CHAPTER II

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1. Materials

1.1. Fruit samples

Aglaia dookoo Griff. (ลองกอง, longkong)

Ananas comosus (สับปะรด, pineapple)

Annona squamosa Linn. (น้อยหน่า, custard apple)

Artocapus heterophyllus Lamh. (ขนุน, jackfruit)

Averrhoa carambola Linn. (มะเฟือง, carambola)

Baccaurea ramiflora Lthe. (มะไฟ, ma-fai)

Bouea macrophylla Griff. (มะปราง, plum mango)

Carica papaya Linn. (มะละกอ, papaya)

Citrullus Inatus Mats. (แตงโม, watermelon)

Citrus aurantifolia (มะนาว, lemon)

Citrus aurantium (ส้ม, orange)

Citrus maxima Merr. (ส้มโอ, pomelo)

Dimocarpus longan Lthe. (ลำไย, longan)

Durio zibethinus Linn. (ทุเรียน, durian)

Eugenia malaccensis Linn. (ชมพู่, rose apple)

Garcinia mangostana (มังคุด, mangosteen)

Litchi chinensis Sonn. (ลิ้นจี่, litchi)

Mangifera indica Linn. (มะม่วง, mango)

Morinda dookoo Griff. (ยอบ้าน, indian mulberry)

Musa sapientum Linn. (กล้วย, banana)

Nephelium lappaceum Linn. (เงาะ, rambutan)

Psidium guajava Linn. (ฝรั่ง, guava)

Pyrus malus Linn. (แอปเปิล, apple)

Salacca edulis Reinw. (สละ, sa-la)

Salacca rumphii wall. (ระกำ, zalacca)

Sandoricum koetijape Merr. (กระท้อน, santol)

Vitis vinifera Linn. (องุ่น, grape)

Zizyphus mauritiana Lamk. (พุทรา, jujube)

1.2. Chemicals

- 2,2'-azobis(2-methylpropionamidine) dihydrochloride ($C_8H_{18}N_6 \cdot 2HCl$, AAPH) (Across organic, USA)
- 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade (Fluka, Switzerland)
- 2-Deoxy-D-Ribose ($C_5H_{10}O_4$) (sigma, USA)
- 2-Thiobarbituric acid anhydrous (TBA) (sigma, USA)
- 95 % Ethanol (C_2H_5OH), commercial grade
- Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) (Fluka, Switzerland)
- Ethyl acetate, analytical grade (J.T. Baker, USA)
- Folin - Ciocalteu's phenol reagent (Merck, Germany)
- Gallic acid monohydrate ($C_7H_6O_5 \cdot H_2O$), HPLC grade (Fluka, Switzerland)
- Hydrochloric acid (HCl) 36.5-38%, analytical grade (J.T. Baker, USA)
- Hydrogen peroxide 30% (H_2O_2), analytical grade (APS finechem, Australia)
- Iron (III) chloride anhydrous ($FeCl_3$) (Merck, Germany)
- L(+)-Ascorbic acid ($C_6H_8O_6$, Vitamin C), analytical grade (Merck, Germany)
- Methanol (CH_3OH), analytical grade (J.T. Baker, USA)
- Nitro Blue Tetrazolium ($C_{40}H_{30}Cl_2N_{10}O_6$, NBT), analytical grade (sigma, USA)
- Phenazine methosulfate ($C_{13}H_{11}N_2CH_3SO_4$, PMS) (sigma, USA)
- Potassium dihydrogen phosphate (KH_2OH), analytical grade (Merck, Germany)
- Potassium hydroxide pellets (KOH), analytical grade (Merck, Germany)
- Sodium bicarbonate ($NaHCO_3$) (Mcgarrett, Thailand)

- Sodium carbonate (Na_2CO_3), analytical grade (Merck, Germany)
- Sodium chloride (NaCl), analytical grade (Merck, Germany)
- Sodium dihydrogen phosphate-2-hydrate ($NaH_2PO_4 \cdot 2H_2O$) (Riedel-de Haen)
- Sodium hydroxide pellet (NaOH), analytical grade (Merck, Germany)
- Titriplex® III (EDTA), analytical grade (Merck, Germany)
- Trichloroacetic acid (TCA), analytical grade (Carlo Erba reagent)
- β-nicotinamide adenine dinucleotide phosphate anhydrous, tetrasodium salt type I, reduced from (β-NADPH) (sigma, USA)

1.3. Equipments

- Analytical Balance
- Homoginizer (Polytron®, Switzerland)
- Lyophilizer (Dura-Dry™ II MP)
- Micro plate reader (Anthos ht II)
- pH meter (Mettler Toledo)
- Refrigerated centrifuge (HITACHI SCR20B, Japan)
- Rotary evaporator (Eyela, Japan)
- Shaking water bath (Hetobirkerod, Denmark)
- UV/VIS spectrophotometer (Spectronic® Genesys™ 5)
- Vortex-Mixer (Scientific Industries, USA)

2. Methods

2.1 Sample preparation and extraction

Fresh fruits were purchased from local supermarkets except grapes, which were collected from the vineyard. All fresh fruits were washed in water and then soaked in a mixture of NaCl and $NaHCO_3$ for 15 minutes, and then rinsed with water and air dry. Fruits were chopped and then homogenized by a homoginizer. The homogenates were extracted according to method of Murthy *et al.*, Singh *et al.*, and Leontowicz *et al.* and left at room temperature (28 °C) in the dark overnight (Murthy *et al.*, 2002; Singh *et al.*, 2002; Leontowicz *et al.*, 2003). The supernatants were separated by centrifugation at 7,000 rpm (5,800 g) for 10 minutes at 4 °C. In some samples, the supernatants were concentrated by a rotary evaporator at 40 °C.

2.2 Determination of total polyphenolic contents in fruit extracts

Total polyphenolic content in fruit extracts was determined according to Folin-Ciocalteu's procedures (Parejo *et al.*, 2002). Briefly, the reaction mixture contained 0.25 ml of each fruit extract, 15 ml of distilled water, 1.25 ml of Folin-Ciocalteu reagent, 3.75 ml of 20% sodium carbonate, and adjusted volume to 25 ml with distilled water. The absorbance was measured at 765 nm after 1 hour incubation at 20 °C. Gallic acid was used as a standard and the total polyphenolic contents were expressed as total gallic acid equivalent (GAE) (mg/g wet weight).

2.3 Determination of free radical scavenging activity using DPPH method

The H-donor activity of fruit extracts was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Abe *et al.*, 1998; Parejo *et al.*, 2002). The DPPH is a stable radical, its methanolic solution shows an absorbency maximum at 517 nm. The H-donor molecules reduce DPPH radical and thus decrease its absorbance at 517 nm.

Briefly, the reaction mixture contained 2 ml of dilutions of the methanolic solution of fruit extracts, 1 ml of 0.5 mM DPPH (in methanol) and 2 ml of distilled water. After standing for 30 minutes, the absorbance of the mixture was measured at 517 nm. The antioxidant compounds presence in fruit extracts can interact with DPPH. The percentage of DPPH radical scavenging activity was calculated by the following formula.

$$\% \text{ radical scavenging activity} = \left[1 - \frac{A_{\text{fruit extract}}}{A_{\text{control}}}\right] X\ 100$$

$A_{\text{fruit extract}}$ is the absorbance value of DPPH containing fruit extract.

A_{control} is the absorbance value of DPPH.

The DPPH scavenging activity was plotted against the concentration of the fruit extract. A logarithmic regression curve was established in order to calculate the IC_{50}, which is the amount of concentration of fruit extract necessary to decrease by 50% the absorbance value of the control.

2.4 Determination of superoxide anion scavenging activity of fruit extracts

Antiradical activity in fruit extracts was determined spectrophotometrically in a 96 well plate reader by monitoring the effect of fruit extracts on the reduction of nitro blue tetrazolium (NBT) to the blue chromogen formazan by superoxide radical ($O_2^{\bullet -}$), at 570 nm. Superoxide anions were generated according to the method of Valentao *et al.* (2001), except NADPH was used instead of NADH. The reaction mixture in the sample wells consisted of β-nicotinamide adenine dinucleotide phosphate (NADPH) (166 μM), NBT (43 μM), phenazine methosulfate (PMS) (5.4 μM), and various concentration of lyophilized fruit extracts, in a final volume of 200 μl then adjusted volume with 19 mM phosphate buffer, pH 7.4. The reaction was conducted at room temperature for 15 minutes and initiated by the addition of PMS. The percentage of superoxide anion scavenging activity was calculated according to the formula below,

$$\% \text{ superoxide anion scavenging activity} = \left[1 - \frac{A_{\text{fruit extract}}}{A_{\text{control}}}\right] X\ 100$$

$A_{\text{fruit extract}}$ is the absorbance value of the reaction mixture containing fruit extract.

A_{control} is the absorbance value of the reaction mixture without fruit extract.

$A_{control}$ is the absorbance value of the reaction mixture without fruit extract.

The superoxide anion scavenging activity was plotted against the concentration of fruit extract. A logarithmic regression curve was established in order to calculate the IC_{50}, which is the amount of concentration of fruit extract necessary to decrease by 50% the absorbance value of the control.

2.5 Determination of hydroxyl radical scavenging activity of fruit extracts

The deoxyribose method was used for determining the scavenging effect of the fruit extract on hydroxyl radicals (Valentao *et al.*, 2002). Briefly, the reaction mixture contained, in a final volume of 1 ml, ascorbic acid (50 µM), $FeCl_3$ (20 µM), EDTA (2 mM), H_2O_2 (1.42 mM), deoxyribose (2.8 mM), and various concentrations of fruit extracts then adjusted volume with 10 mM KH_2PO_4/KOH buffer, pH 7.4. After incubation at 37 °C in the water bath for 1 hour, 1 ml of 2.8% Trichloroacetic acid (w/v) and 1 ml of 1% Thiobarbituric acid (w/v) were added, and the mixture was heated in a water bath at 100 °C for 15 minutes. The absorbance of the resulting solution was measured at 532 nm. This assay was also performed in the same manner but without ascorbic acid or EDTA, to evaluate the pro-oxidant and metal chelation potential of the fruit extracts, respectively.

2.6 Inhibitory effects of fruit extracts on human erythrocyte hemolysis

Erythrocyte oxidative hemolysis was induced by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), a peroxyl radical initiator. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipid and proteins, resulting in hemolysis (Zhu *et al.*, 2002; Hseu *et al.*, 2002).

2.6.1 Preparation of human erythrocyte suspensions

Human red blood cells (RBCs) were isolated from donor's blood kept in citrate by centrifugation at 3,000 rpm (830 g) for 10 minutes. Plasma and buffy coat were carefully removed by aspiration. RBCs were washed four times with phosphate buffer saline (PBS) [154 mM NaCl and 10 mM phosphate buffer, pH 7.4]. During every wash, the erythrocytes were centrifuged at 3,000 rpm (830 g) for 10 minutes to

obtain a packed red cell preparation. After the last wash, the packed erythrocytes were suspended in PBS solution at the final volume of 2 g of hemoglobin / dl.

2.6.2 Preparation of fruit extracts

Fresh fruits were extracted with distilled water for 24 hours and supernatants were separated by centrifugation at 7,000 rpm (5,800 g) for 10 minutes at 4 °C. Supernatants were concentrated by lyophilization.

2.6.3 Inhibitory effect of fruit extracts on human erythrocyte hemolysis

Inhibitory effect of fruit extracts on human erythrocyte hemolysis was determined according to the method of Zhu *et al.* (2002). Briefly, 0.5 ml of RBC suspension in PBS was mixed with 1 ml of PBS solution containing varying amounts of fruit extracts or PBS as a control. The reaction mixture was preincubated at 37 °C and was shaken gently in the water bath for 30 minutes. 1 ml of 150 mM AAPH in PBS was added to the mixture. The reaction mixture was incubated at 37 °C in a shaking water bath for 1 hour. After incubation, the reaction mixture (0.1 ml) was withdrawn into 0.7 ml of ice-cold PBS and centrifuged at 8,000 rpm (7,150 g) for 5 minutes. The supernatant of mixture was read at 540 nm. L-Ascorbic acid was added instead of fruit extract in a refrence tube. The percentage of hemolysis was calculated according to the formula below,

$$\% \text{ Hemolysis} = \left(\frac{A_{\text{fruit extract}}}{A_{\text{control}}} \right) \times 100$$

$A_{\text{fruit extract}}$ is the absorbance value of the reaction mixture containing fruit extract.

A_{control} is the absorbance value of the reaction mixture without fruit extract.

2.7 Statistic analysis

Data were presented as mean ± standard deviation (S.D.) of triplicate examination. One-way ANOVA using SPSS version 11.5 software was used for multiple comparisons. A value of $p < 0.05$ was considered significant.