CHAPTER II

EXPERIMENTAL

2.1 Instruments and Equipments.

Nuclear Magnetic Resonance (NMR) spectra were recorded on Varian Mercury Spectrometer (Varian Company, Palo Alto, CA, USA) operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. ^{1}H and ^{13}C NMR spectra were obtained in deuterated acetone (acetone-*d6*) using tetramethylsilane (TMS) as an internal standard. UV analysis was carried out on UV 2500 UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan), using a quartz cell with 1 cm pathlength. FT-IR spectra were recorded on with Nicolet Fourier Transform Infared spectrophotometer: Impact 410 (Nicolet Instruments Technologies, Inc., Madison, WI, USA). High Resolution Mass Spectroscopy ESI-TOF MS analysis was performed on microTOF: MS/MS (Bruker Daltonics Inc., USA). TLC was performed on aluminium sheets precoated with silica gel (Meck Kieselgel 60 F254) (Merck KgaA, Darmstadt, Germany). The BUCHI rotary evaporator R-200 (BUCHI, Flawil, Switzerland) was used in this study. High Performance Liquid Chromatography (HPLC) performed with Thermo Finnigan spectra SYSTEM (Phenomenex, California, USA) consisting of diode array detector. HPLC was performed on a BDS C18 reverse phase column (4.6 x 100 mm) (Thermo, Electron corporation, Massachusetts, USA), mobile phase consisting of 75 mM citric acid and 25 mM ammonium acetate in milli-Q water (A) and methanol (B) with A: B ratio of 60:40 (v/v) at a flowrate of 1 mL/min. The injection volume was 5 µL (100mg/L) and the detection wavelength was set at 282 nm [75].

2.2 Standards and Reagents.

Naringin, 97 % purity, gallic acid, 98 % and kojic acid were purchased from Acros organics (New Jersey, USA). Folin-Ciocalteu's reagent and ammonium acetate

were obtained from Carlo Erba (Milan, Italy). Citric acid monohydrate was purchased from J.T Baker Chemial (Deventer, Holland). Sodium carbonate anhydrous was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Sodium chloride was purchased from Lab Scan (Bangkok, Thailand). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$ free radical) and L-tyrosine were purchased from Fluka Chemie (Buchs, Switzerland). 2, 6-Di-*tert*-butyl-*p*-hydroxytoluene (BHT) was from Panrea Sintesis (Bacelona, Spian). Mushroom Tyrosinase (EC 1.14.18.1) was obtained from Sigma Chemical Company (St Loius, MO, USA). K_2HPO_4 and KH_2PO_4 were purchased from Merck KdaA (Damstadt, Germany) and Carlo Erba reagent (Milan, Italy) respectively. All Solvents used in extraction were purified from commercial grade solvents prior to use by distillation. Analytical grade solvents used in high-performance liquid chromatography were purchased from Carlo Erba (Milan, Italy).

2.3 Sample preparation.

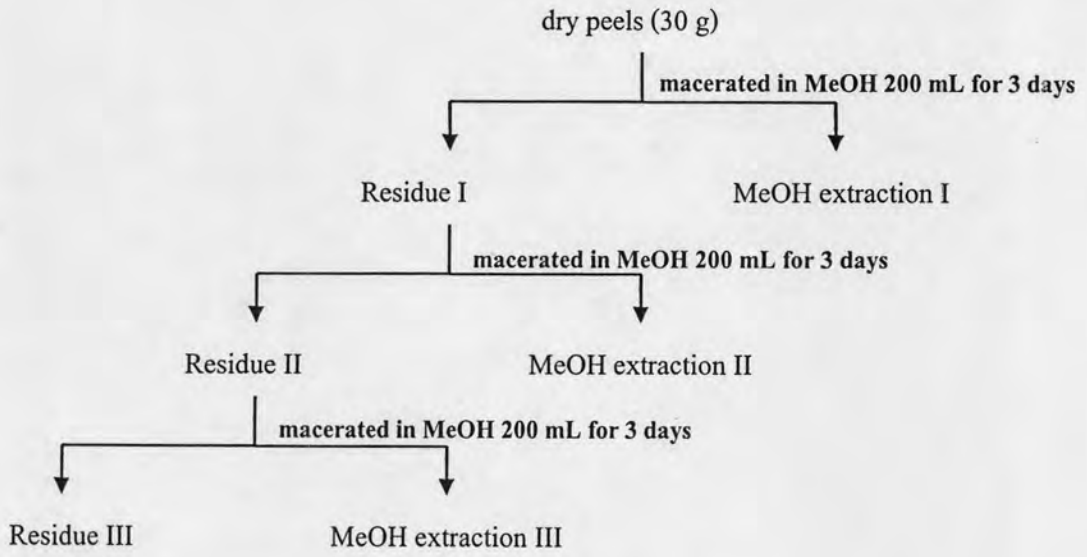
Pomelo peels of the cultivars Kao Taeng-gwa (KT), Kao Yai (KY), Kao Nam Pheung (KN) and Tong Dee (TD), which from Phichit Province, Thailand. Pomelo peels consisting of albedo (white spongy interior) and flavedo (yellow green exterior) were cut into small pieces and sun-dried for 3 days. The sun-dry samples were then put in the oven at 40-60 °C for 24 h before being ground into powder.

2.4 Extraction and isolation of naringin.

Albedo peels of four cultivars, KT, KY, KN, TD and only KT flavedo peel were extracted by maceration in methanol. Each extract was filtered, evaporated under the reduce pressure and tested free-radical scavenging, tyrosinase inhibition and UV-screening activities.

2.4.1 Extraction of peel from four cultivars.

The albedo peels cultivar, KT, KY, KN, TD and flavedo peels of KT were separately extracted using the same procedure. Thirty grams of each peel were macerated in methanol (3x200 mL) for 9 days with stirring during maceration. The slurry was filtered and the methanol extract was concentrated with a rotary evaporator at 35 °C. The obtained crude extract was stored under vacuum.



Scheme 2.1 Extraction of pomelo peels

2.4.2 Isolation of naringin.

The albedo and flavedo were separately extracted using the same procedure. Efficient process to isolate naringin from the peel involved a simple liquid-liquid extraction coupled with crystallization. Initially, the obtained crude methanolic extract of KT, KY, KN and TD albedo peel (3.5 g) was dissolved in water (20 mL) and transferred into a separating funnel. Dichloromethane (40 mL) was added and the mixture was swirled. The mixture was left for 3-4 days at room temperature to allow complete crystallization of naringin. Similar process was carried out with the methanolic extract of KT flavedo peel, the methanolic extract of flavedo peel (2.0 g)

was dissolved in water (16.7 mL) and transferred into a separating funnel. Dichloromethane (33.3 mL) was added and the mixture was swirled. The mixture was left for 7 days at room temperature to allow crystallization.

2.5 Naringin purity determination using HPLC.

Purity of the obtained naringin crystal from albedo peel was determined by reversed-phase HPLC. The chromatographic separation was performed on a BDS C18 column (4.6 x 100 mm) (Thermo Electron Corporation, Massachusetts, USA) with the mobile phase consisting of 75 mM citric acid and 25 mM ammonium acetate in Milli-Q™ water (A) and methanol (B) with A: B ratio of 60:40 (v/v) at a flowrate of 1 mL/min. The injection volume was 5 µL (100mg/L) and the detection wavelength was set at 282 nm [75].

2.6 Determination of total phenolic contents.

Total phenolic content was estimated by the Folin–Ciocalteu method [76]. Two hundred microlitres of methanol crude extract at concentration 500 mg/L were added to 1 ml of 10 % aqueous Folin–Ciocalteu reagent. After 4 min, 800 µL of saturated sodium carbonate solution (75 g/L) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid solutions (20, 40, 60 and 80 mg/L) were used as standards to construct a calibration curve. The results were expressed as miligram of gallic acid equivalent (GAE).

2.7 Determination of Free-radical scavenging activity.

TLC Autographic assay [77].

The assay involves dropping a sample (5 µL) was dropped onto a stationary phase using an analytical syringe. After allowing to dry for about 5 min at room temperature, 0.5 mM DPPH solution (in MeOH) was sprayed over the entire surface of

stationary phase (0.17 mL/cm^2). Only spots with antioxidant activity appeared white against a purple background.

2.8 Determination of Tyrosinase inhibition activity.

The post TLC developing technique [78].

Five microliter of was dropped onto a stationary phase using an analytical syringe. After allowing to dry for about 5 min at room temperature, the 5 mL of the enzyme solution (200 U/mL in 20 mM phosphate buffer, pH 6.8) was sprayed over the entire surface of stationary phase (33.3 units /cm^2). Immediately after that, 2 mM L-tyrosine was sprayed over the same area (33.3 units /cm^2). After appropriate 15-20 min post spraying. Only spots with tyrosinase inhibitor(s) appeared white spots against brownish-purple background.

The Spectrophotometric assay [79].

After isolation and purification, activities from pure compounds were quantified by this method. Briefly, 0.03 mL of tyrosinase solution (333 units/mL) of 50 mM phosphate buffer solution pH 6.8, were added into 96 well –microplate containing 10 µL of sample at various concentrations in methanolic solution. After 5 minutes of incubation at room temperature, 0.1 mL of L-tyrosine solution (2 mM) was added. Absorbances of all the sample solution were measured immediately at 492 nm. Tyrosinase inhibition activity was expressed as the inhibition percentage according to formula:

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

A = absorbance of reaction mixtures without test compounds (control)

B = absorbance of blank of control without enzyme

C = absorbance of reaction mixtures containing test compounds (sample)

D = absorbance of blank of sample without enzyme

2.9 Determination of UV absorption activities

Samples were prepared at concentration of 10 and 50 mg/L, the solution were scanned at wavwlengths between 200-400 nm.