CHAPTER II
EXPERIMENTAL

2.1 Instruments and Equipments

2.1.1 ^{1}H and ^{13}C- Nuclear Magnetic Resonance Spectometer

NMR spectra were recorded with a Varian medel Mecury+ 400 (Varian company, CA, USA) which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual proton in deuterated solvent and using TMS as an internal stadard in some cases.

2.1.2 Mass Spectrometer

ESI-MS analyses were performed with Waters Micromass Quattomicro API ESCi (Waters, MA, USA). Samples were dissolved in CH_3CN and directly injected into the mass spectrometer.

2.1.3 Infrared Spectrometer

The ATR-FTIR spectra was recorded with Nicolet 6700 FT-IR spectrometer equipped with a mercury-cadmium-telluride (MCT) detector (Thermo Electron Corporation, USA): continuμm ™ infraed microscope with 15x Cassegrain infraed objective and 10x glass objective, homemade slide-on germanium (Ge) μATR accessory with a cone shape Ge as IRE.

2.1.4 UV-Visible Spectrophotometer

UV-Visible absorption spectra were obtained with the aid of UV 2500 UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan), using a quartz cell with 1 cm pathlength.

2.1.5 Semi-preparative High Performance Liquid Chromatography (HPLC)

The HPLC performed with ThermoFinnigan spectra SYSTEM (Phenomenex, California, USA) consisting of a diode array detector. A Hypersil HS C18 reverse phase column (250 mm long x 10 mm i.d., 5 μm, Thermo Electron corporation, Massachusetts, USA) was used for analysis. The volume was injected 10 μl. The elution solvent were MeOH:H_2O.

2.1.7 Thin layer chromatography (TLC)

TLC was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 F254) (Merck KgaA, Darmstadt, Germany).

2.1.8 Column chromtography

Column chromatography was performed in silica gel (Merck Kieselgel 60 G) (Merck KgaA, Darmstadt, Germany).

2.1.9 Microtiter plate spectrophotometer

UV-Vis spectrometer, microtiter plate reader, model sunrise (TECAN, Salzburg, Austria).

2.1.10 Rotary Evaporator

The BUCHI rotary evaporator R-200 (BUCHI, Flawil, Switzerland) was used in this project

2.1.11 pH Meter

pH values were determined with pH 211 microprocessor pH meter (HANNA Instrument, Woonsocket, Rhode island, USA).

2.2 Chemicals

Solvents used in spectroscopic techniques, antioxidant activity and anti-tyrosinase assay were reagent or analytical grades purchased from Labscan (Bangkok, Thailand). Solvents used in extraction and column chromatography were purified from commercial grade solvents prior use by distillation. Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma Chemical Company (St Loius, MO, USA). L-tyrosine was purchased from Fluka (Buchs, Switzerland). Kojic acid and glabridin 40% used as standard tyrosianse inhibitor were purchased from Acros organics (Geel Belgium) and HANBUY (Dubai, United Arab Emirates), respectively. Phosphate buffer was prepared from K_2HPO_4 purchased from Merck KgaA (Damstadt, Germany) and KH_2PO_4 purchased from Carlo erba reagenti (Milan, Italy). A 2,2-diphenyl-1-picrylhydrazyl (DPPH) used as stable radical in antioxidant activity assay was purchased from Fluka Chemie (Buchs, Switzerland). A 2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT) used as standard antioxidant was purchased from Panreac Sintesis (Bacelona, Spain).

2.3 Sample materials

The husks of Chai-nat I and Look Daeng Pattani rice strain were obtained from Rice Research Institute of Patthalung, Thailand. The husks of rice of Leb Nok Pattani, Jasmine and Go ko I were obtained from Pattani, Petchaburi and Maehongson, Thailand, respectively.

2.4 Extraction and Isolation

2.41 In screening part extraction

One hundred and seventy grams of each rice husk strains , Chi-nat, Look Daeng Pattani, Lab-nok, Gorkor I, and Jasmine rice were extracted by maceration with hexane, dichlormethane, ethyl acetate and methanol, respectively. The extract of each solvent were filtered and evaporated under reduced pressure. Each dried extract was tested for biological activities, including antioxidant activity, anti-tyrosinase activity and UV-sreening activity. The extract high-yielded with good biological activities was subjected to further isolation of active compounds.

2.4.2 In large bacth part extraction

Chai-nat strain rice husk (3.7 kg) was milled and then extracted by maceration at room temperature with dichloromathane (2x5000 ml) for 10 days and ethyl acetate (2x5000 ml) for 10 days. Each extract was filtered and evaporated under reduced pressure to obtain dried crude extract. These extracts were first run on TLC under the condition 7%MeOH in CH_2Cl_2 and visualized by UV. The extracts containing the same spot were combined together and tested for their antioxidant activity on DPPH radical scavenging assay, anti-tyrosinase activity on post TLC developing technique and UV screening activity.

2.4.3 Isolation of active compounds

The CH_2Cl_2 and MeOH crude extracts were combined and defined as CN-I crude extract. The CN I crude extract (7.0 g) was chromatographed over silica gel column using gradient elution of CH_2Cl_2-MeOH (100:0 to 60:40) and obtained 270 fractions. Each fraction was examined by TLC and combined. The combined fractions were tested for their biological activities. Fractions with good activity were further isolated using chromatographic techniques.

CN I-1 fraction (yellow paste, 400 mg, combined fractions 1-20) was subjected to silica gel open column eluting with gradient hexane-CH_2Cl_2 (50:50 to 0:100). Sixty fractions of 25 ml each were collected and after their examination by TLC, they were combined and tested for anti-tyrosinase activity by using the post TLC developing method [75]. Fraction with good activity was further purified by precipitation in cold MeOH to obtain the pure active compounds.

CN I-2 fraction (white solid, 300 mg, combined fractions 21-66) was subjected to silica gel open column eluting with gradient CH_2Cl_2-MeOH (100:0 to 98:2). Thirty five fractions of 25 ml each were collected and after their examination by TLC, they were combined and test for antioxidant activity by using DPPH radical scavenging method [76] and UV screening activity. Active fraction was further purified by semi-preparative HPLC: Hypersil column with 0.7 ml/min of isocratic 80% MeOH in H_2O as eluent. The fraction was detected at 336 nm. The collected fraction was further characterized by using spectroscopic technique and tested for antioxidant activity.

CN I-8 (purple solid, 150 mg, combined fractions 251-264) was separated using C-18 column chromatography eluting with gradient MeOH-CH_2Cl_2 (100:0 to 20:80). Fifteen fractions 10 ml each were collected and after their examination by TLC, they were combined and tested for biological activities Fraction with good activity was further purified by semi-preparative HPLC: Hypersil column with 0.7 ml/min of isocratic 80% MeOH in H_2O as eluent. The fraction was detected at 544 nm. The collected fraction was dissolved in CH_2Cl_2 to provide two parts, soluble in CH_2Cl_2 part and insoluble in CH_2Cl_2 part. Soluble in CH_2Cl_2 part was further characterized by using spectroscopic technique and tested for its biological activity.

2.5 Biological activities section

(A) Antioxidant activity

The 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging assay was selected for determining the antioxidant activity of rice husk extracts.

DPPH radical scavenging activity

1. TLC Autographic Assay [76]

The assay involves dropping a sample 5µl onto the TLC plate (4 cm x2 cm) and then spraying a entire plate with with a 0.5 mM DPPH solution in MeOH 0.5 ml. After 30 min post spraying, spots of active compounds should occur as yellow spots on a purple background after 30 min post spraying.

After isolation and purification , antioxidant activities of pure compounds were quantified spectroscopically.

2. Spectrophotometric assay [77]

. Various concentrations of samples dissolved in methanol (50µl) were added to methanolic DPPH radical solution (0.25mM, 100µl) in the 96-well microplate. After 30 minutes incubation at room temperature in the dark, the absorbance was

measured at 517 nm. All tests were run in triplicate. The scavenging activity was evaluated from the decrease in absorbance at 517 nm. The activity is shown as percentage of radical scavenging.

% scavenging activity = $[1 - A_{sample}/A_{control}] \times 100$

where

A_{sample} = absorbance at 517 nm of reaction mixtures containing test compounds.

$A_{control}$ = absorbance at 517 nm of reaction mixture without test compounds.

(B) Anti-tyrosinase activity

The post TLC delveloping technique and the conventional spectrophotometric method were selected for determining the tyrosinase inhibition of rice husk crude extracts and pure compounds from rice husk extracts.

1.The post TLC developing technique [75]

The method is suitable for preliminary screening of tyrosinase inhibition from natural product because it is quick and simple. This method involved spraying the TLC plates containing sample spot(s) with tyrosinase (200 unit/ml) solution in 20mM phosphate buffer, pH 6.8 and 2mM of L-tyrosine solution in 20mM phosphate buffer, pH 6.8. Exact amount of sample (2.0-5.0 µl) was dropped onto a stationary phase using an analytical syring. After allowing to dry for about 5 minutes at room temperature, the enzyme solution was sprayed over the entire surface of the stationary phase (0.02 ml/cm^2). Immediately after that, L-tyrosine was sprayed over the same area (0.02 ml/cm^2). After 10-20 min post spraying. A positive result could be visualized directly as white spot(s) against a browning-purple background.

After isolation and purification, anti-tyrosinase activies of pure compounds were quantified spectroscopically.

2.Spectrophotometric assay [78]

After isolation and purification, activities from pure compound were quantified using the following assay. Potassium phosphate buffer (0.07 ml, 50 mM) at pH 6.8, 0.03 ml of tyrosinase (333 units/ml), and 2 µl of the tested sample at various concentration in methanolic solution were added into 96-well microplate. After 5 minutes of incubation at room temperature, 1 ml of L-tyrosine solution (2 mM) was

added. Absorbance at 492 nm was measured immediatly. All tests were run in triplicate. The inhibition of tyrosinase activity was evaluated from the increase in absorbance at 492 nm. The activity is shown as percentage of tyrosinase inhibition.

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

Where

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

B = absorbance of blank of control

C = absorbance of reaction mixture containing test compound (sample)

D = absorbance of blank of sample

(C) UV absorption activity

A stock solution of each sample was prepared in a 10 ml volumetric flask using methanol as a solvent. The resulting stock solution was then diluted with methanol to give the concentration of 25, 50, and 70 ppm. The UV absorbance of each final dilution was recorded by scanning wavelengths between 200 and 800 nm. The molar absorptivity (ε) at the wavelength of maximum absorbance (λ_{max}) was calculated using Beer's law:

$$A = \varepsilon bc$$

where A is absorbance

b is the cell path length (1 cm)

c is the concentration of the absorbing species in mole per liter