the ethanolic extracts, the dried ground fruit materials (300 g) was 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.

Table 2-1 Plants and part of plants used in these study

Plant Species	Source (Amphor, Province)	Part Used
Phyllanthus emblica Linn.	Thongphapoom, Kanjanaburi	fruit
Terminalia chebula Retz.	Thongphapoom, Kanjanaburi	fruit
Terminalia bellerica Gaertn.	Rataphoom, Songkhla	fruit

Figure 2-1 Fruits of *Phyllanthus emblica* Linn.

Figure 2-2 Fruits of *Terminalia chebula* Retz.

Figure 2-3 Fruits of *Terminalia bellerica* Gaertn.

2.4 Assay for antioxidant activity

The antioxidant activity of each plant 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 a stable free 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, at 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 the sample solution (500 µl) was mixed with an equal volume of 6×10^{-5}M DPPH (Fluka, 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, Fluka), a well

known synthetic antioxidant, was tested in the same system as a positive standard. The scavenging activity of the samples corresponded to the reduction in the intensity of DPPH. The result was expressed as percentage inhibition:

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

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 lattter 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 The TBA test is one of the most frequently used tested 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 coloured product (Figure2-4).

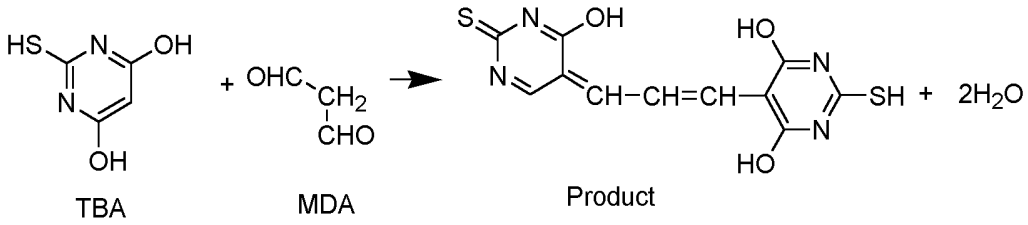

Figure 2-4 The reaction between thiobarbituric acid (TBA) and malonaldehyde (MDA) to produce the pink coloured product, which can be measured spectrophotometrically at 532 nm. The yield of the coloured product is proportional to the MDA formed and thus to the extent of lipid peroxidation. Lipid peroxidation can thus be quantified, as can the protective effect of any compound that acts to prevent it form occuring (Halliwell and Gutteridge, 1999)

In acidic solution, the product formed adsorbs 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 liposome which were prepared from a bovine brain extract suspension in phosphate bufferred saline (PBS), $FeCl_3$, ascorbic acid, PBS and sample 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 TBA test was performed after the 20 minutes incubation by adding butylated hydroxytoluene (BHT) in ethanol followed by thiobarbituric acid (TBA) and hydrochloric acid (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 at least 0.5-1 hour. At the end of this incubation period at 85^{o}C, TBA should have formed a coloured adduct with MDA. As the lipid suspension would be turbid and unsuitable for spectroscopic analysis, the chromogen was extracted into 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 96-well plate for each sample. Absorbance was read at 532 nm for all replicates of all reactions and mean absorbance were calculated (Uchiyama and Mihara, 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 follwed:

$$\% \text{ inhibition} = 100 \times [(FRM - B) - (ET - B - EA)]/(FRM - B)$$

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

B = Absorbance of the blank mixture (liposome 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 mean and standard error of mean (SEM) values.

2.5 The content of total phenolic compounds

It is a colorimetric oxidation/reduction that measures all phenolic compounds. The Folin-Ciocalteu reagent is a solution of polymeric complex ions formed from phosphomolybdic acid and phosphotungstic heteropoly acid. It oxidizes phenolates (alkali salts), reducing the heteropoly acid to a phosphowolframate-phosphomolybdate complex. The phenolates are only present in alkaline solution but the reagent and products are alkali unstable.

The content of total phenolic compounds was determined by Folin-Ciocalteu method (1927). This method is used gallic acid as a standard, for the preparation of calibration curve 20 µl aliquots of 5, 10, 20, 40, 80 and 100 µg/ml ethanolic gallic acid solutions were mixed with 100 µl Folin-Ciocalteu reagent (diluted ten-fold) and 80 µl (75 g/l) sodium carbonate. The absorption was read after 30 minutes at room temperature at 765 nm and the calibration curve was drawn. 20 µl of plant extract (100 µg/ml) was mixed with the same reagents as described above, and after 30 minutes the adsorption was measured for the determination of plant phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in plant extracts are expressed as gallic acid equivalents (GAE). GAE was calculated by the following formula (Miliauskas *et al*., 2004).

$$C = c \bullet V/m$$

C = total content of phenolic compounds (mg/g plant extract) in GAE

c = the concentration of gallic acid established from the calibration curve (µg/ml)

V = the volume of extract (ml)

m = the weight of plant extract (mg)

2.6 *In vitro* assay for cytotoxic activity

The antiproliferative assay, SRB (sulphorhodamine B) assay, was performed according to the method of Skehan *et al*. (1990). This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. The principle of SRB, which is a bright pink aminoxanthene dye, is that it is an anionic protein stain containing two sulphonic groups, which bind electrostatically to basic amino acid residues of cellular protein under mildly acidic condition. The protein-bound dye is extracted from cells and solubilized for spectrophotometry by weak bases. This colorimetric assay can be used to estimate cell number indirectly only for monolayer by providing a sensitive index of total cellular protein content which is linearly related to cell density (Skehan *et al.,* 1990). This assay was found to give good results over both high and low cell densities (Freshney, 1994).

2.6.1 Human cell lines

Three different kinds of human cancerous cell lines and one normal cell line were used in this study. The human breast adenocarcinoma cell line (MCF-7) and the human cervical cancer cell line (Hela) were established and kindly provided by Cancer Research Institutes of Thailand, the human androgen-insensitive prostate cancer cell line (PC3) was obtained from Dr. Chavaboon Dechsukum, Faculty of Medicine, Prince of Songkla University, Thailand and one type of normal cell line (MRC5) which was a non-cancerous cell line was

kindly provided by Prof. Houghton King College, London, England. The MCF-7 were cultured in Minimum Essential Media (MEM) with Earle's salt (without glutamine) (GIBCO^{TM}) supplement with 10% heat-inactivated foetal bovine serum (GIBCO^{TM}) , 50 IU/ml penicillin and 50 µg/ml streptomycin (GIBCO^{TM}) and 1% non-essential amino acid (GIBCO^{TM}). Hela were cultured in MEM with 10% heat-inactivated foetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. PC3 were cultured in RPMI 1640 medium (GIBCO^{TM}) supplement with 10% heated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin (Keawpradub *et al.,* 1997). The human normal cell was grown in Dulbecco's modified Eagle's (DMEM) culture medium (GIBCO^{TM}) containing 10% foetal bovine serum and 1% of 10,000 U penicillin and 10 mg/ml streptomycin. The cells were maintained at 37^{o}C in an incubator with 10% CO_2 and 95% humidity.

2.6.2 Testing procedure

According to their growth profiles, the optimal plating densities of MCF-7, Hela, PC3 and MRC5 were determined to be $3x10^3$, $3x10^3$, $1x10^3$ and $5x10^3$ cells/well, respectively to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay (Skehan, *et al*., 1990). Cells growing as monolayer in a 25 cm^3 flask were washed with magnesium and calcium free phosphate buffer saline (PBS) pH 7.4 (AMRESCO®). PBS was decanted and cells detached with 0.025% trypsin-EDTA (GIBCO^{TM}) to make a single cell suspension. The viable cells were counted by trypan blue (GIBCO^{TM}) exclusion in haemacytometer (Freshney, 1994) and diluted with medium to give a final concentration of $3x10^3$, $3x10^3$, $1x10^3$ and $5x10^3$ cells/ml for MCF-7, Hela, PC3 and MRC5 respectively. 100 µl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 hr the cells were treated with the extracts and pure compounds. Each extract was initially dissolved in a quantity of DMSO (Sigma) for ethanolic extracts, or sterile distilled water for water extracts and vinblastine sulphate (Sigma) used as positive control. The first screening was 50 µg/ml of each extract, which was tested against all cancer cells, and the results of the percentage of cell survival less than 50 % at an exposure time of 72 hours were considered to be active. According to National Cancer Institute

guidelines (Boyd, 1997) extracts with IC_{50} values < 20 µg /ml were considered active. The active extracts were further diluted in medium to produce the required concentrations. 100 µl/well of each concentration was added to the plates to obtain final concentrations of 1, 10, 50, 100 µg/ml for the active extract, 0.1, 1, 10, 50 µM for pure compound and 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100 nM for vinblastine sulphate, the final mixture used for treating the cell contained not more than 0.1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure time of 72 hours. At the end of each exposure time, the medium was removed. The wells were then washed with medium, and 200 µl of fresh medium were added to each well. The plates were incubated for a recovery period for 72 hours. On the seventh day of culture period, cells were fixed by 100 µl of ice-cold 40% trichloroacetic acid (TCA, Aldrich Chemical) per well, incubated at 4 $^{\circ}C$ for 1 hour in the refrigerator and washed 5 times with tap water to wash non viable cells, so viable cells were fixed as monolayer in each well. 50 µl of SRB solution (0.4% w/v in 1% acetic acid, Sigma) was added to each well and left in contact with the cells for 30 min; then the plates were washed 4 times with 1% acetic acid until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM Tris base [tris (hydroxy methyl) aminomethane, pH 10.5] (Sigma) was added to each well to solubilize the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (6 replicate) was read on a Power Wave X plate reader at 492 nm as an indication of cell number. Cell survival was measured as the percentage absorbance compared with the control (non-treated cells). The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine.

2.7 Bioassay-guided fractionation

Water and ethanolic extracts from the three medicinal plants and Triphala formula were studied preliminarily for antioxidant activity (section 2.4) and cytotoxic activity (section 2.6).

Results from the preliminary assays for free radical scavenging activity (section 3.1.1) and cytotoxic activity (section 3.1.4) of three medicinal plants and Triphala formula found that the ethanolic extracts of *P. emblica* gave the strongest evidence of active ingredients with both activity. So separation of the two active extracts was undertaken.

An aliquot of the ethanolic extract of the pulp of *P. emblica* (40 g) was separated by vacuum liquid chromatography (VLC), using Hexane (20×100 ml), hexane:chloroform (40× 100 ml), chloroform (40×100 ml), chloroform:methanol (45×100 ml) and methanol (65×100 ml). Drying and evaporation of each fraction yielded residues of 0.0579, 0.4744, 0.3998, 25.5978 and 1.7061 g, respectively these fractions being denoted as FA1, FA2, FA3, FA4 and FA5. These five fractions were tested for free radical scavenging activity (section 3.2.1) and cytotoxic activity (section 3.2.2).

2.8 Isolation of chemical constituents from *Phyllanthus emblica* Linn.

2.8.1 FA2

An aliquot (0.450 g) of FA2 was chromatographed over a silica gel column using hexane:ethyl acetate (0.5:9.5) as eluent finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure.

Fractions 103-104 were combined and obtained C1 (a pure compound), as white crystals (1.9 mg). It was analysed chemical structure in the future.

2.8.2 FA3

An aliquot (0.300 g) of FA3 was chromatographed over a silica gel column using hexane:ethyl acetate (1:9) as eluent finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure.

Fraction 1 was obtained C2 (a pure compound), as white solids (1.7 mg). It was identified by using ^{1}H-NMR as ß-sitosterol.

2.8.3 FA4

2.8.3.1 An aliquot (5.00 g) of FA4 was chromatographed over a silica gel column using chloroform:methanol:water (9:3:0.5) as eluent finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure as follows:

Fractions 1-7 were obtained C3 (a pure compound), as white solids (3.8 mg). It was identified by using ^{1}H-NMR as ß-sitosterol-3-*O*-ß-D-glucopyranoside.

Fractions 8-35 were obtained as yellow brown sticky solid (241.2 mg). It was further separated by 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.

Subfractions 1-10 were obtained C4 (a pure compound), as yellow sticky solid (14.0 mg). Further chemical analysis by using ^{1}H- and ^{13}C-NMR showed in the result of structure elucidation part.

Fractions 95-155 were obtained as brown sticky solid (372.2 mg). The total brown sticky solid was further separated using sephadex^{TM}LH-20 column and methanol as elution system. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure.

Subfractions 26-29 were obtained as yellow crystals (24.0 mg). The total yellow crystals was washed with methanol to obtain C5 (a pure compound), as white crystals (23.9 mg). Further chemical analysis by using ^{1}H- and ^{13}C-NMR showed in the result of structure elucidation part.

2.8.3.2 An aliquot (5.00 g) of FA4 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:

Fractions 24-29 were obtained as brown crystals (730.0 mg). The total yellow crystals was washed with methanol to obtain C5 (a pure compound), as white crystals (726.8 mg). Further chemical analysis by using ^{1}H- and ^{13}C-NMR showed in the result of structure elucidation part.

Fractions 30-32 were obtained as brown sticky solid (509.6 mg). The total brown sticky solid was further separated by silica gel column using chloroform:methanol:water (7:2.8:0.2) as eluent finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure.

Subfractions 30-40 were obtained as yellow liquid (54.6 mg). The total yellow liquid was further separated by silica gel column using chloroform:methanol (7.5:2.5) as eluent finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure.

Subfractions 1-6 were obtained as yellow liquid (15.5 mg). The total yellow liquid was further separated by silica gel column using chloroform:methanol (7.5:2.5) as eluent finally being washed with methanol. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure as follows:

Subfractions 14-21 were obtained C6 (a pure compound), as yellow liquid (6.0 mg). It was analysed chemical structure in the future.

Subfractions 19-24 were obtained C7 (a pure compound), as yellow liquid (4.0 mg). It was analysed chemical structure in the future.

The further study, three compounds (C1, C6 and C7) were elucidated chemical structure by ^{1}H- and ^{13}C-NMR and MS.

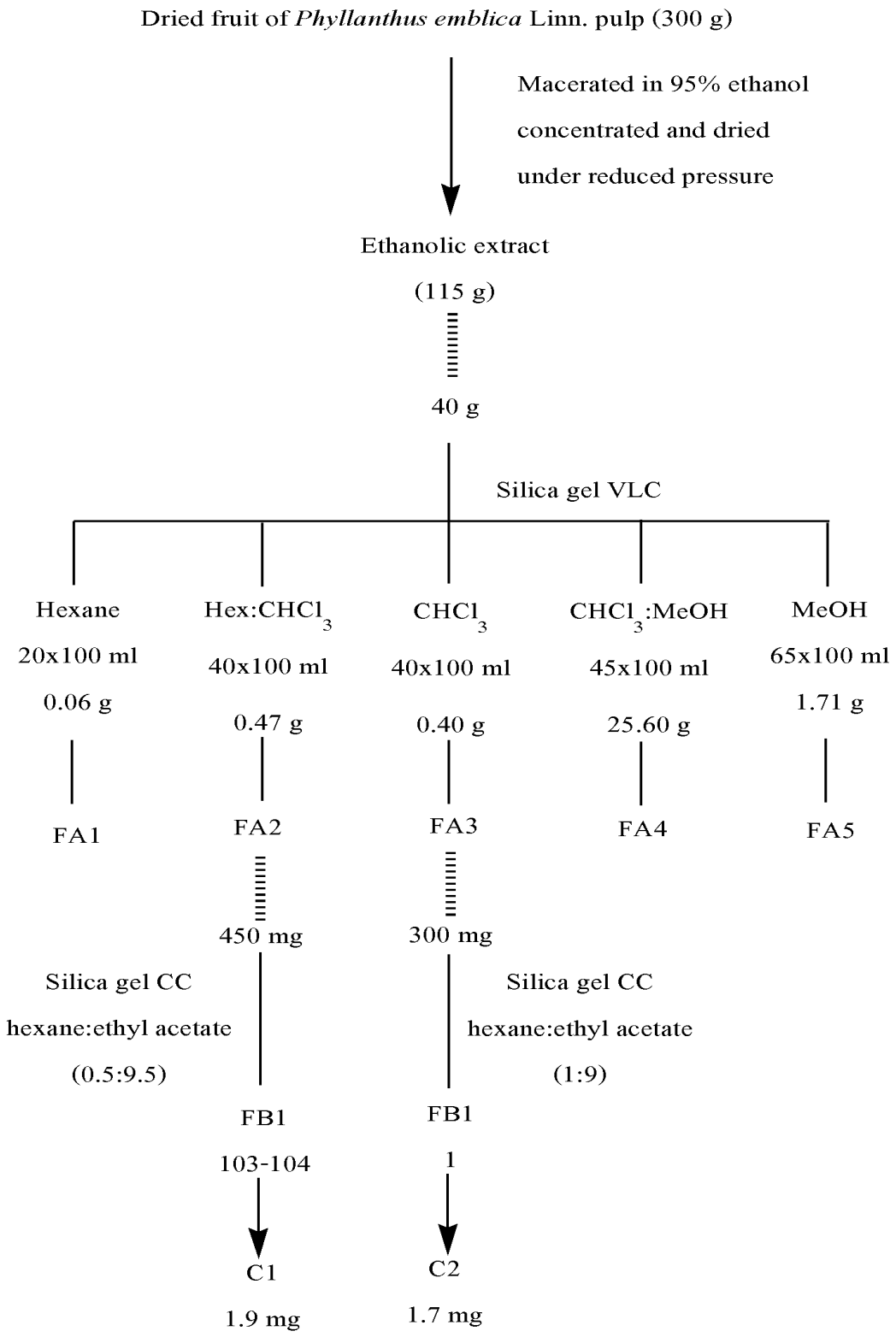

Figure 2-5 Flow chart for separation compounds from FA2 and FA3 of the ethanolic extract of *Phyllanthus emblica*

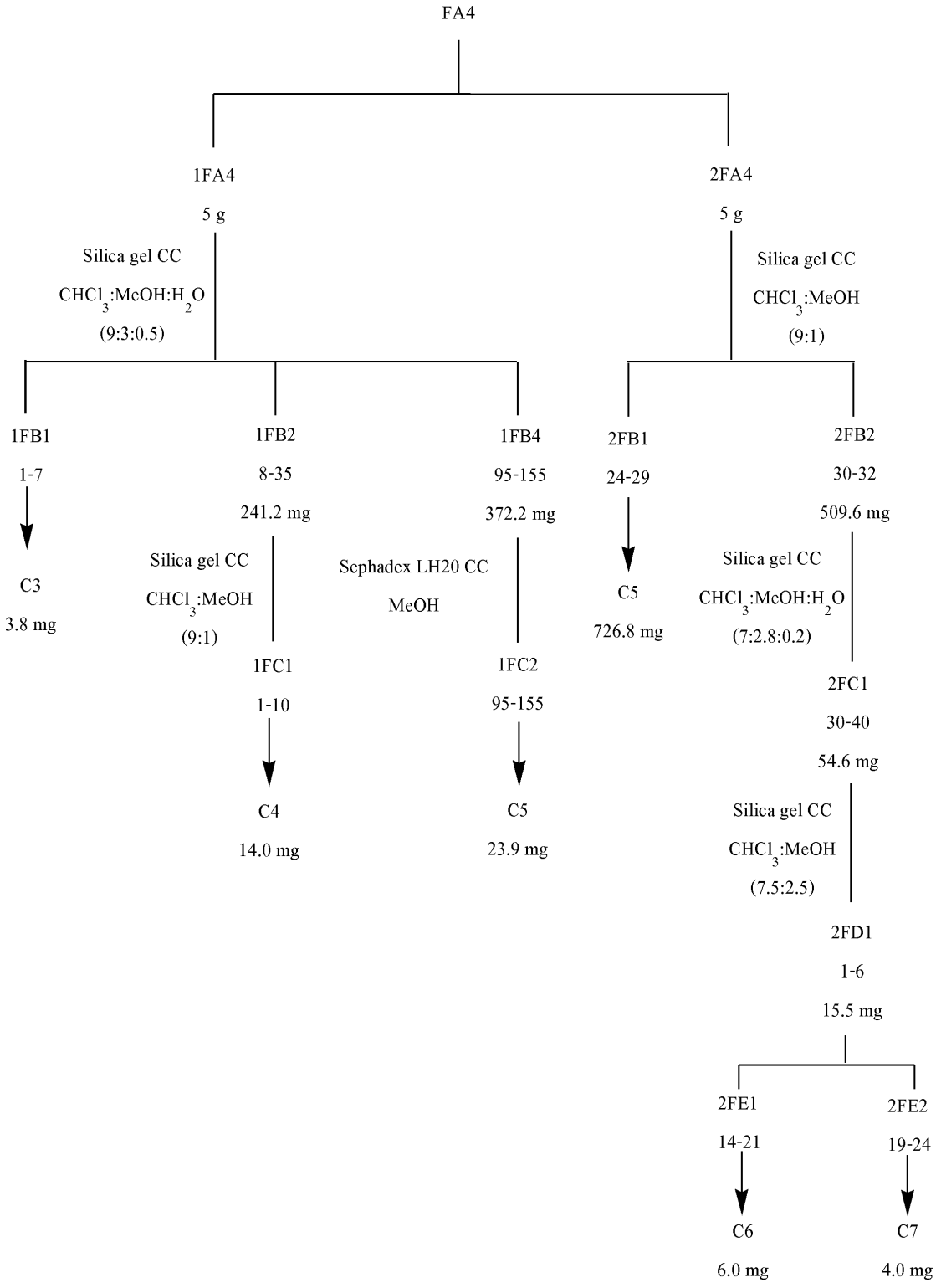
FA4
1FA4
5 g
Silica gel CC
CHCl3:MeOH:H2O
(9:3:0.5)
2FA4
5 g
Silica gel CC
CHCl3:MeOH
(9:1)
1FB1
1-7
C3
3.8 mg
1FB2
8-35
241.2 mg
Silica gel CC
CHCl3:MeOH
(9:1)
1FC1
1-10
C4
14.0 mg
1FB4
95-155
372.2 mg
Sephadex LH20 CC
MeOH
1FC2
95-155
C5
23.9 mg
2FB1
24-29
C5
726.8 mg
2FB2
30-32
509.6 mg
Silica gel CC
CHCl3:MeOH:H2O
(7:2.8:0.2)
2FC1
30-40
54.6 mg
Silica gel CC
CHCl3:MeOH
(7.5:2.5)
2FD1
1-6
15.5 mg
2FE1
14-21
C6
6.0 mg
2FE2
19-24
C7
4.0 mg

Figure 2-6 Flow chart for separation compounds from FA4 of the ethanolic extract of *Phyllanthus emblica*

2.9 Quantitative determination of gallic acid using HPLC

2.9.1 Reagents

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

Stock solution of gallic acid; 1.0 mg/ml, was prepared by dissolving weighted quantities of standard compounds into methanol and was diluted to the desired concentration. By using the stock solutions, a series of standard solutions were prepared with the concentrations of 1.0-5.0 µg/ml.

2.9.2 Chromatographic conditions

The mobile phase consisted of water (A)-glacial acetic acid (B)-methanol (C) with isocratic elution as follows: 90%A - 5%B - 5%C. The flow rate was 1.0 ml/min. The sample volume injected was 20 µl. UV spectra were set at 280 nm for gallic acid.

2.9.3 Preparation of plant samples

The samples were prepared by accurately weighting 20.0 mg of each plant extracts, adjustively to 10 ml with methanol into a volumetric flask and the solution were then sonicated for 15 min. The solution were diluted with methanol for the ethanol plant extracts and methanol-water (1:1) for the water plant extracts to give final concentration in a range of 50-200 µg/ml and filtered through a 0.45 µm filter membrane before analysis. Twenty microliters of the plant samples were directly injected into the HPLC column and separated under above chromatographic conditions.