CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this research including the materials, apparatus and the experiments. The details of each part are given below.

Materials

1. Artocarpin standard (purified artocarpin was kindly supported by Prof. Dr.Kuniyoshi Shimizu, Faculty of Agriculture, Kyushu University, Japan)
2. Ascorbic acid (ACS reagent, Lot No. 60780, Riedel-deHaen, Germany)
3. B16F1 mouse melanoma cell line (CRL-6323, Lot No. 2634963, American Type Culture Collection, USA.)
4. Butylhydroxytoluene; BHT (A.R. grade, Lot No. 78H0689, Merck, Danstadt, Germany)
5. 2,2-Diphenyl-1-picrylhydrazyl; DPPH (A.R. grade, Lot No. 083K0830, Sigma-Aldrich , Germany)
6. 3,4-Dihydroxyphenylalanine; L-DOPA (A.R. grade, Lot No.023K7024, Sigma-Aldrich, Germany)
7. Diethyl ether (A.R. grade, Batch No. 04090204, LabScan Asia Co. Ltd., Thailand)
8. Dimethylsulfoxide; DMSO (A.R. grade, Lot No. 0320064, Sigma chemical Co. Ltd.,USA)
9. Dimethylsulfoxide (99.5% GC Plant cell culture tested, CAS No. 67685, Merck, Germany)
10. Disodium hydrogenorthophosphate (A.R. grade, Lot No. F1262781344, Merck, Germany)
11. Disodium hydrogenorthophosphate anhydrous (A.R. grade, Lot No. F1262781, BDH Laboratory Supplies, England)

12. Dulbecco's Modified Eagle's Medium; DMEM (A.R. grade, Lot No. 054K8302, Sigma-Aldrich, Germany)
13. Fetal bovine serum;FBS (A.R. grade, Lot No. 40f3634K, GIBCO, UK)
14. Hydrochloric acid (A.R. grade, CAS No. 7647010, J.T. Baker, USA)
15. Kojic acid (A.R. grade, Lot No. 0832534, Sigma-Aldrich, Germany)
16. Methanol (HPLC grade, Batch No. 03040021, LabScan Asia Co. Ltd., Thailand)
17. Mushroom tyrosinase enzyme (A.R. grade, Lot No. 023K7024, Sigma-Aldrich Germany)
18. Propylene glycol (Cosmetic grade, Vidhyasom Co., Ltd., Thailand)
19. Sodium carbonate (A.R. grade, Lot No. 043K0015, Merck, Germany)
20. Sodium hydroxide (A.R. grade, Lot No. B916398648, Merck, Germany)
21. Sodium dihyrogen orthophosphate (A.R. grade, Lot No. A403421322, Merck, Germany)
22. Sodium dihydrogen orthophosphate 1-hydrate (A.R. grade, Lot No. A403421322, BDH Laboratory Supplies, England)
23. Tyrpsin EDTA (A.R. grade, Lot No. 1212385, GIBCO, Canada)
24. Trypan blue solution (R&D grade, Lot No. 55K2342, Sigma chemical Co. Ltd., USA)

Apparatus

1. Diode array detector (model SPD-10M10AVP, Shimadzu, Japan)
2. High performance liquid chromatographer ;HPLC (model LC-10ATVP, Shimadzu, Japan)
3. HPLC column (model Alltima C18, 5 µm, 4.6x250 mm I.D., Alltech Associates Inc. Corperation, USA)
4. HPLC system controller (model SCL-10A, Shimadzu, Japan)
5. Incubator (model 311,ThermoForma, USA)
6. Inverted microscope (model TS100, Nikon Eclipse, Japan)

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7. Microplate reader (model CeresUV900C, Bio-Tek Instrument, USA)
8. Microplate spectrophotometer (model Spectra Count®, Perkin Elmer, USA)
9. Rotavapor (model R153, Buchi, Switzerland)
10. Vacuum system B-169 (NESLAB Instruments Inc., USA)
11. Vertical larminar flow cabinet (model BHG2004S, Faster s.r.l., Italy)
12. Water bath (model LWB-211A, Daihom LabTech Co. Ltd., Korea)

The Experimental Methods

1. The Preparation of the Extract of *A. incisus* Heartwood

1.1 Plant Materials

The heartwood of *A. incisus* was collected in May 2005 from Phitsanulok Province, Thailand. The heartwood was chipped in the size of 1x1x10 cm and exposed to the sun for 2 days. Then the chipped heartwoods were dried at 50°C for 2 days by using hot-air oven and ground into powder by using mill. The obtained powder was kept in the tight container at room temperature before being used.

1.2 Extraction Process

Two solvent systems, diethyl ether or methanol was used for preparation of *A. incisus* extract with modified method (Shimizu et al., 1998. pp. 408-409) as shown in Figure 10.

Ether Extract : Five-hundred grams of the *A. incisus* powder was filled in a percolator and then soaked with 800 mL of diethyl ether at room temperature for 2 days. The solution of *A. incisus* ether extract was filtered through woven cloth filter and evaporated to concentrate under reduced pressure with a vacuum evaporator set at 30°C. After that the extracts were dried in desiccator. The obtained ether extract was stored at -20°C in tight-amber glass before being used.

Alcohol Extract : One-kilogram of *A. incisus* powder was filled in the glass container and then soaked with 2 L of methanol. The tight container containing the extracts was shaken at room temperature for 1 week. The solution of *A. incisus* extract was filtered and evaporated to concentrate under reduced pressure with a vacuum evaporator at 50°C. After that the extract was dried in desiccator. The obtained methanol extract was stored at -20°C in tight-amber glass before being used.

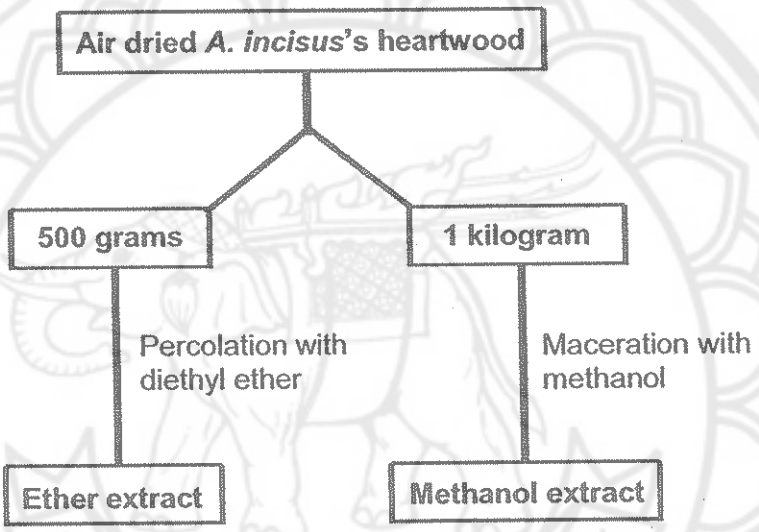

Figure 10 The Extraction Procedure of *A. incius*'s Heartwood with Diethyl Ether and Methanol.

2. The Determination of the Artocarpin Content by HPLC Analysis

2.1 HPLC Condition

High performance liquid chromatography (HPLC) was used to determine the artocarpin, a major component contained in the *A. Incisus* heartwood's extract. The HPLC apparatus consists of UV- detector, system controller and C18 column with 5 µm and 250x4.60 mm diameter. The chromatographic conditions are as follows:

Mobile phase : Methanol:Water (85:15) by volume

Flow rate : 1.0 mL/min

Injection volume : 50 µL

Pressure : 1700 Psi

Run time : 20 min

UV absorption : 282 nm

2.2 Calibration Curve

The stock solution obtained from dissolving the standard artocarpin to a concentration of 1 mg/mL of methanol. The stock solution was then diluted with mobile phase to the concentrations of 0.04, 0.08, 0.12, 0.16 and 0.2 mg/mL. Three calibration curves were performed by plotting the peak areas under the chromatogram versus the concentrations.

2.3 Sample Preparation

The stock of sample solutions obtained from dissolving the extracts to a concentration of 1 mg/mL of methanol. The sample stock solution was then diluted with mobile phase to the concentrations of 0.5 mg/mL. The sample solutions were well mixed by using Vortex and then filtered through a 0.45 µm of Millipore before injection.

3. Study the Effective of *A. incisus* Ether Extract on the Tyrosinase Inhibitory Activity using the DOPAchrome Method

This experiment was determined by spectrophotometry with modified method (Baurin et al., 2002; Likhitwittayawuid, 2001; Masamoto, 1980).

3.1 Sample Preparation

The stock solution of *A. incisus* extract was prepared by dissolving the crude extract to the concentration of 10 mg/mL of DMSO or propylene glycol.

The stock solution of kojic acid was prepared by dissolving kojic acid to the concentration of 10 mg/mL of DMSO or propylene glycol. Kojic acid was used as a positive control in this study.

3.2 DOPAchrome Method

For each concentration of the sample solution, fours well were designed as A, B, C and D. Each contained a reaction mixture (180 μL) as follows;

(A) 20 μL of mushroom tyrosinase (426 units/mL), 140 μL of 20 mM phosphate buffer (pH 6.8) , and 20 μL of DMSO or propylene glycol

(B) 160 μL of 20 mM phosphate buffer (pH 6.8) and 20 μL of DMSO or propylene glycol

(C) 20 μL of mushroom tyrosinase solution (426 units/mL) , 140 μL of 20 mM phosphate buffer (pH 6.8) , and 20 μL of the sample solutions

(D) 160 μL of 20 mM phosphate buffer (pH 6.8) and 20 μL of the sample solutions.

The mixed solution was incubated at room temperature for 10 min and then 20 μL of 0.85 mM L-DOPA was added into each well. After incubation at 25°C for 20 min, an amount of DOPAchrome produced in each well was measured an absorbance at 490 nm by using 96-well microplate reader. The processes of the DOPAchrome method are illustrated in Figure 11.

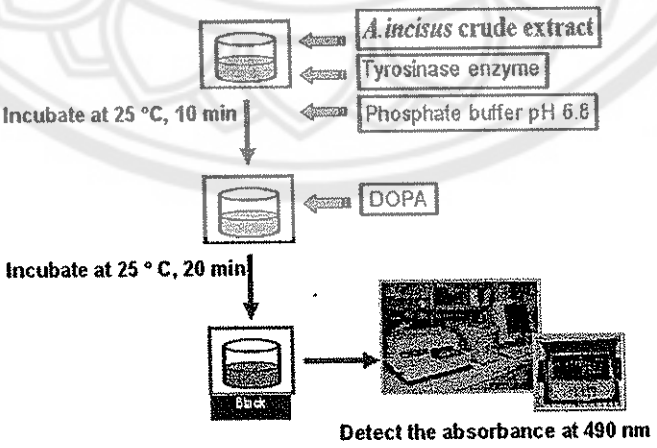

Figure 11 The Diagram of DOPAchrome Method

Tyrosinase inhibitory activity was calculated by using the following equation:

$$\% \text{ tyrosinase inhibition} = [\ (A-B) - (C-D) / (A-B)\ ] \times 100$$

Where; A is an absorbance of the mixture well (A)

B is an absorbance of the mixture well (B)

C is an absorbance of the mixture well (C)

D is an absorbance of the mixture well (D)

IC_{50}, the 50% inhibition of tyrosinase activity was calculated as the concentration of test samples that inhibit 50% of tyrosinase activity under experimental conditions by using GraphPadPrism 3.02 (GraphPad Software, Inc. USA).

3.3 Statistic Analysis

The assay to determine enzyme activity and the tyrosinase inhibitory effect were conducted at least tree times with different sample preparations. All data were expressed as mean±SD by using Microsoft Excel® 2003 (Mirosoft Corp., USA).

4. **The Determination of the Melanin Content in Melanocyte Cells Culture**

B16-F1 (ATCC No. CRL-6323) mouse melanoma cells were purchased from ATCC. This cell line was isolated from the melanocyte of mouse strain C57BL/6J and deposited at ATCC by the Naval Biosciences Laboratory. The following cell line descriptions are given by:

B16-F1 Organism : *Mus musculus* (mouse)

Source : skin (melanoma)

Strain : C57BL/6J

Morphology : fibroblast-like

Depositors : Naval Biosciences Laboratory

Tumorigenic : yes, in syngeneic mice

Comments : Confirmed as a murine cell line by ATCC. Test for microbial contamination were negative. This cell line produces melanin which may cause the culture medium to turn brown/black. This is normal and should not be misinterpreted as contamination.

4.1 Cultivation of Cells

B16F1 melanoma cells were initially cultured in 25 cm^2 flask ($3.2x10^6$ cells/mL) with DMEM supplement with 10% FBS at air containing 5% CO_2 and temperature of 37°C. The medium was changed every 2 days. The passage numbers of 5 to 8 were used in this study.

4.2 The Study of B16F1 Growth Curve

Cell number versus time (day) was plotted to elucidate the graph pattern of cells without the interfering of the samples and then to select the optimum harvesting time for the sample testing. The cell morphological was observed under a light microscope.

4.3 The Treatment of Cells

Before being tested, the cell suspension was transferred from 25 cm^2 flask into a 24-well plate ($1x10^5$ cells/well) and kept in incubator for overnight to complete adhering of the cells on the plate. After 24 hours of cultivation, the old medium was replaced with 1.0 mL of new DMEM medium containing the various concentrations of *A. incisus* extract or artocarpin containing DMSO not more than 0.1% (v/v). The control cells were treated with 0.1% (v/v) DMSO without the extract. Kojic acid and hydroquinone were used as positive control.

4.4 Cell Viability

After incubation for 4 days, the proliferation of cell was measured by directly count the number of cells treated with trypan blue. Hemocytometer was used for counting viable cell that not being stained with blue dye.

4.5 Melanin Content Assay

Melanin content assay was performed by using the modified method (Mun, et al., 2004. p. 807) and with triplicate run.

After incubation for 4 days, the treated cells were harvested by using trypsinization and washed twice with phosphate buffer saline (PBS). The samples were air-dried and dissolved in 200 µL of 1 N NaOH containing 10% of DMSO. The samples were heated at 80 °C for 1 hr and then cooled down at room temperature. The absorbance of melanin was measured at wavelength of 490 nm. The melanin content per cell was calculated by comparing to the absorbance of control adjusted to 100%.

4.6 Statistic Analysis

All experimental data were analyzed using analysis of variance (ANOVA) and significant difference among mean from triplicate analysis at $p<0.05$ were determined by Duncan's multiple range test using SPSS 12.0 for window (SPSS Inc., USA).

5. Study the Antioxidant Property of *A.incisus* Ether Extract by using DPPH Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to measure the free radical scavenging activity of the extract. The degree of DPPH decoloration indicated the scavenging efficiency of the added sample. This DPPH assay was performed with triplicate under modified method (Juliano et al., 2005; Amarowicz et al., 2004).

5.1 Sample Preparation

The sample solutions of *A. incisus* ether extract, BHT and L-ascorbic acid were prepared by dissolving each of them with DMSO, methanol and deionized water, respectively, to obtain the concentration of 10 mg/mL. BHT and L-ascorbic acid were used as positive control in this study.

5.2 DPPH Radical Reduction

The reaction mixture consisted of 150 µL DPPH (0.2 mM, stored at -20°C until use) and 75 µL of the sample solution. This sample solution was replaced with methanol, DMSO or deionized water for acting as a blank solution. The mixture was mixed using vortex for 15 seconds and left to stand for 30 min. The absorbance of remaining DPPH was measured after incubation by determination of radical scavenging activity on DPPH at wavelength of 515 nm. The processes of DPPH assay are shown in Figure 12.

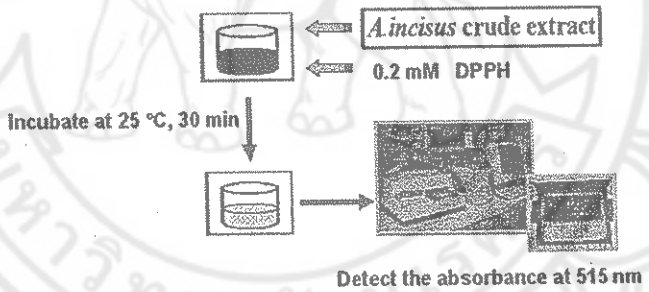

Figure 12 The Diagram of DPPH Assay

The radical scavenging activity was calculated as a percentage of DPPH discolouration using the following equation:

$$\%\text{Radical Scavenging Activity} = [1-(A_S/A_B)] \times 100$$

Where; A_S is an absorbance of DPPH with tested sample

A_B is an absorbance of DPPH without tested sample

EC_{50}, the equivalent concentration to give 50% effect, was determined by log-probit analysis using 6 to 10 different final concentrations of samples by using GraphPadPrism 3.02 (GraphPad Software, Inc. USA).

5.3 Statistic Analysis

The assay to determine antioxidant were conducted at least tree times with different sample preparations. All data were expressed as mean±SD by using Microsoft Excel® 2003 (Mirosoft Corp., USA).