CHAPTER III

RESEARCH METHODOLOGY

Materials

The chemicals used in this study are listed in Table 14 (Appendix A). Two kinds of grape seed (GS) extract were used: commercial grape seed extract (GSE) from Arkopharma and another was from *Vitis vinifera* cv. Ribier (Pok Dum). The pine barks extract (PBE) containing the oligomeric proanthocyanidins was purchased from Maritime Prime. Molisch's reagent was kindly supported by Department of Biochemistry, Faculty of Medicine, Chiangmai University.

Instruments

The instruments used in this study are listed in Table 15 (Appendix A).

Methods

The overviews of methods in this study are shown in diagram (Figure 16). There were three series of methods for the different purpose. One was the preliminary antioxidant measurement of tamarind seed husk (TaSH). The second was aimed to prepare the small size of polyphenolic compounds and use them in the biological test. The last was aimed to purify polymeric proanthocynidins which was the major compound in tamarind seed husk (TaSH).

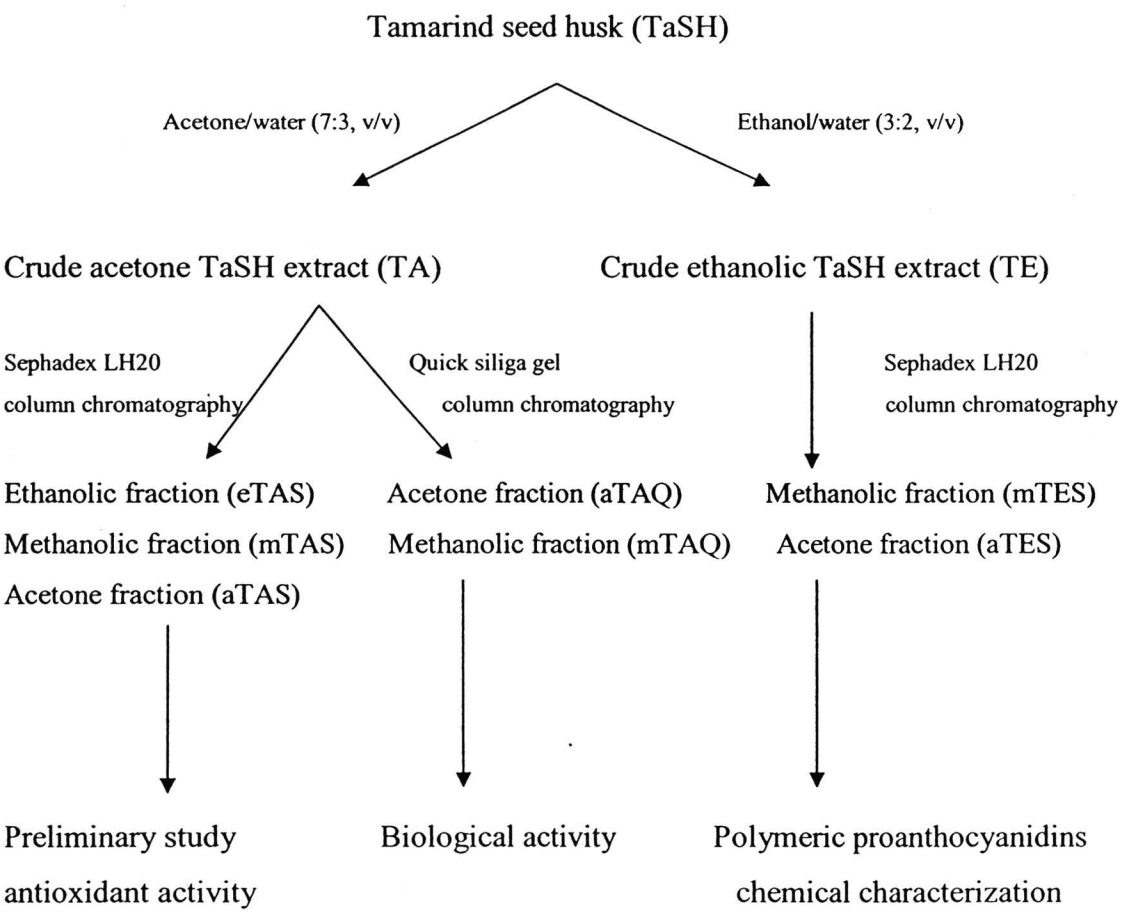

Figure 16 Diagram of the planned methods used in the present study

Preparation and extraction of polyphenolic compounds from TaSH.

1. Preparation of TaSH powder

2. The seeds from sour tamarinds (TaSH), which were locally purchased, were dried in a hot air oven at 100-140 °C for 1 hour. The seeds were crushed to separate the inner part from the seed husk after cooling. Brown-red seed husk was collected and ground, powder of TaSH was kept at 4°C.

3. Ethanol extraction of polyphenols from TaSH

Mature TaSH and young TaSH were extracted by ethanol/water (7:3, v/v) at room temperature according to the study of Pumthong (1999). The insoluble materials were filtered out. The crude TaSH extract was obtained by evaporating under the reduced pressure at 60-70°C using rotating evaporator.

4. Acetone/water extraction of polyphenols from TaSH

Acetone and water in ratio of acetone: water, 7:3 v/v, was added into tamarind seed husk powder and stay at room temperature for 24 hours at an extraction ratio of 15. The insoluble materials were filtered out. The crude TaSH extract (TA) was obtained by evaporating under the reduced pressure at 60-70°C using rotating evaporator.

5. Ethanol extraction of polymeric proanthocyanidins from TaSH

Ground TaSH was extracted using ethanol/water (3:2, v/v) at 60°C and mixed for 24 hours (Ezure Y., CMU, pers. comm.). The insoluble materials were filtered out. The crude ethanolic TaSH extract (TE) was collected after having removed the ethanol by rotary evaporation.

Purification of polyphenols from TaSH extracts.

1. Sephadex LH20 column chromatography

1.1 Preliminary purification method

Preliminary column was done using according to Strumeyer, et al. (1975). TA (from 1.2) was eluted with ethanol/ water (19:1, v/v) and acetone/water (1:1, v/v). The fractions were collected and their absorbance was measured at 280 nm. The elutions of fractions were monitored using thin-layer chromatography (TLC).

1.2 Purification of tannins from TA

The second method, tannins needed to be purified from low molecular weight phenolics and pigments that were present in the crude plant extracts. The TA (from 1.2) was partially purified by chromatography on Sephadex LH20 (2.5 x 34 cm) using mobile phases of ethanol/ water (19:1, v/v), methanol/water (19:1, v/v) and acetone/water (1:1, v/v). The ethanolic fractions (eTAS), methanolic fractions (mTAS) and acetone fractions (aTAS) were collected and their absorbance was measured at 280 nm. The elutions of fractions were monitored using thin-layer chromatography (TLC).

1.3 Purification of polymeric proanthocyanidins

The standards of polymeric proanthocyanidins were prepared according to a partially modified method of Peng *et al.* (2000). SephadexLH20 gel preswollen in methanol/water (3:2, v/v) was slurry-packed into a glass column (2 cm i.d. x 14 cm). The column was equilibrated with methanol/water (3:2, v/v) prior to sample loading. The TE was loaded onto the column, and the first fraction was eluted with 5 column volumes of methanol/water (3:2, v/v) at a flow rate of 1.2 ml/min. The extracting solution was evaporated to dryness to give the powder (mTES). Polymeric PA was eluted with acetone/water (3:2, v/v). The aqueous acetone fraction (aTES) was concentrated under the reduced pressure at 60°C to remove acetone. The polymeric proanthocyanidins from a grape seed extract (GE) was purified with SephadexLH20 in the same way as TaSH. After purification by Sephadex LH20, the acetone fraction of GE (aGES) was used to be a standard for polymeric proanthocyanidins determination.

2. Silica gel column chromatography

Tamarind seed husk extract (TA) was loaded to the silica gel column. The column was developed successively using solvent of hexane, dichloromethane, ethyl acetate, ethyl acetate/acetone (4:1, v/v), ethyl acetate/ acetone (1:1, v/v), acetone and methanol. Each fraction developed with each solvent was evaporated to powder under reduced pressure. Each fractions was determined the antioxidant activity. The acetone fractions (aTAQ) and methanolic fractions (mTAQ) which represented the highest antioxidant activity were collected for further biological experiments.

Identification of polyphenols in TaSH fractions.

1. Determination of polyphenols

1.1 Determination of tannin by various basic chemical tests

The experiments were done following the methods of Farnsworth (1966). The 2 ml of 1 mg/ml tested samples were added in 5 experiment test tubes. The first was the control tube. The second tube was added with 2-3 drops of 1% ferric chloride, the occurrence of blue green color was the positive result of tannin. The third one was added with 2-3 drops of gelatin solution, the occurrence of white and tubidity was the positive results of tannin. The fourth tube was added with 5-6 drops of bromine solution, the occurrence of buff color was the positive result of tannin. The last tube was added with 1 ml of vanillin reagent and 1 drop of conc. HCl, the occurrence of crimson color was the positive result of flavan in terminal unit of condensed tannins.

1.2 Determination of flavonoids by cyanidins reaction

The reaction was carried using 1 ml sample with 0.1g of magnesium powder and conc. HCl 10 drops. After cooling, 1 ml water and 1ml octyl alcohol were added. Flavonoids with γ-benzopyrone nucleus such as flavonol, flavanone, flavanonol and xanthone give the red colour, while the other type such as flavones, chalcone and aurone give the orange colour (Fornsworth, 1966).

1.3 Determination of flavanonol by Pew test

0.5 g Zn-dust and 2 drops 2N HCl were added into 1 ml sample for 1 minute. Then 10 drops conc. HCl were added. The flavanonol or flavonol-3-glycoside gave the red color immediately (Fornsworth, 1966).

1.4 Determination of carbohydrate by Molish's test

Molisch's test is a chemical test for presenting carbohydrates, based on the dehydration of carbohydrate by sulfuric acid to produce an aldehyde, which condenses with two molecules of phenol (α-naphthol) resulting in a red- or purple-colored compound. The test solution is combined with a small amount of Molisch's reagent (α -naphthol dissolved in ethanol) in a test tube. After mixing, a small amount of concentrated sulfuric acid was slowly added besides the sloping test-tube (without mixing) to form a bottom layer. A positive reaction which has carbohydrate is

indicated by appearance of a purple ring at the interface between the acid and test layers.

1.5 Determination of total phenolic compounds by Folin-Ciocalteu method

This method determines the total free phenolic groups and is a method to determine total soluble phenolics. The analysis is based on the reduction of phosphomolybdic acid by polyphenolic compound in aqueous alkali solution. The method of Slinkard (1977, pp. 49-55) was slightly modified. The sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 ml of water and heated to a boil. After cooling, a few crystals of sodium carbonate were added. The solution was filtered out and brought to 1 liter with water. Twenty milliliter of samples was added in 100 µl of Folin Ciocalteu. The reactions were mixed and stand for between 30 seconds - 8 minutes before adding 300 µl of sodium carbonate solution. The solution was mixed and left for 2 hours at room temperature. The absorbance was determined at 765 nm against blank, and graph between absorbance and concentration was plotted.

1.6 Determination of condensed tannin by butanol/HCl method

Butanol/HCl assay was carried out according to a slightly modified method of Vemerris and Nicholson (2006). In brief, 0.1 ml of tested samples was added with 1 ml portion of an acidic solution of ferrous sulfate [7.7 mg of $FeSO_4.7H_2O$ dissolved in 50 ml of conc. HCl- n-butanol (2:3, v/v)]. The loosely sealed mixtures in the test tubes were placed in the water bath at 95°C for 15 min. The absorbance was read at 550 nm.

1.7 Determination of condensed tannin by vanillin method

Vanillin assay was carried out according to a slightly modified method of Butler, et al. (1982, pp. 1087-1089) using sulfuric acid/water (7:3, v/v) as a solvent. Briefly, a 100 µl aliquot of a freshly prepared solution of vanillin (1g/100ml) in sulfuric acid/ water (7:3, v/v) was added to 50 µl of aqueous plant extract. After 15 minutes, the absorbance at 500nm was measured. The concentration of PA was expressed as (+)-catechin eqauivalents (used for the standard curve).

2. Thin layer chromatography (TLC)

TLC was used to separate and detect the presence of antioxidative proanthocyanidins in samples according to Lea (1978). The monomer and oligomer up to heptamer move by mobile phase of solvent system while the polymeric proanthocyanidins still remain in the origin of the TLC plate. The separation was done on silica gel GF254 plate, using the solvent system of toluene: acetone: formic acid (6: 6: 1, v/v/v). TA (from 1.1), aTAS, and mTAS (from 2.1) were used in this study. The detection was performed by spraying with DPPH solution (1, 1-diphenyl-2-picrylhydrazyl), 4 mg in 25 ml ethanol. The decoloration indicates the presence of antioxidant compound. The retention factor (R_f) was calculated by measuring the distance of sample traveled up a chromatographic plate divided by the distance traveled by the solvent front.

3. High performance liquid chromatography (HPLC)

Three eluting solvent systems of HPLC were used in this study as follows.

3.1 Eluting solvent system 1

HPLC system 1 was used for hydrolysate of TA and its fractions from Sephadex LH20 (aTAS and mTAS, from 2.1). The samples were refluxed in 30 ml methanol and 30 ml 1.5N HCl for two hours and the hydrolysate analyzed by using a reverse phase C18 column. The mobile phase consisted of 2% acetic acid (A) and acetonitrile (B) utilizing the following gradient over a total run time of 60 min: 0% B for 10 min, 0-60% B for 20 min, 60-80% B for 10 min, 80-100% B for 10 min and hold 100% B for 10min. The chromatogram was monitored at 254nm. The details are in Appendix B.

3.2 Eluting solvent system 2

HPLC system 2 was used for the fractions from silica gel chromatography. The TA (from 1.1) and its fractions from quick silica gel column (aTAQ and mTAQ from 2.2) were analyzed by HPLC using a reverse phase C18 column according to Soong and Barlow (2006). The chromatogram was recorded by using wavelength at 280 nm. The mobile phase solvents A and B were the gradient of 3% acetic acid and methanol. The linear gradient elution was used as follows: 0-10% B over 10 min; 10-30% B over 10 min, 30 -50% B over 10 min, 50-60% B over 10 min, 60-80% B over 20 min, 80-60% B over 5 min, 60-20% B over 5 min, 20-0% B

over 5 min and hold 0% B for 5 min. The flow rate was 1 ml/min, and the eluted fractions were collected for 70 min.

3.3 Eluting solvent system 3

HPLC system 3 was used for the identification of polymeric proanthocyanidins. The TE, aTES, GE and aGES was characterized by the reverse-phase HPLC according to the method of Weber, et al. (2007) using a Shimadzu SCL-10AVP system consisting of a LC-10ADvp Pump, an SIL-10ADvp autosampler and SPD-10AV detector (278 nm). The column used was an Allima C18 5U column (250mm x 4.6 mm I.D., 5 μm particle size, Altima, Mandel Scientific Company, Canada). The mobile phase solvents A and B were 0.3% trifluoroacetic acid and acetonitrile. The linear gradient elution was used as follows: 10-15% B over 45 min; 15-60% B over 15 min, hold for 20 min; 60 to 10% B over 1 min; column equilibration at 10% B for 20 min. The flow rate was 0.7 ml/min, and the data were collected for 80min. The column temperature was ambient, with an injection volume of 20 μl. The HPLC analysis of aTES and aTES at the concentration of 1, 2, 3, 4 and 5 mg/ml dissolved in methanol/water (1:1, v/v) was repeated three times at each concentration under the conditions described in quantitative HPLC analysis to give a five point calibration curve.

4. Ultraviolet (UV) spectrophotometry

UV spectrometry was used for detection of proanthocyanidins in the samples (aTES from 2.3) which were dissolved in methanol. The concentrations were 20, 40, 60, 80, and 100 μg/ml for aTES and 10, 20, 40, 50 and 60 μg/ml for aGES. UV spectra were recorded on UV-1700 spectrophotometer (Pharma Spec, Shimadzu, Japan).

5. Infrared (IR) spectrophotometry

IR spectra was used for detection functional groups of proanthocyanidins in TA, aTAS and GSE in an automatic recording IR spectrophotometer. The sample was mixed with potassium bromide (1:10). The range of measurement is from 400-4000 cm^{-1}.

6. Nuclear magnetic resonance (NMR) spectrophotometry

^{13}C NMR spectroscopy was performed according to a slightly modified method of Kennedy, et al. (2001). The aTES and mTES samples (100 mg/ml, 1:2

acetone-d_6/D_2O) were characterized by ^{13}C NMR (100 MHz, Bruker AVANCE™ spectrometer), with chemical shifts in ppm referenced internally with acetone-d_6. The proton-decoupled, inverse-gated sequence, with 90° pulse length (14 μs), 25125 Hz spectral width, 10K data points, 0.65 s acquisition time, relaxation delay of 4 seconds, 12 K scans, and 5 Hz line broadening was carried out at a temperature of 295.8 K.

Antioxidant activity

1. ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) method

1.1 ABTS/ metmyoglobin/ H_2O_2 method

The principle of the reaction was as described by George and Irvine (1952) and Miller *et al.* (1993). The details for preparation of reagents are in Appendix F. The mixture containing 500 μl of 500 μM ABTS, 70 μl of 76 μM metymyoglobin, 980 μl of 5 mM phosphate buffer saline (PBS) pH 7.4 and 20 μl test samples was added in plastic cuvette. The reaction was initiated by adding 450 μl of 500 μM H_2O_2. The reaction rate was recorded on the time course program of spectrophotometer for 180 seconds, and the absorbance at 414 nm measured. The amount of putative antioxidant required to suppress absorbance of the ABTS radical is compared to the amount of Trolox. The antioxidative activity was calculated as Trolox equivalent antioxidant activity (TEAC) per 1 mg of the substance. (See the detail in Appendix F)

The post- addition assay was kinetically determined to elucidate the antioxidant mechanisms according to the method of Strube, et al. (1997). All chemical concentration and system are the same as the ABTS method above. Metmyoglobin and ABTS were mixed, and the reaction was initiated by adding of hydrogen peroxide. ABTS radicals were allowed to accumulate until the absorbance reached 0.5 (at the time 90 second), then the test compound was added. The decrease in absorption caused by the test compound reflects ABTS radical scavenging capacity rather than inhibition of radical formation. The decrease in slope after addition of the test compound gives an indication of the effect of the test compound on the formation of radicals.

1.2 ABTS persulfate decolorization method

The antioxidant activity was measured according to ABTS persulfate decolorization assay (Re, et al., 1999). ABTS was dissolved in water to a 7mM

concentration. For the ABTS radical production, the ABTS solution was mixed to 2.45 mM potassium persulfate (final concentration) at the ratio of ABTS/potassium persulfate (1: 0.5, mol/mol) and allowed the mixture to stand in the dark at room temperature for 24 hours before use. The ABTS solution was diluted with water to an absorbance of 0.7±0.02 at 734 nm. The diluted ABTS solution (200 µl) was added to 2 µl of samples in 24 wells-plates, and then the absorbance at 734 nm was measure after 10 minutes incubation at room temperature. The antioxidative activity was calculated as the half maximal inhibitory concentration (IC_{50}) of the tested samples. The data from the experiments were calculated and shown by mean ± SD.

2. DPPH method

The DPPH assay was carried out according to the method of Liu, et al. (2008) with a modification. Briefly, 167 µM DPPH of methanol solution (180 µl) was mixed with 20µl of the sample solution dissolved in ethanol. The mixture was incubated at 37°C for 15 minutes, and then the absorbance was measured at 540nm using a Multimode Detector (Model DTX 880, USA). The negative control without the sample was measured in the same manner. All measurements were performed in triplicate. The percentage of DPPH radical scavenging activity of the sample was determined at five sample concentrations within the range of 10-90% reduction in absorbance, and the percentage was calculated by the following equation,

%DPPH radical-scavenging
= [(absorbance of control – absorbance of sample)/ absorbance of control] ×100, where 150.3 µM DPPH solution in 95% ethanol was used as a control.

Chemical interaction between TaSH and iron

1. Chelation method

This procedure was modified from Srichairatanakool, et al. (2006). The 10 µl of 1% TA in 0.1%HCl was added with 1 ml of HEPES buffer. Comparing spectrum of TA with or without ferric chloride (0.25-50 mg/dl) was determined using UV-2401PC spectrophotometer (Shimadzu, Japan). The absorption was monitored between 400-800 nm against a reagent blank (TA solution only)

2. Autoxidation of ferrous ion

The Fe^{2+} concentration was determined by the bathophenanthroline disulfonate method (Yoshino, et al., 1998). The 20 µl of samples were added to 960 µl of 10mM Tris-HCl (pH 7.1) and 20 µl of 0.05 mM Fe_2SO_4 and maintained at 37°C at 0, 5, 10, 15, 20, 25,.., 95 minutes . Aliquots of 0.2 ml were mixed with 0.1ml of 1mM bathophenanthroline disulfonate, and the absorbance at 540nm was measured as a function of time. The ascorbic acid (0.05 mM and 50 mM) was used as standard.

Biological effects of polyphenols from TA on cell viability

1. Cell viability assay on normal cells by neutral red

Cultures of retinal ganglion cells (RGC-5) and the mouse hippocampal ganglion cells (HT-22 cells) were maintained in Dulbecco's modified Eaggle's medium (DMEM) containing 10% fetal calf serum and 100 units/ml of penicillin and 100 mg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells had a doubling time of about 18-20 hours. The details for preparation of reagents are in Appendix F. Cell viability was measured using neutral red assay. RGC-5 cells and HT-22 cells were seeded in 24-well plates (approximately 2,500 or 5,000 cells per well) with 10% fetal bovine serum and 1% penicillin and streptomycin. All cells were incubated at 37°C with TA or standard flavonoids at different treatment time and doses. After incubation, the cultured media was emptied from wells and the wells were washed twice with HEPES buffer pH 7.2 (see the preparation in Appendix E). Then 1 ml of HEPES buffer and 33µl of 1% neutral red dye were added to each well. After 2 hours incubation, all wells were washed 3 times with HEPES buffer and allowed to air dry overnight. After that, 500 µl of solubilization buffer (50% ethanol and 1% glacial acetic acid) were added and incubated for 20 minutes at room temperature. The absorbance at 570 nm was determined. The data from the experiments were calculated and shown by mean ± SD.

2. Cytotoxicity assay of TA on cancer cells by MTT

This method was done according to Loetchutinat, et al. (2005). The two cancer cell lines, the GLC4 human small cell lung carcinoma cell line (CS) and K562 human erythromyelogenous leukemia cell line (KS) were used in this study. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and

1% penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cytotoxicity assay was performed as follows; the cells were placed in a 24-well plate at initial density of $5x10^4$ cells per ml and incubated with the tested samples for 72 hours. After, 2mM MTT was added for 4 hours. The collected formazan was dissolved in DMSO and was measured at 560 nm. Cell viability was measured by MTT assay based on the reduction of MTT to purple-colored formazan (560nm absorption) by live, but not dead cells. The concentration of TA required to inhibit cell growth by 50% when measured at 72 h (IC_{50}) was determined by plotting the percentage of cell growth inhibition versus the TA concentration. The calculation was as follow.

$\%IC = [Abs_{560}(control)_{t72} - Abs_{560}(CM)_{t72}]/[Abs_{560}(control)_{t72} - Abs_{560}(CM)_{t0}] \times 100$

IC_{50} = The concentration of samples that inhibit cell proliferation by 50%

Protective effects of TA, aTAQ and mTAQ on oxidative hemolysis and their mechanisms.

1. Hemolysis method.

The hemolysis method was carried out according to the method of Ajila, et al. (2008) with a modification. Human blood samples were collected in heparin treated tubes by venipuncture from healthy human volunteers, after obtaining the informed consent, and the hematocrits were measured. The erythrocytes, in which plasma, platelets, and buffy coat were removed, were obtained by the consecutive centrifugation at 600 g for 10 minutes and wash three times with PBS (pH 7.4). The 5% erythrocytes suspension (v/v) was prepared by adding the PBS to the centrifuged RBCs. The volumes of PBS to be added in the following experiments were calculated by the hematocrit. Oxidant and treated compound were added in various concentrations. The 100 µl of 5% erythrocytes, 10 µl of test samples and 100 µl of 25 mM H_2O_2 (or 10 µl of 25 mM diethyl meleate (DEM) or 10 µl of 25mM phenyl hydrazine (PHZ)) were added to 96-wells plate, and the final volume was adjusted to be 250 µl by adding PBS buffer. The mixtures were incubated in 37°C for 3 hours. Hypotonic solution (5 mM phosphate buffer) was added and calculated as positive control (100% hemolysis).The percentage of hemolysis was determined by measuring the optical

density at 540nm. The data from hemolysis method was calculated as mean ± SE. The statistical analyses of the experimental results were by t-test.

2. Measurement of methemoglobin (metHb)

RBCs obtained by consecutive centrifugation were added the test samples dissolved in PBS, and then the mixtures were made up to 2% (v/v) of RBCs concentration by adding PBS. The mixtures were placed for 1 h while gently shaking from time to time at 37°C. Then 10 mM H_2O_2 (final concentration) in PBS was added, and the mixtures were incubated for 2 h while gently shaking from time to time at 37°C. The mixtures were centrifuged at 600×g for 10 min. 100 µl of the supernatants and 100 µl of PBS were mixed, and then the absorbance at 540, 560, 577, and 630 nm, correcting for background absorbance measured at 700 nm, were measured. The concentrations of metHb were calculated according to Murphy, et al. (1996) following equation:

$$[\text{MetHb}] = 0.041828A_{577} - 0.079247A_{560} + 0.318075A_{630}$$

3. Determination of glutathione level in erythrocytes

Glutathione level in erythrocyte was determined using glutathione kit (Sigma). The details for preparation of reagents were in appendix H. The treated erythrocytes were separated by centrifugation at 600×g for 10 minutes and washed twice with 3 volume of PBS. The 200µl aliquot of erythrocytes were taken and deproteinized with 200µl of 5% SSA solution. Each sample was shaken vigorously and left for 10 minutes at 4 °C. The precipitated protein was removed by centrifuge at 10,000xg for 10 minutes, the volume of the supernatant was measured and was used this as the original sample volume in the calculation for glutathione determination. Keep the sample at 4°C. For the assay of red blood cells, dilute the sample approximately 10-fold to stay in the detection range. The measurement of GSH uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of DTNB to the yellow product TNB as measured spectrophotometrically at 412 nm.

4. Determination of intracellular ROS

This method has been done following the method of Tedesco, et al. (2001). The dichlorofluorescin (DCFH) reacts with intracellular ROS to form the highly fluorescent 2', 7'-dichlorofluorescein which exits the cells. The erythrocytes were treated with an antioxidant sample for 10 minutes at 37°C. After incubation with 10 μM DCFDA, the erythrocytes were centrifuged, resuspended in PBS and treated with oxidant (10 μM hydrogen peroxide or phenylhydrazine) for 5 minutes at room temperature. ROS production was measured fluorometrically with excitation and emission setting at 495 and 530 nm, respectively.

Protective effects of TA, aTAQ and mTAQ on DNA damage using DNA nicking assay

The pUc18 plasmid DNA (molecular weight = 1,800 kDa, 2,686 bp) were used to be a model DNA that consisted of a multiple-cloning site, a modified ampicillin-resistance gene and an origin of replication derived from pBR322 ligated to a portion of the lacZ gene of E. coli strain Top 10F. Plasmid pUc18 DNA was dissolved in de-ionized water (50 ng/ml). The details for preparation of reagents were in appendix I. This method has been done following the method of Moriwaki *et al.* (2008) with a modification. Aliquots of plasmid DNA (5 μl) were incubated with 4 μl of 25 mM hydrogen peroxide (final concentration was 6.66 mM) and 3 μl of 8 mM ferrous sulfate (final concentration was 1.6 mM) with or without 3 μl samples for 60 min at 37°C. Samples were loaded onto a 1% agarose gel. Following electrophoresis (2 h, 90 V) in TBB, gels were strained with ethyldium bromide. The gels were viewed under UV light and photographed and images were stored in TIFF format on floppy disk. Densitometry analysis of individual DNA bands was achieved using the Image Quant software.