CHAPTER 3
METHODOLOGY

Conceptual frame work

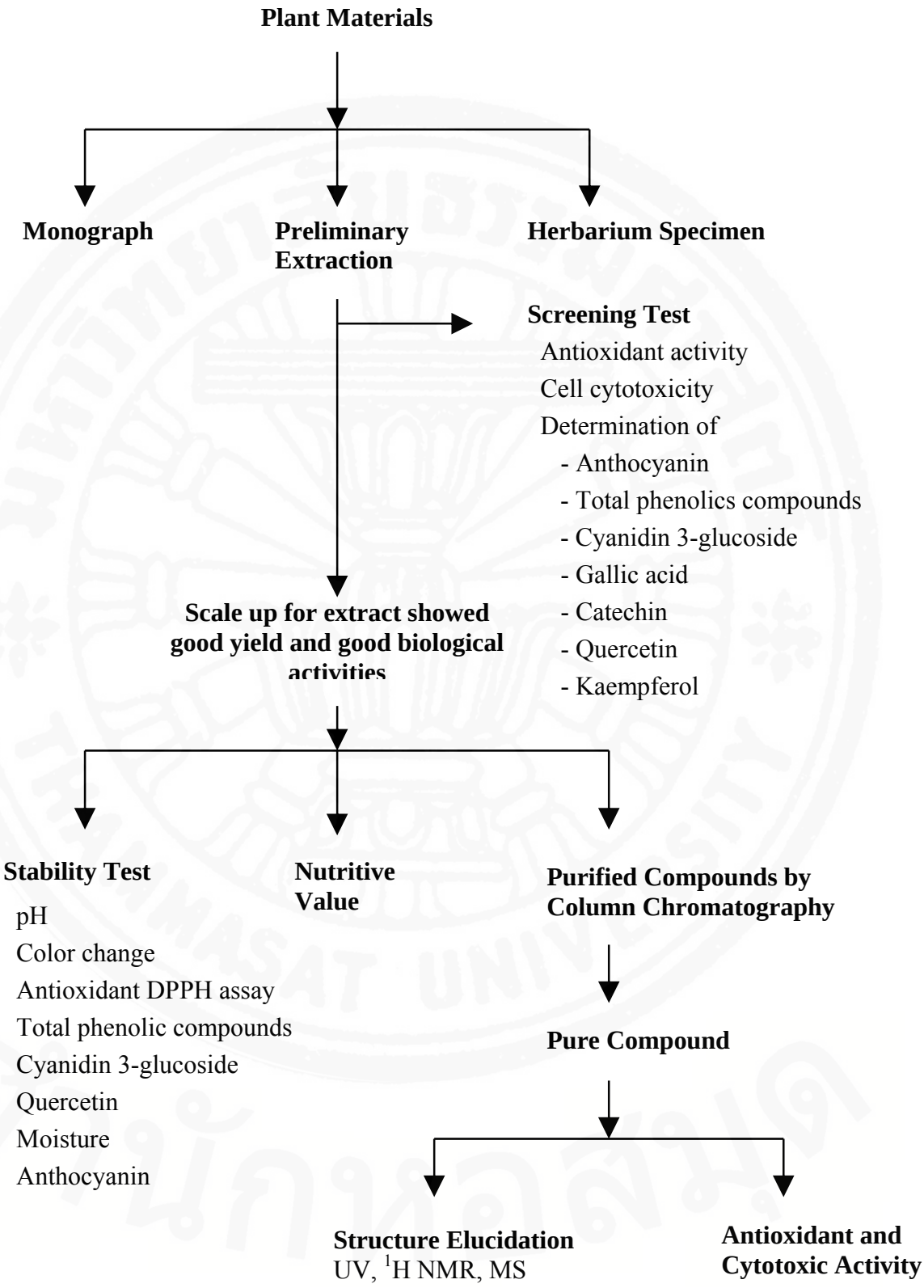
The ripe berries of *Cleistocalyx nervosum* var. *paniala* (Ma-kiang) were collected and the authentications of plant materials were carried out at the herbarium of the Department of Forestry Bangkok (BKF), Thailand, where the herbarium vouchers have been kept to specify plant and species identified. The herbarium of this plant was used for identify by botanist at BKF, the other specimen was also kelp at there

The dried berries of *C. nervosum* var. *paniala* were powdered and sieved through sieve no. 60. The dried powder plant material was determined for physical properties of dried plants such as ethanol soluble extractive value, water soluble extractive value, total ash and acid insoluble ash. The fresh ripe berries of *C. nervosum* var. *paniala* were preliminary extracted with several different methods such as squeezed to give juice, macerated with 50% ethanol, 95% ethanol or decocted with water, and dried by freeze dry, vacuum dry and spray dry methods.

The chemical properties was studied by the preliminary phytochemical screening of plant extracts and thin layer chromatography technique was also used for confirmed the detection of major constituents. UV-Vis and HPLC fingerprints of plant extracts by different methods were used for determination active ingredients from extracts.

The total monomeric anthocyanin and polymeric anthocyanin content in crude extracts were determined with spectrophotometric method. The amount of total phenolics compound was measured by Folin–Ciocalteu method. The content of ascorbic acid, catechin, cyanidin 3-glucoside, gallic acid, kaempferol and quercetin in the crude extracts were evaluated by HPLC. Antioxidant activity was determined by DPPH scavenging method and lipid peroxidation of liposomes assay**.** The crude extracts of *C. nervosum* var. *paniala* from different extraction methods were also tested with two different types of human carcinoma cell lines ; cervical and lung (HeLa and COR L23) and one type of human normal cell lines (MRC-5, lung fibroblast) .

Each extraction method which showed the highest antioxidant activity, were selected for preparing in large scale (2 - 3 kg) for the isolation of pure compounds by bioassay guide fractionation, complete analysis (nutritive value) such as crude protein, crude fiber, crude fat, heavy metals (lead and iron), minerals (sodium, potassium, calcium and magnesium), moisture content, total ash, carbohydrate. The stability test of the extract was also investigated by keeping at accelerate condition (45 °C with 75% relative humidity for 120 days) and ambient temperature (25 - 32 ^{o}C with 55-60% relative humidity for 180 days). Physical parameters such as color (using Chrome meter), pH (using pH meter), and moisture were determined. The amounts of chemical compounds were also evaluated such as total phenolic compounds, cyanidin-3-glucoside and quercetin content and antioxidant activity by DPPH method. The diagram of work plan was shown below (Scheme 1).



Scheme 1 Conceptual framework

Part I Preparation of plant materials

The ripe berries of *C. nervosum* var. *paniala* were collected from Amphor Ban-hong, Lamphun Province, Thailand. This plant was identified and confirmed by botanist (Thanongsak Jonganurak), and comparing with voucher specimens of known identities (SN 040553, BKF 47870, Chantaranothai, P., 1993) in the Forest Herbarium (BKF), Three sets of voucher herbarium specimens (Jansom 1, BKF 146529) were prepared and deposited at the Forest Herbarium (BKF), National Park, Wildlife and Plant Conservation Department, Bangkok, Thailand (Figure 7).

The ripe berries of *C. nervosum* var. *paniala* were cleaned with water and kept at -20 ^{o}C until use.

Figure 7 Voucher herbarium specimens of *C. nervosum* var. *paniala* (Jansom 1, BKF 146529)

Part II Monograph

1. Chemicals

Methanol, Analytical grade	(Merck, Germany)
Deionized water, Milli-Q, ≥ 18 MegaOhm	(Milford, USA)
Ethanol, Analytical grade	(Merck, Germany)
2,2-diphenyl-1-picrylhydrazyl (DPPH)	(Fluka, USA)
Hexane, Analytical grade	(Merck, Germany)
TLC plate (GF_{254}), precoated	(Merck, Germany)
Formic acid, Analytical grade	(Merck, Germany)
Chloroform, Analytical grade	(Merck, Germany)
Sulfuric acid, Analytical grade	(Merck, Germany)
Hydrochlorric acid, Analytical grade	(Merck, Germany)
Potassium hydroxide, Analytical grade	(Merck, Germany)
Ferric chloride, Analytical grade	(Merck, Germany)
Vanillin, Analytical grade	(Fluka, USA)
Bismuth subnitrate, Analytical grade	(Fluka, USA)
Potassium iodide, Analytical grade	(Fluka, USA)
Hexachloro platinic(IV) acid, Analytical grade	(Fluka, USA)
Anisaldehyde, Fluka , Analytical grade	(Fluka, USA)
2-aminoethyl diphenyl borinate, Analytical grade	(Fluka, USA)
Polyethylene glycol– 400, Analytical grade	(Fluka, USA)
3,5-dinitrobenzoic acid, Analytical grade	(Merck, Germany)

2. Equipments

UV chamber (254 nm and 366 nm)	(Camag, Switzerland)
Analytical balance	(Boeco, Germany)
Inverted microscope (with digital camera)	(Nikon, USA)
Muffle furnace	(Thermolyne, USA)
Hot air oven	(Memmert, Germany)
Micropipettes	(Boeco, Germany)
High performance liquid chromatography (HPLC)	(TSP, USA)
Spraying apparatus	(Camag, Switzerland)
Spectrophotometer	(Shimadzu, Japan)

3. Method

3.1 Macroscopic and microscopic method for ground plants (Thai Herbal Pharmacopoeia, 1995; WHO, 2000)

To interpreted morphological and anatomical descriptions of *Cleistocalyx nervosum* var. *paniala* (Ma-kiang), the fruit was identified both macroscopic characters for shape, texture, fracture, marking, color, odor and taste and also microscopic ones for cell and tissue components such as starch grain, crystal, oil globule, pigment. In addition the chemical components as alkaloid, glycoside, tannin, resin and steroid etc. were also examined.

Due to the annual fruiting only in June, the Ma-kiang fruits had to be kept in freezing condition for using throughout the experiment. The frozen fleshly parts were free-hand cut into thin section by means of sharp razor. Then mounted onto a slide in water or other staining reagents and examined under microscope. The dried powdered fruits were also performed. The pictures were recorded by using the digital camera.

3.2 Ethanol soluble extractive value and water soluble extractive value (Thai Herbal Pharmacopoeia, 1995; WHO, 2000)

The ground berries was weighed (2 g) and macerate in 40 ml 95% ethanol (or chloroform-water) with stirring for 24 hrs. The extract solution was filtrated. Filtrate (20 ml) was transferred into evaporating dish and kept in an air oven at 100–110 °C for 4-5 h, cooled and weighed. This was repeated consequently till the weight became constant. The weight was regarded as a measure of extractive value.

3.3 Total ash and acid insoluble ash (Thai Herbal Pharmacopoeia, 1995; WHO, 2000)

The ground berries was weighed in a silica crucible. The crucible was heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 3–5 h at 600 °C. Sample was cooled in desiccator and weighed. To ensure completion of ashing, it was heated again in the furnace for half an hour, cooled and

weighed. This was repeated consequently till the weight became constant (ash became white or grayish white). Weight of ash gave the total ash content. Ash obtained above was dissolved in 25% hydrochloric acid and filtered. The filter paper was dried in oven and weighed. Weight of ash gave acid insoluble ash.

3.4 Preliminary phytochemical screening (Khandelwal, 2004)

3.4.1 Alkaloids

Twenty grams of the ground berries was macerated with 95% ethanol for 24 h and the solution was filtered. The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2 N HCl. After cooling, the mixture was filtered and the filtrate was divided into two equal portions. One portion was treated with a few drops of Mayer's reagent and the other with equal amounts of Dragendroff reagent. The samples were then observed for the presence of turbidity or precipitation.

3.4.2 Anthraquinones

About 5 g of the ground berries was extracted with boiling diluted HCl solution for 15 minute. The solution was filtered and extracted with chloroform. The organic phase was treated with diluted KOH solution. The presence of anthraquinones was indicative if the KOH phase was pink-red color.

3.4.3 Coumarins

About 5 g of the ground berries was taken in test tube, and wet with water. The tube was closed with a cork which had filter paper hanging damped with NaOH solution. Put the tube in a boiling water bath for 3-5 minutes. The filtered paper was treated under ultraviolet light at 365 nm wavelength for 3 minute. The presence of coumarins was a glowing blue-green color.

3.4.4 Flavonoids

Twenty grams of the ground berries was macerated with 95% ethanol for 24 h and the solution was filtered. The alcoholic extract was treated with a few drops of concentrated HCl and magnesium turnings (0.5 g). The presence of flavonoids was indicative if pink or magenta-red color developed within 3 min.

3.4.5 Saponins

About 2.5 g of the ground berries was extracted with boiling water. After cooling, the extract was shaken vigorously to froth and was then allowed to stand for 15-20 min and the samples were then observed for the presence of froth.

3.4.6 Tannins- phenolics compound

Twenty grams of the ground berries was macerated with 95% ethanol for 24 h and the solution was filtered. The alcoholic extract was evaporated and the residue was extracted by 10 ml of hot 0.9% NaCl solution, filtered and divided into 3 equal portions. A sodium chloride solution was added to one portion of the text extract, 1% gelatin solution to a second portion and the gelatin-salt reagent to a third portion. Precipitation with the latter reagent or with both the second and third reagent is indicative of the presence of tannins. Positive tests are confirmed by the addition of $FeCl_3$ solution to the extract and should result in a characteristic blue, blue-black, green or blue-green color and precipitate.

3.5 Thin layer chromatography (TLC) confirmation (Wagner et al., 1984)

Thin layer chromatography technique was used for confirm the detection of number of major constituents in the berries. Juice, water, ethanol, chloroform and hexane extracts of all the samples were run on the TLC plates pre-coated and manually prepared with silica gel-GF_{245}. The resolution of plates was tried in different solvent system. Qualitative phytochemical evaluation was also carried out to test the presence of

alkaloids, anthraquinones, antioxidant, coumarins, flavonoids, phenolics, saponins and tannins (Table 4). The R_f values were calculated as

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

Table 4 Thin layer chromatography (TLC) confirmation of phytochemical

Test	Mobile phase	Spraying reagent	Detection
Alkaloids	Hexane/chloroform (8:2) Hexane/chloroform (2:8) Chloroform/methanol(9:1) Chloroform/methanol/ water (9:3:0.5)	Dragendroff	Visible
Anthraquinones		KOH	UV 366
Antioxidant compounds		DPPH	Visible
Coumarins		KOH	UV 366
Flavonoids		Natural products-polyethyleneglycol	UV 366
Phenolics compound		Ferric chloride	Visible
Saponins		Vanillin-sulfuric acid reagent	Visible
Tannins		Ferric chloride	Visible

3.6 High performance liquid chromatography (HPLC) chromatogram (fingerprint)

The dried berries of *C. nervosum* var. *paniala* were ground in a no. 60 sieve to prepare the powdered. The plant powder was extracted with 95% ethanol. The ethanolic extract solution was monitor with HPLC (Thermo Separation Products-TSP, Riviera Beach, CA, USA). The HPLC apparatus was consisting of a pump ConstaMetric 4100, a SpectroMonitor 4100, a reversed-phase column, C18 (25 x 0.46 cm), 5 µm particle size (phenomenex, USA) with a guard column of the same material was used. Injection was performed by a 100 µl fixed loop coupled to an autosampler.

Data acquisition and processing were performed by PC 1000 (TSP) software. The elution was performed under gradient conditions.

Chromatographic separation for wavelength 254 and 365 nm; solvent A (0.3% acetic acid) and solvent B (methanol), the gradient profile was linear gradient 0 to10% B for 40 min, linear gradient 10 to 70% B for 40 min, isocratic 80% B for 10 min and isocratic 100% A for 10 min. The flow rate was 0.8 ml/min, and monitored at wavelengths 254 and 365 nm.

Chromatographic separation for wavelength 510 nm; solvent A (acetonitrile) and solvent B {1%phosphoric acid/10% acetic acid/5% acetonitrile (1:1:1, v/v/v)}, the gradient profile was isocratic 0% A for 6 min, linear gradient 0 to 20% A for 18 min, linear gradient 20 to 40% A for 6 min and isocratic 0% A for 6 min. The flow rate was 1.0 ml/min, and monitored at wavelength 510 nm.

3.7 UV-Vis chromatogram (finger print)

The dried berries of *C. nervosum* var. *paniala* were ground in a no. 60 sieve to prepare the powdered. The powder was extracted with 95% ethanol. The ethanolic extract solution was monitor with UV-Vis spectrophotometer (Shimadzu UV-2550, Japan). The wavelength scan was applied from 200 to 800 nm constituents in the fingerprint.

Part III Preparation of plant extracts

1. Chemicals

95% ethanol, commercial grade	(Excise Department, Thailand)
Distilled water	(Milford, USA)

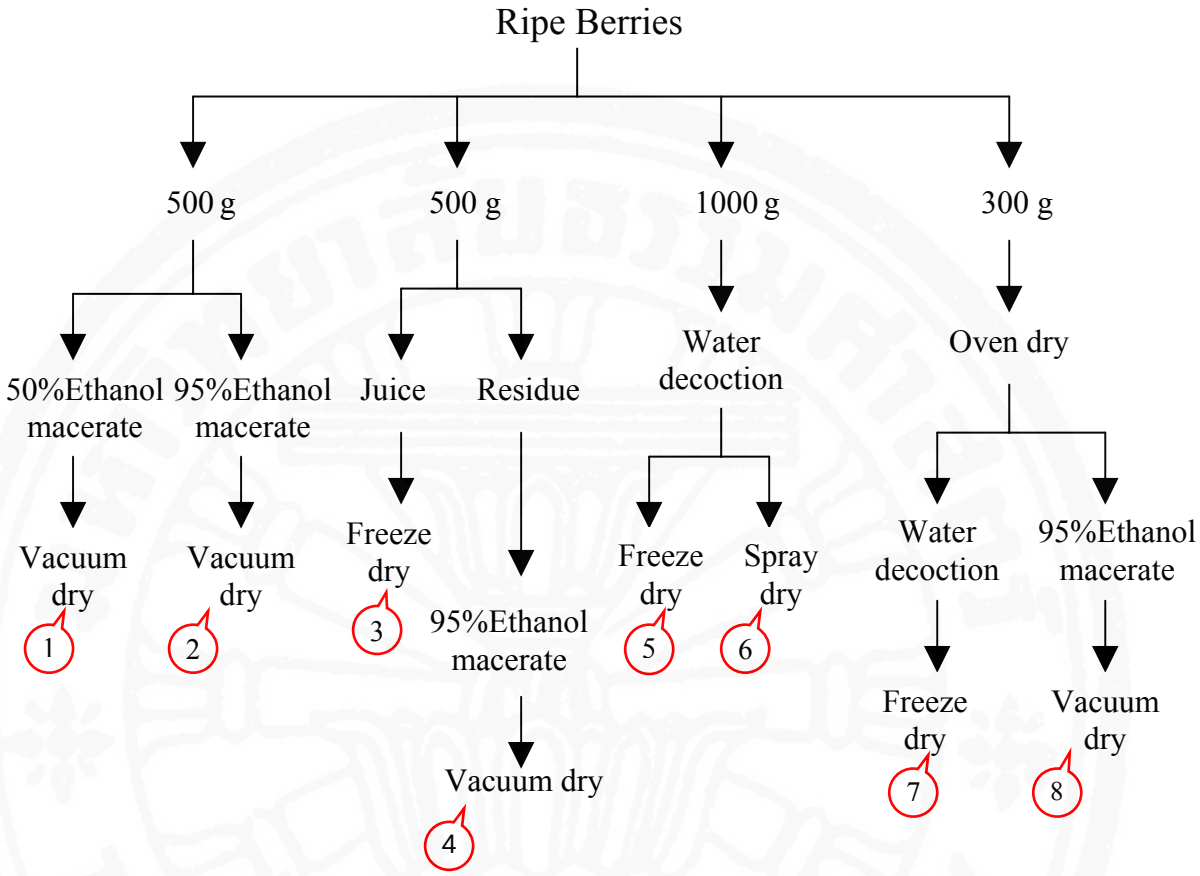
2. Equipments

Rotary evaporator	(Buchi, Japan)
Analytical balance	(Boeco, Germany)
Hot plate	(Thermolyne, USA)
Hot air oven	(Memmert, Germany)
Freeze dryer	(Telstar, Spain)
Spray dryer	(Buchi, Japan)

3. Method

3.1 The preliminary method of extraction on biological screening

Fresh ripe berries of Ma-kiang were squeezed and dried by freeze dry and residue was decoction with 95% ethanol and dry by evaporator. Fresh and dry fruits which dry at 50°C, powdered and extracts obtained by methods similar to those practiced by local people, e.g. water extraction and ethanolic extraction. In brief, for water extraction, fresh and dried ground plant materials were boiled for 30 minutes in distilled water, and the extracts were filtered and dried by freeze dry and spray dry. For ethanolic extraction, fresh and dried ground plant materials were macerated with 50% and 95 % ethanol for 3 days. The ethanolic extracts was filtered and concentrated to dryness under reduced pressure. The percentages of yields were calculated. The diagram of extraction was showed below (Scheme 2).



Scheme 2 Extraction of *Cleistocalyx nervosum* var. *paniala*

1. 50%Ethanol-freeze dry (E50FD)

   Fresh berries were macerated with 50% ethanol for 3 days. The ethanolic extracts was filtered and concentrated under reduced pressure and dryness with freeze dry

2. 95%Ethanol-freeze dry (E95FD)

   Fresh berries were macerated with 95% ethanol for 3 days. The ethanolic extracts was filtered and concentrated under reduced pressure and dryness with freeze dry.

3. Juice-freeze dry (JuFD)

   Fresh berries were squeezed to give juice. The juice was filtered and concentrated to dryness with freeze dry.

4. Residue-ethanol freeze dry (REFD)

Berries meal from JuFD method was macerated with 95% ethanol for 3 days. The ethanolic extracts was filtered and concentrated under reduced pressure and dryness with freeze dry.

5. Water-Freeze Dry (WFD)

Fresh berries were boiled for 30 minutes in distilled water, and the extracts were filtered and dried by freeze dry.

6. Water-spray dry (WSD)

Fresh berries were boiled for 30 minutes in distilled water, and the extracts were filtered (4 brix), filtrate was added maltodextrin and magnesium stearate to give 10 brix and dried by spray dry.

7. Dry-water freeze dry (DWFD)

Fresh berries were dried at 50°C, powdered and dried materials were boiled for 30 minutes in distilled water, and the extracts were filtered and dried by freeze dry.

8. Dry-ethanol freeze dry (DEFD)

Fresh berries were dried at 50°C, powdered and dried materials were macerated with 95% ethanol for 3 days. The ethanolic extracts was filtered and concentrated under reduced pressure and dryness with freeze dry.

3.2 Scale up of plant extracts

95% Ethanol-freeze dry (E95FD) and water-freeze dry (WFD) were the method of extraction which showed the highest biological activity and both were selected for preparing in large scale (more than 3 kg) for the isolation of pure compounds, stability test and nutritive value of extract was also investigated.

Part IV Determination of chemical constituent

1. Chemicals

Methanol, Analytical grade	(Merck, Germany)
Deionized water, Milli-Q, ≥ 18 MegaOhm	(Milford, USA)
Potassium chloride, Analytical grade	(Merck, Germany)
Sodium acetate, Analytical grade	(Merck, Germany)
Ethanol, Analytical grade	(Merck, Germany)
Formic acid, Analytical grade	(Merck, Germany)
Phosphoric acid, Analytical grade	(Merck, Germany)
Acetonitrile, HPLC grade	(Lab scan, Thailand)
Acetic acid, Analytical grade	(Merck, Germany)
Hydrochloric acid, Analytical grade	(Merck, Germany)
Sodium acetate trihydrate, Analytical grade	(Merck, Germany)
Potassium metabisulfite, Analytical grade	(Merck, Germany)
Sodium carbonate, Analytical grade	(Merck, Germany)
Cyanidin 3-glucoside (kuromanin)	(Fluka, Switzerland)
Quercetin	(Aldrich, USA)
Folin-Ciocalteu reagent	(Fluka, USA)
Catechin	(Sigma, USA)
Gallic acid	(Fluka, USA)
Keampferol	(Fluka, USA)
Ascorbic acid	(Fluka, USA)

2. Equipments

Analytical balance	(Boeco, Germany)
Hot plate	(Thermolyne, USA)
Spectrophotometer	(Shimadzu, Japan)
High performance liquid chromatography (HPLC)	(TSP, USA)
pH meter	(WTW inolab, Germany)
Centrifuge	(Boeco, Germany)
Sonicator	(Elma, Germany)
Micropipettes	(Boeco, Germany)

3. Method

3.1 Determination of total monomeric anthocyanin and polymeric anthocyanin (Ronald, 2001)

3.1.1 Determination of total monomeric anthocyanin by pH-difference method

Twenty five milligrams of crude extract was dissolved in 25 ml distilled water. Appropriate dilution factor (DF) for sample by diluting with 0.025 M potassium chloride buffer (pH 1.0), until the absorbance of sample at $\lambda_{vis\text{-}max}$ is within the linear range of the spectrophotometer (for most spectrophotometers the absorbance should be less than 1.2). Final volume of sample was divided by initial volume to obtain the dilution factor (DF). The spectrophotometer was tarred with distilled water at all wavelengths that will be used ($\lambda_{vis\text{-}max}$ and 700 nm). The sample will be prepared to two dilutions, one with 0.025 M potassium chloride buffer (pH 1.0), and the other with 0.4 M sodium acetate buffer (pH 4.5), diluting each by previously determined dilution factor. These dilutions were let to equilibrate for 15 min. Each dilution was measured the absorbance at $\lambda_{vis\text{-}max}$ and at 700 nm (to correct for haze), against a blank cell filled with distilled water. All measurements should were made between 15 min and 1 h after sample preparation, since longer standing times tend to increase observed readings. The absorbances of diluted samples were calculated (A) as follows:

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5}$$

Calculated the monomeric anthocyanin pigment concentration in original sample using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/l)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

Where; MW = Molecular weight

DF = Dilution factor (if 0.2 ml is diluted to 3 ml, DF = 15)

ε = Molar absorptivity

Note: The MW and ε used in this formula correspond to the predominant anthocyanin in sample. Use ε reported in the literature for the anthocyanin pigment in acidic aqueous solvent. If ε of the major pigment is not available, or if the sample composition is unknown, calculate pigment content as cyanidin 3-glucoside, where MW = 449.2 and ε = 26,900.

3.1.2 Determination of pigment degradation, polymeric anthocyanin by subtractive method

Twenty five milligrams of crude extract was dissolved in 25 ml distilled water. Appropriate dilution factor (DF) for sample by diluting with 0.025 M potassium chloride buffer (pH 1.0), until the absorbance of sample at $\lambda_{vis\text{-}max}$ was within the linear range of the spectrophotometer (for most spectrophotometers the absorbance should be less than 1.2). Final volume of sample was divided by initial volume to obtain the dilution factor (DF). The spectrophotometer was tarred with distilled water at all wavelengths that were used (420 nm, $\lambda_{vis\text{-}max}$, 700 nm). The samples were diluted with distilled water using the dilution factor already determined. 2.8 ml of diluted sample was transfer to each of two cuvettes, and then add 0.2 ml of bisulfite solution to one and 0.2 ml distilled water to the other. These samples were let to equilibrate for 15 min, and measure the absorbance of both samples at 420 nm, $\lambda_{vis\text{-}max}$, and 700 nm (to correct for haze), against a blank cell filled with distilled water. The color density of control sample (treated with water) was calculated as follows:

$$\text{Color density} = [(A_{420\ nm} - A_{700nm}) + (A_{\lambda vis\text{-}max} - A_{700\ nm})] \times DF$$

Where; DF = dilution factor (if 0.2 ml sample diluted to 3 ml, DF = 15)

Calculated the polymeric color of bisulfite bleached sample as follows:

$$\text{Polymeric color} = [(A_{420\ nm} - A_{700\ nm}) + (A_{\lambda vis\text{-}max} - A_{700\ nm})] \times DF$$

Calculated the percent polymeric color using the formula:

$$\text{Percent polymeric color} = (\text{polymeric color/color density}) \times 100$$

3.2 Determination of total phenolic compounds (Hopia et al., 1999)

Twenty five milligrams of crude extract was dissolved in 25 ml methanol. 200 µl of these samples were mixed with 200 µl of methanol, 2 ml of Folin-Ciocalteu reagent and 1.6 ml of sodium carbonate solution (7.5 g/100 ml), and the mixture were measured at 765 nm after 30 minutes. Gallic acid was used as a standard and the total phenolics were expressed as GAE (gallic acid equivalents) in milligrams per gram dry weight of extracts.

3.3 HPLC determination of ascorbic acid, cyanidin 3-glucoside, gallic acid, catechin, quercetin and kaempferol.

3.3.1 Determination of ascorbic acid (Leong et al., 2002)

The analysis was carried out using high performance liquid chromatography (HPLC) system, with UV–Vis detector. A reversed-phase column, Luna C18 (250 x 4.6 mm), 5 µm particle size, with guard column of the same material was used. Injection was performed by a 100 µl fixed loop coupled to an autosampler. The elution was performed under gradient conditions. solvent A; Acetonitrile and solvent B; 0.3% acetic acid, The gradient profile were linear gradient 0 to 6.7% A for 10 min, isocratic 0% A for 5 min and isocratic 0% B for 10 min. The flow-rate was 1.0 ml/min. The eluate was monitored at wavelength 245 nm. Ascorbic acid will be used as reference standard.

3.3.2 Determination of cyanidin 3-glucoside (Arusa et al., 2004)

The analysis was carried out using high performance liquid chromatography (HPLC) system, with UV–Vis detector. A reversed-phase column, Luna C18 (25 x 0.46 cm), 5 µm particle size, with a guard column of the same material was used. Injection was performed by a 100 µl fixed loop coupled to an autosampler. The elution was performed under gradient conditions. solvent A; acetonitrile and solvent B; 1%phosphoric acid/10% acetic acid (glacial)/5% acetonitrile (1:1:1, v/v/v), The gradient profile were isocratic 0% A for 6 min, linear gradient 0 to 20% A for 18 min, linear gradient 20 to 40% A for 6 min and isocratic 0% A for 6 min. The

flow-rate was 1.0 ml/min. The eluate was monitored at wavelength 510 nm. Cyanidin 3-glucoside (kuromanin) was used as reference standard.

3.3.3 Determination of catechin, gallic acid, kaempferol and quercetin (Paul et al., 2006)

The analysis was carried out using high performance liquid chromatography (HPLC) system, with UV–Vis detector. A reversed-phase column, Luna C18 (25 x 0.46 cm), 5 µm particle size, with a guard column of the same material was used. Injection was performed by a 100 µl fixed loop coupled to an autosampler. The elution was performed under gradient conditions. solvent A; Acetonitrile and solvent B; methanol, The gradient profile were linear gradient 0 to 10% B for 40 min, linear gradient 10 to 70% B for 40 min, isocratic 80% B for 10 min and isocratic 100% A for 10 min. The flow-rate was 0.8 ml/min. The eluate was monitored at two difference wavelengths, 280 nm for gallic acid and catechin, and 365 nm for kaempferol and quercetin. Catechin, gallic acid, kaempferol and quercetin were used as reference standards.

3.3.4 Validation of HPLC method

3.3.4.1 Linearity

The linearity of the method was evaluated by analyzing a series of each standard. Ten microliters of three standard concentrations (containing 50 - 150, 500 - 2000, 10 - 40, 10 - 100, 50 - 200 and 50 - 200 µg/ml of standard ascorbic acid, catechin, cyaniding 3-glucoside, gallic acid, kaempferol and quercetin, respectively) were injected into the HPLC. The elution was carried out as described above and standard curves were obtained by plotting the concentration of each standard concentration versus peak area. The slope and intercept values were calculated using the least-square linear regression method. The correlation coefficient was also calculated.

3.3.4.2 Precision

The precision of the assay was determined by repeatability (intra-day) and reported as % R.S.D. for a statistically significant number of replicate measurements. Six solutions of each standard concentrations (containing 100, 1000, 20, 50, 100 and 100 µg/ml of ascorbic acid, catechin, cyaniding 3-glucoside, gallic acid, kaempferol and quercetin, respectively) were prepared and assayed. The results were reported as standard deviation and % R.S.D.

3.3.4.3 Accuracy

The accuracy was determined by recovery of known amounts of each reference standard added to the samples at the beginning of the process. An accuracy weight of extract (200 mg) was transferred to a test tube and 2 ml of solvent was added (final concentration = 100 mg/ml). Aliquots of 800 µl of this solution were separately transferred into a vial containing 100 µl of each standard solution and the solvent was added to make up to 1000 µl to give final concentrations containing 100, 1000, 20, 50, 100 and 100 µg/ml of standard ascorbic acid, catechin, cyaniding 3-glucoside, gallic acid, kaempferol and quercetin. The accuracy of the method was tested by the proposed method. The percentage recovery was calculated by the following equation:

$$\% \text{ Recovery} = \frac{C(spiked) - C(sample)}{C(std)}$$

Although it is desirable to achieve the recovery closed to 100%, recovery values should be 80.0 - 120.0%

3.3.4.4 LOD/LOQ

Limit of detection and limit of quantitation of each reference standard were determined by injecting the solvent and noise was determined. The LOD and LOQ by considering (S:N) ratio were analyzed.

3.4 Statistical analysis

Each experiment was tripicately done. The results were expressed as mean and standard deviation (SD). The chemical constituent in the crude extracts of *C. nervosum* var. *paniala* from different extraction methods were statistically analyzed using one-way ANOVA with Duncan's new multiple range test (DMRT) by SPSS program version 11.5 for Windows. The statistical probability (*p*-value) less than 0.05 indicated a statistical significant difference between groups.

Part V Determination of antioxidant activity

1. Chemicals

Methanol, Analytical grade	(Merck, Germany)
Deionized water, Milli-Q, ≥ 18 MegaOhm	(Milford, USA)
Ethanol, Analytical grade	(Merck, Germany)
2,2-diphenyl-1-picrylhydrazyl (DPPH)	(Fluka, USA)
Butylate hydroxyl toluene (BHT)	(Merck, Germany)
Cyanidin 3-glucoside (kuromanin)	(Fluka, Switzerland)
Quercetin	(Aldrich, USA)
Catechin	(Sigma, USA)
Propyl gallate	(Fluka, USA)
Thiobarbituric acid	(Fluka, USA)
Liposome, from bovine brain (B3635)	(Sigma, USA)
Sodium hydroxide, Analytical grade	(Merck, Germany)
Butanol, Analytical grade	(Merck, Germany)
Ferric chloride, Analytical grade	(Merck, Germany)
Ascorbic acid, Analytical grade	(Merck, Germany)

2. Equipments

Analytical balance	(Boeco, Germany)
Water bath	(Memmert, Germany)
Hot air oven	(Memmert, Germany)
Spectrophotometer	(Shimadzu, Japan)
pH meter	(WTW inolab, Germany)
Centrifuge	(Boeco, Germany)
Sonicator	(Elma, Germany)
Micropipettes	(Boeco, Germany)

3. Method

3.1 Reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and spectrophotometric assay (Yamasaki et al., 1994)

Scavenging effect of extract or pure compound on DPPH radical was examined based on the method of Yamasaki *et al*., 1994. This antioxidant activity testing method is based on chemical test. Butylated hydroxytoluene

(BHT) was used as reference standard and positive control. Samples for testing were dissolved in methanol to obtain a high concentration of 200 μg/ml. Each sample was further diluted for at least 4 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of sample solution (500 μl) was mixed with an equal volume of 6 x 10^{-5} M DPPH (in methanol) and allowed to stand at room temperature for 20 min. The absorbance (A) was measured at 520 nm. BHT as a positive standard was tested in the same system. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage inhibition; %inhibition = $[(A_{control} - A_{sample})/A_{control}]$ x 100. Effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) were obtained by linear regression analysis of dose-response curve plots of % inhibition versus concentration and calculated EC_{50} by prism program.

3.2 Inhibition of lipid peroxidation on liposome assay (Uchiyama et al., 1978 and Farrukh et al., 2006)

Determination of the antioxidative activity using the Thiobarbituric acid (TBA) assay is based on the prevention of the formation of malondialdehyde, a degradation product of lipid peroxidation. Preparation of liposome solution and the TBA assay as follow.

0.2 ml of liposome was mixed with 0.1 ml $FeCl_3$ (1mM), 0.1ml ascorbic acid (1mM), 0.5 ml PBS and 0.1 ml ethanolic or water extracts (six different concentrations were prepared from each sample) into test tube. The mixture was incubated at 37 ^{o}C for 20 min, and added TBA reagent (0.1 ml 2% BHT, 0.5 ml 1% TBA (in 50 mM NaOH), 0.5 ml 1% HCl) to all tubes. The mixture were heated at 85-90 ^{o}C for 30 min, and allowed to cool to room temperature. 2.5 ml of butanol was added to all tube, mix and centrifuge at 3500 rpm for 10-20 min. Butanol was removed and placed in cuvettes and the extinction were determined at 532 nm on spectrophotometer. Propylgallate was used as positive control, and percent inhibition was calculated.

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

The data were presented as means of triplicates, and the amount required for a 50% reduction (IC_{50}) were determined graphically.

3.3 Statistical analysis

Each experiment was duplicately done. The results were expressed as mean and standard deviation (SD). The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) and the amount required for a 50% reduction (IC_{50}) of the crude extracts of *C. nervosum* var. *paniala* from different extraction methods were statistically analyzed using one-way ANOVA with Duncan's new multiple range test (DMRT) by SPSS program version 11.5 for Windows. The statistical probability (*p*-value) less than 0.05 indicated a statistical significant difference between groups.

Part VI *In vitro* cytotoxicity Assay (Skehan et al., 1990; Keawpradub et al., 1997)

1. Human cell lines

Two different types of human carcinoma cell lines; cervical cancer cell lines (HeLa) and large cell lung carcinoma (COR L23), and one type of human normal cell lines (MRC-5, lung fibroblast) were used in this study.

2. Chemicals

Minimum essential medium (MEM)	(Gibco, USA)
Dulbeco's modified eagle medium (DMEM)	(Gibco, USA)
RPMI medium 1640	(Gibco, USA)
Fetal bovine serum (FBS)	(Gibco, USA)
Penicillin	(Gibco, USA)
Streptomycin	(Gibco, USA)
Methanol, Analytical grade	(Merck, Germany)
Deionized water, Milli-Q, ≥ 18 MegaOhm	(Milford, USA)
Ethanol, Analytical grade	(Merck, Germany)
Acetic acid, Analytical grade	(Merck, Germany)
Cyanidin-3-glucoside (kuromanin)	(Fluka, Switzerland)
Quercetin	(Aldrich, USA)
Catechin	(Sigma, USA)
Propyl gallate	(Fluka, USA)
Thiobarbituric acid	(Fluka, USA)
Trypsin	(Sigma, USA)
Trypan blue	(Gibco, USA)
Sodium hydroxide, Analytical grade	(Merck, Germany)
Butanol, Analytical grade	(Merck, Germany)
Ferric chloride, Analytical grade	(Merck, Germany)
Ascorbic acid, Analytical grade	(Merck, Germany)
Phosphate buffer saline (PBS)	(Gibco, USA)
Sulforhodamine B	(Sigma, USA)
Dimethylsulfoxide (DMSO)	(Fluka, USA)
Trichloroacetic acid (TCA)	(Fluka, USA)

3. Equipments

Laminar air flow	(Boss tech, Thailand)
Analytical balance	(Boeco, Germany)
Auto clave	(Hirayama, Japan)
Hot air oven	(Memmert, Germany)
pH meter	(WTW inolab, Germany)
Centrifuge	(Boeco, Germany)
Sonicator	(Elma, Germany)
Micropipettes	(Boeco, Germany)
CO_2 humidified incubator	(Shel lab, USA)
Haemocytometer	(Costar, USA)
96-well microtiter plates	(Costar, USA)
ELISA plate reader	(Bio Tek, USA)
Bio medical freezer	(Sanyo, Japan)
Ultra-low temperature freezer	(Sanyo, Japan)
Inverted microscope	(Nikon, USA)
Pipette boy	(Brand, USA)
Multi-channels pipette	(Costar, USA)

4. Method

Hela cells were cultured in MEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin, 1% streptomycin. MRC-5 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin, 1% streptomycin and COR L23 cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin, 1% streptomycin. All cell lines were maintained at 37 °C in 5% CO_2 humidified incubator and were sub-cultured weekly. The culture medium will be changed twice a week.

Cells at the exponential growth phase were detached with phosphate buffer saline (PBS, pH 7.4) to make single-cell suspensions. The viable cells were counted by trypan blue exclusion in a haemocytometer and diluted with medium to give final concentrations. Each cell suspensions were seeded in 96-well microtiter plates in the volume of 100 μl /well. According to their growth

profiles, the optimal plating density of each cell line was determined ($3x10^3$,$1x10^3$ and $5x10^3$ cells/well for COR L23, HeLa and MRC-5 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.

The plates were incubated for 24 h (day 1) at 37 °C in 5% CO_2 humidified incubator. Then, the cells were treated with plant extracts by adding 100 µl/well of each concentration in quadruplet to obtain final concentration of 2, 20, 100 and 200 µg/ml. The cells were treated with the extracts or pure compounds. Each extract was initially dissolved in DMSO for ethanolic extracts and in sterile distilled water for water extracts. The cells with same final concentration of DMSO were used as the solvent-control wells (The final mixture used for treating the cell contained not more than 1% of the solvent). The plates were incubated for 72 h (day 4) at 37 °C in a 5% CO_2 humidified incubator. At the end of exposure time, the medium was removed. The wells were then washed with medium, and 200 µl of fresh medium were added. The plates were incubated for a recovery period for 3 days, and cell numbers analyzed by SRB assay. Two replicate plates were used to determine the cytotoxicity of each extract.

The antiproliferative assay, SRB (sulphorhodamine B) assay was used to assess growth inhibition. This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. For the assay, cells were fixed by layering 100 ml of ice-cold 40% trichloroacetic acid on top of the growth medium. Cells were incubated at 4^oC for 1 hour, after which plates were washed five times with cold water, excess water drained off and the plates left to dry in air. SRB stain (50 µl; 0.4% in 1% acetic acid) was added to each well and allowed to be in contact with the cell for 30 minutes. Subsequently, to remove excess dye, they were washed with 1% acetic acid, rinsed 4 times 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] was added to each well to solubilised the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. Absorbance (OD) of each well (quadruplet for each concentration) was

measured by using ELISA plate reader at 492 nm. The intensity of color developed in each well was corresponded to the cell number. Percentage of growth inhibition was calculated by using equation below.

$$\% \text{ growth inhibition} = [(OD_{\text{solvent control}} - OD_{\text{test sample}})/ OD_{\text{solvent control}}] \times 100$$

IC_{50} value was expressed as concentration of extract in microgram per milliliter that caused a 50% growth inhibition comparing with controls.

According to National Cancer Institute guidelines the extracts with IC_{50} values < 20 μg/ml were considered "active".

Statistical analysis

Each experiment was done as quadruplicate. The results were expressed as mean and standard error of mean (SEM). The percentage of growth inhibition of the crude extracts of *C. nervosum* var. *paniala* from different extraction methods were statistically analyzed using one-way ANOVA with Duncan's new multiple range test (DMRT) by SPSS program version 11.5 for Windows. The statistical probability (*p*-value) less than 0.05 indicated a statistical significant difference between groups.

Part VII Determination of nutritive value

1. Chemicals

Methanol, Analytical grade	(Merck, Germany)
Deionized water, Milli-Q, ≥ 18 MegaOhm	(Milford, USA)
Ethanol, Analytical grade	(Merck, Germany)
Sodium hydroxide, Analytical grade	(Merck, Germany)
Butanol, Analytical grade	(Merck, Germany)
Nitric acid, Analytical grade	(Merck, Germany)
Boric acid, Analytical grade	(Merck, Germany)
Sulfuric acid, Analytical grade	(Merck, Germany)
Hydrochloric acid, Analytical grade	(Merck, Germany)
Sodium hydroxide, Analytical grade	(Merck, Germany)
Sodium (Na), AA grade	(Merck, Germany)
Potassium (K), AA grade	(Merck, Germany)
Calcium (Ca), AA grade	(Merck, Germany)
Magnesium (Mg), AA grade	(Merck, Germany)
Lead (Pb), AA grade	(Merck, Germany)

2. Equipments

Crucible	(HCT, Germany)
Muffle furnace	(Thermolyne, USA)
Analytical balance	(Boeco, Germany)
Water bath	(Memmert, Germany)
Hot air oven	(Memmert, Germany)
Atomic absorption spectrophotometer (AAS)	(Hitachi, Japan)
pH meter	(WTW inolab, Germany)
Centrifuge	(Boeco, Germany)
Sonicator	(Elma, Germany)
Micropipettes	(Boeco, Germany)
Moisture analyzer	(Scaltec, Germany)
Gas chromatography (GC)	(Buchi, Switzerland)
Kjeldahl apparatus	(Buchi, Switzerland)
Protein analyzer	(Buchi, Switzerland)

Fat extractor	(Buchi, Switzerland)
Fiber analyzer	(VELP, Italy)

3. Methods

3.1 Determination of ash and mineral content (AOAC, 1995)

Each sample was weighed in a silica crucible. The crucible was heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 3–5 h at 600°C. Sample was cooled in desiccator and weighed. To ensure completion of ashing, it was heated again in the furnace for half an hour, cooled and weighed. This was repeated consequently till the weight became constant (ash became white or grayish white). Weight of ash gave the ash content. Ash obtained above was dissolved in 5% HCl to obtain the solution ready for determination of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and lead (Pb) through atomic absorption spectroscopy (AAS). Standard solution of each element was prepared and calibration curves were drawn for each element.

3.2 Determination of moisture (AOAC, 1995)

The sample material was taken in a flat-bottom dish and kept in an air oven at 100–110°C for 4-5 h, cooled and weighed. This was repeated consequently till the weight became constant. The loss in weight was regarded as a measure of moisture content.

3.3 Determination of crude fat (Pendl et al., 1997)

The sample was extracted with butanol in soxhlet extractor and determined fatty acid by gas chromatography (GC) with flame ionization detector (FID)

3.4 Determination of crude protein (AOAC, 1995)

Sample was taken in a Kjeldahl flask, and conc. H_2SO_4 was added followed by the addition of selenium mixture. The mixture was heated first gently and then strongly once the frothing had ceased. When the solution

became colorless or clear, it was heated for another hour, allowed to cool, diluted with distilled water and transferred to Kjeldahl flask, washing the digestion flask. 40% caustic soda was added and the flask was connected with the splash heads of the distillation apparatus. Next H_3BO_4 was taken in the receiving flask and distilled. When two-thirds of the liquid had been distilled, it was tested for completion of reaction. The flask was removed, and titrated against 0.1 N H_2SO_4 solution using indicator for determination of Kjeldahl nitrogen, which the result return to gave the protein content. Crude fiber was determined to be reported along with the nutritive value.

3.5 Determination of crude fiber (AOAC, 1995)

Sample was treated with 1.25% H_2SO_4. After filtration and washing, the residue was treated with 1.25% NaOH. It was the filtered, washed with hot water and then acetone and again with hot water. The residue was ignited and the ash weighed. Loss in weight gave the weight of crude fiber.

3.6 Percentage carbohydrate

Percentage carbohydrate was given by the formula as follow

%carbohydrate = 100 – (%ash+ %moisture+ %fat+ %protein+ %crude fiber)

3.7 Energy

Energy was finally calculated by the formula as follow

Energy (Kcal) = (4 x protein) + (9 x fat) + (4 x carbohydrate)

3.8 Statistical analysis

Each experiment was duplicately done. The results were expressed as mean and standard deviation (SD). The nutritive value of the crude extracts of *C. nervosum* var. *paniala* from E95FD and WFD extraction methods were statistically analyzed using paired sample t-test by SPSS program version 11.5 for Windows. The statistical probability (p-value) less than 0.05 indicated a statistical significant difference between groups.

Part VIII Purified compounds by column chromatography with bioassay-guided fractionation

1. Chemicals

Methanol, Analytical grade	(Merck, Germany)
Deionized water, Milli-Q, ≥18 MegaOhm	(Milford, USA)
Ethanol, Analytical grade	(Merck, Germany)
Acetonitrile, HPLC grade	(Lab scan, Thailand)
2,2-diphenyl-1-picrylhydrazyl (DPPH)	(Fluka, USA)
Butylate hydroxyl toluene (BHT), Analytical grade	(Merck, Germany)
Cyanidin-3-glucoside (Kuromanin)	(Fluka, Switzerland)
Formic acid, Analytical grade	(Merck, Germany)
Phosphoric acid, Analytical grade	(Merck, Germany)
Chloroform, Analytical grade	(Merck, Germany)

2. Equipments

Column (11 x 12.5 cm, glass)	(VN supply, Thailand)
Silica gel 60 (9733)	(Merck, Germany)
High performance liquid chromatography (HPLC)	(TSP, USA)
Analytical balance	(Boeco, Germany)
Spectrophotometer	(Shimadzu, Japan)
Sonicator	(Elma, Germany)
Micropipettes	(Boeco, Germany)

3. Method

An extract that exhibited the best antioxidant activity on DPPH assay was pre-purified by vacuum liquid chromatography (VLC) on silica (Merck Silica gel 60, No 9733), and eluted with a mobile phase of increasing polarity: chloroform, chloroform /methanol (1:1), methanol and 50% methanol. All fractions will be tested antioxidant activity (reduction of DPPH Radical).

The highest antioxidant activity fraction was selected. This fraction (200 mg/ml) was subjected to high performance liquid chromatography (HPLC) system. A reversed-phase column, Luna C18 (25 x 0.46 cm), 5 µm particle size, with a guard column of the same material was used. The elution was performed under gradient conditions. solvent A; acetonitrile and solvent B; 1% phosphoric

acid/10% acetic acid (glacial)/5% acetonitrile (1:1:1, v/v/v), The gradient profile were isocratic 0% A for 6 min, linear gradient 0 to 20% A for 18 min, linear gradient 20 to 40% A for 6 min and isocratic 0% A for 6 min. The flow-rate was 1.0 ml/min. The eluate was monitored at wavelength 510 nm. A fraction (pure compound) was isolated by collecting the eluated at retention time = 22.8 min, and dried by freeze dry method. This fraction was tested for antioxidant activity by DPPH assay and lipid peroxidation of liposomes assay. The structure elucidation of this compound (pure compound) was determined by spectroscopic method (UV-Vis, ^{1}H NMR and MS spectroscopy).

Part IX Stability test (Koseywattana, 2002; The European Agency for the evaluation of medicinal products, 2003)

1. Chemicals

Sodium chloride, commercial grade	(Thailand)

2. Equipments

Analytical balance	(Boeco, Germany)
Hot air oven	(Memmert, Germany)
pH meter	(WTW inolab, Germany)
Chrome meter	(Konica Minolta, Japan)
Thermometer with hygrometer	(Barico, Germany)
Desiccator	(Thailand)

3. Method

3.1 Accelerated condition

The extracts (weigh 3 g of each extract sample in vial with screw cap) were monitored under accelerated test conditions (45°C, 75% RH for 4 months). Sign of physical change such as color change (using Chrome meter), pH (using pH meter), moisture and determined in two samples were determined after various storage times at 0, 8, 15, 22, 30, 45, 60, 75, 90, 105 and 120 days. At the same time, samples were assayed for total phenolic compounds, cyanidin 3-glucoside and quercetin content and antioxidant activity by DPPH method.

3.2 Ambient temperature condition

The extracts (weigh 3 g of each extract sample in vial with screw cap) were monitored under ambient temperature condition (25 - 32°C, 55 - 60% RH for 6 months). Sign of physical change such as color change (using Chrome meter), pH (using pH meter), moisture and determine in two samples were determined after various storage times at 0, 8, 15, 22, 30, 45, 60, 90, 120, 150 and 180 days. At the same time, samples were assayed for total phenolic compounds, cyanidin 3-glucoside and quercetin content and antioxidant activity by DPPH method.

3.3 Statistical analysis

The results were expressed as mean and standard deviation (SD). The chemical constituent in the extracts of *C. nervosum* var. *paniala* after various storage times were statistically analyzed using one-way ANOVA with Duncan's new multiple range test (DMRT) by SPSS program version 11.5 for Windows. The statistical probability (*p*-value) less than 0.05 indicated a statistical significant difference between groups.