Chapter 3

Methodology

1. Conceptual frame work

The rhizome of *Dioscorea membranacea* Pierre, Hua-Khao-Yen-Tai, was reported to be used against cancer by folk doctors in Thailand, were collected from Amphor Pa-tue, Chumporn Province, Thailand. The identification of plant material was carried out by comparison with authentic voucher specimens have been kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. Microscopic description was also used to identify plant powder and compare its characteristics to the previous report (Itharat, 2002).

The rhizomes of *D. membranacea* were washed, sliced thinly, dried in an oven at 50°C and powdered. The extracts of *D. membranacea* obtained by three methods, namely decoction, maceration and soxhlet extraction methods. The percentage of yield from all extracts was calculated.

The chemical characteristics of each extract of *D. membranacea* that obtained from different extraction methods were studied using HPLC technique as HPLC fingerprint and content of dioscorealide B, cytotoxic agent of *D. membranacea* (Itharat *et al*., 2003).

The biological activity characteristic of each extract of *D. membranacea* was also studied as cytotoxic activity against MCF-7 human breast cancer cell line using sulphorhodamine assay (SRB assay).

The ethanolic extract obtained from single solvent maceration which is commonly used by Thai folk doctors to treat cancer was investigated the stability under heat-accelerated conditions (60, 70 and 80°C, 75% RH for 1 month) and accelerated condition (45°C, 75% RH for 4 months). In various storage times, all extracts obtained by all tested conditions was determined the remaining dioscorealide B content as a chemical characteristic change and the cytotoxic activity against MCF-7 by SRB assay as a biological activity characteristic change.

The diagram of work plan was shown below (Figure 3.1).

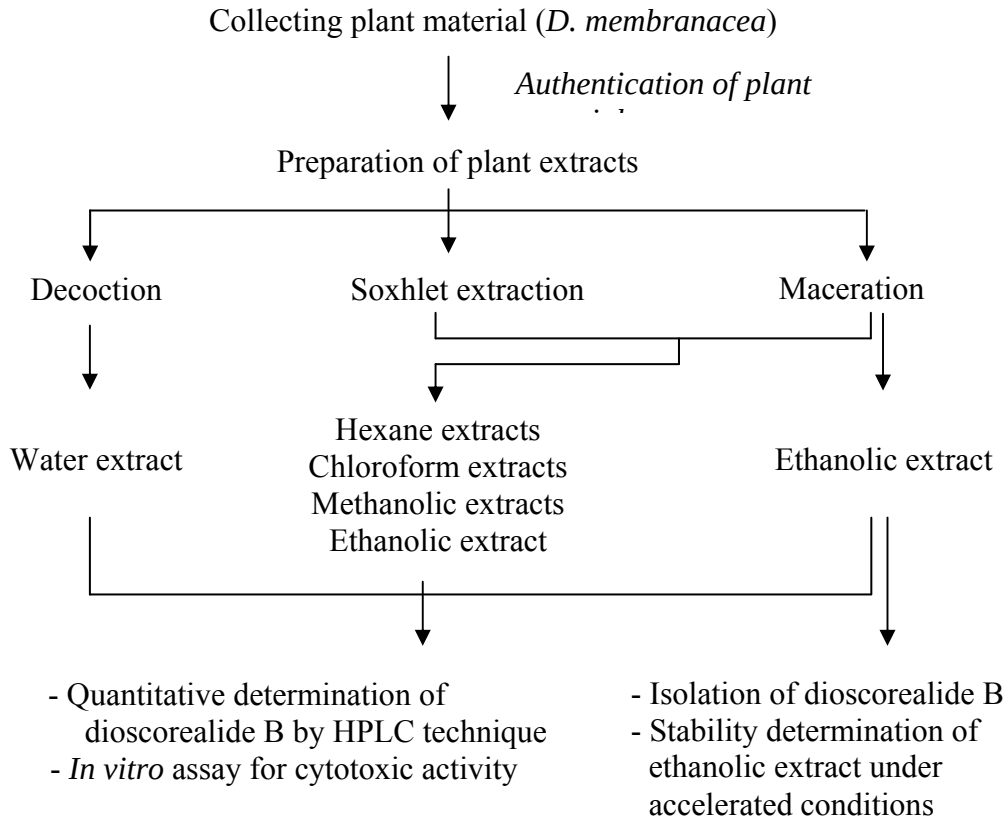

Figure 3.1

Conceptual framework

2. Plant material

The rhizomes of *Dioscorea membranacea* Pierre were collected from Amphor Pa-tue, Chumporn Province, Thailand. The identification of plant material was carried out by comparison with authentic voucher specimens have been kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. Microscopic description was also used to identify plant powder and compare its characteristics to the previous report (Itharat, 2002).

3. Preparation of plant extracts

3.1 Chemicals

Hexane (analytical grade)	(Lab-Scan, Thailand)
Chloroform (analytical grade)	(Lab-Scan, Thailand)
Methanol (analytical grade)	(Lab-Scan, Thailand)
95% Ethanol (commercial grade)	(C.M.J., Thailand)
Distilled water (Milli-Q, ≥ 18 MegaOhm)	(Milford, USA)

3.2 Equipments

Analytical balance	(Boeco, Germany)
Hot air oven	(Memmert, Germany)
Hot plate	(Thermolyne, USA)
Water bath	(Memmert, Germany)
Lyophillizer	(Telstar, Spain)
Rotary evaporator	(Buchi, Japan)

3.3 Methods

The rhizomes of *D. membranacea* were washed, sliced thinly, dried in an oven at 50°C and powdered. Its extracts were obtained by three extraction methods such as decoction, maceration and soxhlet extraction, using different solvents e.g. hexane, chloroform, methanol, ethanol and water.

Figure 3.2

Rhizome of *D. membranacea* in dried powder

3.3.1 Decoction

Dried plant material (5 kg) was boiled in distilled water (3 L) at 70°c for 30 minutes, filtered and dried by using a lyophillizer. This part was called as water extract.

3.3.2 Maceration

Dried plant material (300 g) was soaked with solvent (1 L) for 3 days by single solvent maceration and polarity sequencing solvent maceration. The extracts from each solvent were filtered and dried by an evaporator.

3.3.2.1 Single solvent maceration

The 1.2 kg dried plant powder was divided to be 4 portions. Each portion of plant powder was macerated by hexane, chloroform, methanol, and 95% ethanol. Thus, we obtained hexane, chloroform methanolic and ethanolic extracts.

3.3.2.2 Polarity sequencing solvent maceration

Dried plant powder (300 g) was macerated by solvents continuously from non-polar solvent to polar solvent (hexane, chloroform and methanol, respectively) of the same plant powder. Thus, we obtained hexane, chloroform and methanolic extracts, respectively.

3.3.3 Soxhlet extraction

Soxhlet extractor was used to extract dried plant material (75 g) by single solvent soxhlet extraction and polarity sequencing solvent soxhlet extraction for 4 hours of each solvent. The extracts from each solvent were filtered and dried by an evaporator.

3.3.3.1 Single solvent soxhlet extraction

The 300 g plant powder was divided to be 4 portions. Each portion of plant powder was extracted by hexane, chloroform, methanol, and 95% ethanol. So, we obtained hexane, chloroform methanolic and ethanolic extracts.

3.3.3.2 Polarity sequencing solvent soxhlet extraction

The plant powder (75 g) was extracted by soxhlet apparatus using solvents continuously from non-polar solvent to polar solvent (hexane,

chloroform and methanol, respectively) of the same plant powder. We obtained hexane, chloroform and methanolic extracts.

The percentage of yield of all extracts was calculated. The diagram of extraction was showed below (Figure 3.3).

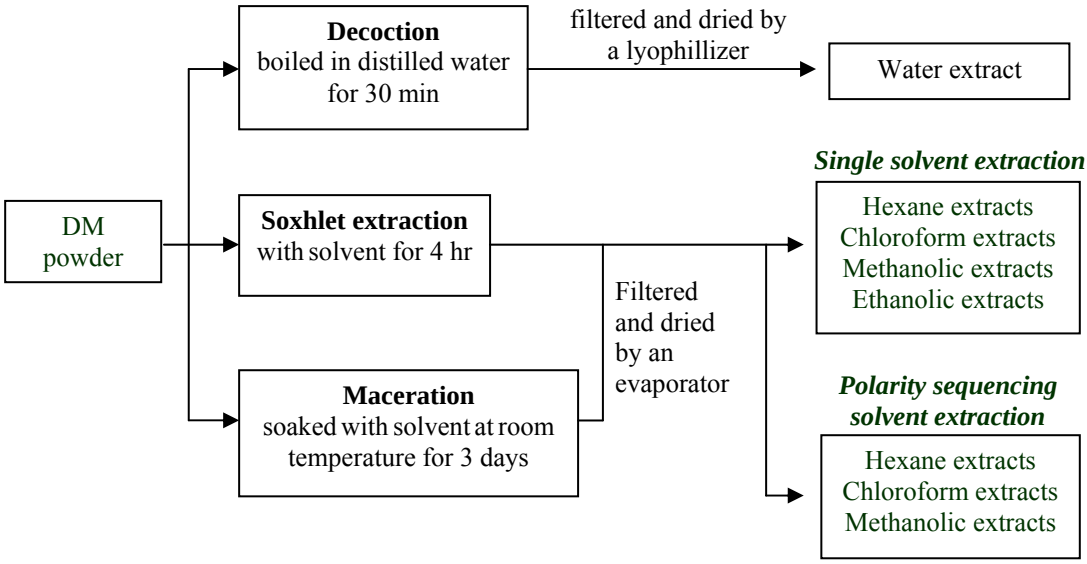

Figure 3.3

Extraction of *D. membranacea*

4. Isolation of dioscorealide B from *D. membranacea* by column chromatography

4.1 Chemicals

Hexane (analytical grade)	(Lab-Scan, Thailand)
Chloroform (analytical grade)	(Lab-Scan, Thailand)
Methanol (analytical grade)	(Lab-Scan, Thailand)
Ethylacetate (analytical grade)	(Lab-Scan, Thailand)

4.2 Equipments

Analytical balance	(Boeco, Germany)
Column (11 x 12.5 cm, glass)	(VN supply, Thailand)
Silica gel 60 (7734)	(Merck, Germany)
Silica gel 60 (9385)	(Merck, Germany)
Sonicator	(Elma, Germany)
Rotary evaporator	(Buchi, Japan)
TLC plates (silica gel 60GF254)	(Merck, German)

4.3 Methods

An aliquot (40 g) of ethanolic extract obtained by maceration of *D. membranacea* was separated by vacuum liquid chromatography (VLC) using a gradient of solvents. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure. The fraction which had dioscorealide B-like spot of TLC chromatogram was continued isolated by column chromatography (CC) until obtained pure compound with similar TLC chromatogram to dioscorealide B standard . The details of isolated Dioscorealide B stepwise showed below.

The ethanolic extract was chromatographed by VLC using a gradient of solvents, hexane (2000 ml), $CHCl_3$:Hexane (9:1) (2000 ml), $CHCl_3$ (250 ml/fraction, 12 fractions) and MeOH (1000 ml), respectively. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure. The 2.20 g of the third (fraction 5-7, DMC3) from 4 $CHCl_3$ fractions was chosen to separate by CC using a gradient of solvents, $CHCl_3$ (13.5 ml/fraction, 112 fractions), $CHCl_3$:MeOH (7:3) (1000 ml) and MeOH (1000 ml), respectively. Then, the fractions were combined following TLC examination (silica gel GF254/ mobile phase as $CHCl_3$/ detection with acidic anisaldehyde spraying reagent). The precipitate of the seventh fraction (fraction 46-61, DMCC7, 29.9 mg) of 9 $CHCl_3$ fractions was continuously separated by CC with Ethylacetate:Hexane (1:1) (8 ml/fraction, 3 fraction and 2.5 ml/fraction, 69 fraction), Ethylacetate (100 ml), Ethylacetate:MeOH (1:1) (100 ml) and MeOH (100 ml), respectively. The fraction 3-

10 of Ethylacetate:Hexane (1:1) were combined to yield 21.05 mg (% yield = 0.053) of pure compound with dioscorealide B-like spot of TLC chromatogram.

Figure 3.4

Isolation of dioscorealide B from *D. membranacea* by column chromatography

The isolated pure compound was identified by comparison its TLC chromatogram characteristic with dioscorealide B standard and its proton NMR spectrum with authentic proton NMR spectrum of dioscorealide B.

5. Quantitative determination of dioscorealide B by HPLC technique

5.1 Chemicals

Acetonitrile (HPLC grade)	(Lab-Scan, Thailand)
Water (HPLC grade)	(Lab-Scan, Thailand)
Methanol (HPLC grade)	(Lab-Scan, Thailand)
Helium gas	(Chattakorn Lab Center, Thailand)

5.2 Equipments

HPLC apparatus

- Quarternary pump (ConstaMetric 4100 Bio) (TSP, USA)
- Autosampler (SpectroSystem AS 3500) (TSP,USA)
- UV-vis detector SpectroMonitor 4100 (LDC Analytical, USA)
- TSP PC1000 software (TSP, USA)

Analytical column

- C18, 5u, 250 mm×4.6 mm (Luna) (Phenomenex, USA)

Guard column

- Cartridge holder (Phenomenex, USA)
- C18 guard cartridge 4x3.0 mm (Phenomenex, USA)

Sonicator (Elma, Germany)

Micropipettes 20, 200, 1000 µl (Rainin, USA)

Filter membrane (0.45µm) (Sartorius, Germany)

Syringe (Nipro, Thailand)

Screw cap vial 12x32 mm, 1.8 ml Cleer (Microliter Analytical Supplies, USA)

Syringe filter nylon 13 mm, 0.45 µm (GAT, Thailand)

Analytical balance (Mettler-Toledo, USA)

Analytical balance (Boeco, Germany)

5.3 Methods

The analysis of dioscorealide B, a cytotoxic marker, in the *D. membranacea* extracts was performed using HPLC system including a quaternary pump,, autosampler, and UV-vis detector. Chromatographic separation was achieved at room temperature using a reversed-phase analytical column, C_{18} column (250×4.6 mm, 5 µm), with a guard column of the same material. Data acquisition and analysis were performed by TSP PC1000 software.

5.3.1 Chromatographic condition

The mobile phase was consisted of water (A) and acetonitrile (B) with gradient elution as followed: 70-55% A at 0-10 min, 55% A for 5 min, 55-30%

A at 15-30 min, 30% A for 5 min, 30-70% A at 35-37 min, 70% A for 8 min. The flow rate of the mobile phase was 1.0 ml/min and the injection volume was 10 µl. Chromatographic separations were achieved at room temperature. The quantitation was performed using peak area. The UV detector was used to detect the peak of dioscorealide B with the fixed wavelength at 270 nm (Sirikatitham *et al*., 2007).

5.3.2 Preparation of dioscorealide B standard

A stock solution of the standard dioscorealide B; 1.0 mg/ml, was prepared by dissolving weighed quantity of standard compound in acetonitrile and diluting to be serial concentration. The stock solution was serially diluted to the concentrations of 25.0-200.0 µg/ml. All solutions were stored under refrigeration. The sample solutions, 10 µl, were directly injected into the HPLC column and separated under above chromatographic condition.

5.3.3 Preparation of plant extract samples

Extract sample solutions were prepared by accurately weighing; 10.0 mg of plant extract and adding of acetonitrile. The solutions were sonicated for 15 min and allowed to cool to room temperature before being filtered through a 0.45-µm filter membrane before analysis. The sample solutions, 10 µl, were directly injected into the HPLC column and separated under above chromatographic condition. The analysis was performed in triplicate.

5.3.4 Validation of HPLC method

The development of a method for separation and quantitation of the active compound in plant extract is very important for quality control of such preparations (Penissi *et al*., 2003). Therefore, the quantitative analysis method development and validation were required. The validation parameters addressed were specificity, precision, accuracy and linearity.

5.3.4.1 Specificity

Specificity is the ability to find and quantify the compound of interest also in the presence of other compounds. This refer to chromatographic methods that the analyze can be separated with sufficient resolution from other

accompanying peaks and that it can be detected with a suitable instrument (Meyer, 2004). The specificity of the developed HPLC method for dioscorealide B was carried out in the presence of the other components in the crude extract.

5.3.4.2 Linearity and range

Linearity of the method was studies by injecting 5 concentrations of standard solutions prepared in acetonitrile in the range of 12.5-200 μg/ml (12.5, 25, 50, 100 and 200 μg/ml) in triplicate into the developed HPLC system. The linearity test was carried out for 3 consecutive days. The peak areas versus concentration data were treated by least-squares linear regression analysis. Under most circumstances, regression coefficient (r^2) is $\geq$ 0.999 (Analytical Methods Technical Committee, 1994). The SD value for the slope and Y-intercept of the calibration curve was calculated.

5.3.4.3 Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions (Analytical Methods Technical Committee, 1994). The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analyses of three different concentrations of the standard solutions in triplicate on the same day. Intermediate precision of the method was checked by repeating the studies on three different days.

5.3.4.4 Accuracy

Accuracy is the value of the experimental measurement which close to the true value (Analytical Methods Technical Committee, 1994). The accuracy of the developed method was tested by fortifying a mixture of crude extract solutions with six concentrations of the standard solutions (0, 12.5, 25, 50, 100 and 200 μg/ml) and determining the recovery of added analyse. 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%.

5.3.5 Quantification of dioscorealide B in extract samples

The calibration curve of dioscorealide B standard was created by plotting concentrations versus corresponding mean peak areas. The peak areas versus concentration data were treated by least-squares linear regression analysis. The concentration of dioscorealide B in extract samples was calculated from linear equation of calibration curve.

5.3.6 Statistical analysis

The quantitative determination of dioscorealide B was done as triplicate. The results were expressed as mean and standard error of mean (SEM) of dioscorealide B content. The dioscorealide B content of each extract obtained from different extraction methods was statistically analyzed using One-Way ANOVA with LSD of Post Hoc Multiple Comparison by SPSS program version 13 for Windows. The statistical probability (*p*-value) less than 0.05 indicated a statistical significant difference between groups.

6. *In vitro* assay for cytotoxic activity of *D. membranacea* extracts

6.1 Human cell line

MCF-7 human breast cancer cell line was kindly provided by Assoc. Prof. Dr. Arunporn Itharat, Faculty of Medicine, Thammasat University.

6.2 Chemicals

Dulbeco's modified eagle medium (DMEM)	(Gibco, USA)
Fetal bovine serum (FBS)	(Gibco, USA)
Penicillin	(Gibco, USA)
Streptomycin	(Gibco, USA)
Acetic acid (analytical grade)	(Merck, Germany)
Trypsin	(Sigma, USA)

Trypan blue	(Gibco, USA)
Sodium hydroxide (analytical grade)	(Merck, Germany)
Phosphate buffer saline (PBS)	(Gibco, USA)
sulphorhodamine	(Sigma, USA)
Dimethylsulfoxide (DMSO)	(Fluka, USA)
Trichloroacetic acid (TCA)	(Fluka, USA)
Deionized water (Milli-Q, ≥ 18 MegaOhm)	(Milford, USA)

6.3 Equipments

Analytical balance	(Boeco, Germany)
Autoclave	(Hirayama, Japan)
Hot air oven	(Memmert, Germany)
CO_2 humidified incubator	(Shel lab, USA)
Laminar air flow	(Boss tech, Thailand)
Microplate reader	(Bio Tek, USA)
Bio medical freezer	(Sanyo, Japan)
Ultra-low temperature freezer	(Sanyo, Japan)
Inverted microscope	(Nikon, USA)
pH meter	(WTW inolab, Germany)
Centrifuge	(Boeco, Germany)
Sonicator	(Elma, Germany)
Haemocytometer	(Costar, USA)
96-well microtiter plates	(Costar, USA)
Pipette boy	(Brand, USA)
Multi-channels pipette	(Costar, USA)
Micropipettes	(Rainin, USA))
Glass bottles	(Schott Duran, Germany)
Centrifuge tube 15, 50 ml	(Corning, Mexico)
Pipette tips 200, 1000 μg/ml	(Corning, Mexico)
Microcentrifuge tubes 1.7 ml	(Corning, USA)
Cell culture flask, canted neck, 75cm^2	(Corning, USA)
Disposable pipette 5, 10, 25 ml	(Corning, USA)

6.4 Methods

6.4.1 Human cell line culture

MCF-7 cell line was cultured in DMEM culture medium supplement with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (Keawpradub *et al*., 1999) and maintained at 37°C in an incubator with 10% CO_2 and 95% humidity.

6.4.2 Cytotoxicity assay

According to growth profile of MCF-7, the optimal plating densities of MCF-7 cell line was determined to be 3×10^3 cells/well 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 75 cm^3 flask were washed by magnesium and calcium-free phosphate buffer saline (PBS, pH 7.4). PBS was decanted. Cells were detached by 0.025% trypsin-EDTA to make a single cell suspension. The viable cells were counted by trypan blue exclusion in haemacytometer (Freshney, 1994) and diluted with medium to give a final concentration of $3x10^4$ cells/ml. A hundred microlitres of cell suspension were seeded in each well of 96-well microplates and incubated at 37°C to allow for cell attachment. After 24h, the cells were treated with the plant extracts. Each extract was initially dissolved in either DMSO, for hexane, chloroform, methanolic and ethanolic extracts, or sterile distilled water for water extract. The extracts were diluted in medium to produce required concentrations. A hundred microlitres of each concentration were added to each well of plates to obtain final concentrations of 1, 10, 50, 100 μg/ml in quadruplicate. The final mixture used for treating the cells contained not more than 1% of the initial solvent; the same as in solvent control wells. The plates were incubated for 72 h. 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 to each well. The plates were incubated for a recovery period for 72 h. Then, the cell growth was analyzed using the SRB assay.

6.4.3 Sulphorhodamine B (SRB) assay (Skehan *et al.,* 1990)

The antiproliferative SRB assay was performed to assess growth inhibition by a colorimetric assay which was estimated cell number indirectly by staining total cellular protein with the SRB dye. The viable cells were fixed by layering 100 µl of ice-cold 40% trichloroacetic acid (TCA) per well, incubated at 4°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. Fifty microlitres 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] 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 read on a microplate reader at 492 nm as an indication of cell number. The intensity of color developed in each well was corresponded to the survival cell number. Cell survival was measured as the percentage absorbance compared with the control (non-treated cells).

Percentage of growth inhibition was calculated by using equation below.

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

The IC_{50} value of the percentage of growth inhibition was expressed as concentration of extract in microgram per millilitre that caused a 50% growth inhibition comparing with controls. The IC_{50} value was calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine.

According to National Cancer Institute guidelines (Boyed, 1997), the extracts with less than 20 µg/ml IC_{50} value were considered active.

6.4.4 Statistical analysis

The results of the crude extracts of *D. membranacea* from different extraction methods were expressed as the percentage of growth inhibition and IC_{50} by mean and standard error of mean (SEM) by Prism program.

7. Stability study of ethanolic extract under accelerated conditions

7.1 Chemicals

Sodium chloride (analytical grade)	(APS Finechem, Australia)

7.2 Equipments

Analytical balance	(Boeco, Germany)
Hot air oven	(Memmert, Germany)
Thermometer with hygrometer	(Barico, Germany)
Dessiccator (30 cm vacuum)	(Roongsub Chemical, Thailand)

7.3 Methods

The ethanolic extract of *D. membranacea* obtained by single solvent maceration was investigated its stability under accelerated conditions. The changes of chemical and biological activity characteristics of the ethanolic extract in various storage times were determined.

7.3.1 Stability conditions

The ethanolic extract (10 mg of extract in vial with screw cap) was monitored under heat-accelerated condition (60, 70, 80^{o}C, 75% RH for 1 month) and accelerated condition (45^{o}C, 75% RH for 4 months).

7.3.2 Stability determinations

After various storage times, the samples were assayed for remaining dioscorealide B content by HPLC, determination of chemical characteristic change,

and assayed for cytotoxic activity by SRB assay against MCF-7 at 10 μg/ml of extract, determination of biological activity characteristic change, as described above.

7.3.3 Statistical analysis

The experiment was done as triplicate for quantitative determination of dioscorealide B content and quadruplicate for cytotoxic activity determination by SRB assay against MCF-7. For the determination of dioscorealide B content, the results were expressed as mean and standard error of mean (SEM) of the percentage of dioscorealide B remaining while the determination of cytotoxic activity, the results were expressed as the percentage of growth inhibition by mean and standard error of mean (SEM). The percentage of dioscorealide B remaining and growth inhibition of ethanolic extract of *D. membranacea* from different sampling times of stability determination were statistically analyzed using One-Way ANOVA with LSD of Post Hoc Multiple Comparison by SPSS program version 13 for Windows. The statistical probability (*p*-value) less than 0.05 indicated a statistical significant difference between groups.