CHAPTER III

MATERIALS AND METHODS

The experiments were divided into three parts:

1. Extraction and isolation of active constituent (oxyresveratrol) from aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad)
2. Stability evaluation of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) solutions
3. Determination of antioxidant and free radical scavenging activities of *Artocarpus lakoocha* heartwood extract (Puag-Haad)

Crude Drugs

1. Dried aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) (80% w/w content of oxyresveratrol as assayed by HPLC), E.A.R. drugstore, Chiangmai, Thailand

Materials

1. Absolute ethanol, AR grade, Mallinckrodt, Mexico
2. 2, 2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), Sigma-Aldrich, Inc., USA
3. Butylated hydroxyanisole (BHA), Nikko Chemicals Co., Ltd., Japan
4. Chloroform, AR grade, BDS Laboratory Supplies, UK
5. Disodium ethylene diamine tetraacetic acid (EDTA), T Chemical Co., Ltd., Thailand
6. Disodium hydrogen orthophosphate, E. Merck, Germany
7. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Sigma-Aldrich, Inc., USA
8. Hematoporphyrin, Sigma-Aldrich, Co., USA
9. Hydrogen peroxide solution (30%), VWR International, Ltd., UK
10. Iron (III) chloride-6-hydrate, VWR International, Ltd., UK

11. L-3, 4-dihydroxyphenylalanine (L-DOPA), Fluka Chemie GmbH, Germany
12. L-methionine (2-Amino-4(methylmercapto) butyric acid), Fluka Chemie GmbH, Germany
13. Magnesium ascorbyl phosphate liposome (Rovisome®), ROVI GmbH & Co., Germany
14. Methanol, AR grade, Apex Chemical Co., Ltd., Thailand
15. Mushroom tyrosinase (T-7755), Sigma-Aldrich Inc., USA
16. Nitro blue tetrazolium (NBT), Sigma-Aldrich Co., USA
17. Potassium dihydrogen orthophosphate, APS Finechem, Australia
18. Propylene glycol, Srichand United Dispensary Co.,Ltd., Thailand
19. Riboflavin (Vitamin B2), Fluka Chemie GmbH, Germany
20. Rovisome®, P. C. Intertrade Co., Ltd., Thailand
21. Sephadex™ (LH-20), Amersham Pharmacia Biotech AB, Sweden
22. Sodium chloride, E. Merck, Germany
23. Sodium dihydrogen phosphate dihydrate, E. Merck, Germany
24. Sodium metabisulfite, VIV Interchem Co., Ltd., Thailand
25. Whole blood, Animal Husbandry department, Faculty of Veterinary, Chulalongkorn University, Thailand

Reference Antioxidants

1. (-)-Epigallocatechin gallate (EGCG) (458.4 g/mol, 99.0% purity), Sigma-Aldrich, Inc., USA
2. 6-Hydroxy-2, 5, 7, 8,-tetramethylchroman-2-carboxylic acid (Trolox®) (250.3 g/mol, 97.0% purity), EMD Biosciences, Inc., USA
3. L-ascorbic acid (176.13 g/mol, 99.75% purity), DSM Nutritional Product Co., Ltd., Switzerland
4. Pine bark extract (96.88% proanthocyanidins), Human Province Resources Products Imp & Exp, Corp., China
5. Oxyresveratrol (244 g/mol), purified from Puag-Haad in this study (purity > 95%)

Reference Antityrosinase Agent

1. Licorice extract (PT-40), Maruzen Pharmaceutical Co., Ltd., Japan

Apparatus

1. Analytical balance, AG 285, Mettler Toledo, Switzerland
2. Centrifuge, Centrifugette 4206, ALC, USA
3. Fluorescent Lamp, PL 9W, Sylvania, Thailand
4. FT-NMR spectrometer, DPC-300, Bruker, Germany
5. Micropipette, Gilson, France
6. Microplate reader, Model 450, Bio-Rad, USA
7. Multi-channel micropipette, Gilson, France
8. pH meter, Model 420A, Orion, USA
9. Photometer, Tes digital lux meter 1332, Tes, Taiwan
10. Radiometer (UVB probe), IL1700, International light, Inc., USA
11. Rotary evaporator, Rotavapor RE-120, Buchi, Switzerland
12. Sonicator (Transonic digitals), Elma, Germany
13. UVB lamp, 290-320 nm, Model TL20W/12, Phillips, Netherlands
14. UV- visible spectrophotometer, UV-1601, Shimadzu, Japan
15. Vacuum Pump, CB 169 Vacuum System, Buchi, Switzerland
16. Vortex mixer, Vortex Genie-2, Scientific Industries, Inc., USA

Others

1. Quartz cell, Starna, UK
2. 96-well microplates, Nunc, Denmark
3. Parafilm, American National Can TM, USA
4. TLC Alumina sheet silica gel 60 F_{254} 20 x 20 cm, E. Merck, Germany
5. Whatman filter paper No.1, 150 mm, Whatman International Ltd., UK

Methods

Part 1. Extraction and Isolation of Active Constituent (oxyresveratrol) from Aqueous Extract of *Artocarpus lakoocha* Heartwood (Puag-Haad)

The Puag-Haad powders or the dried aqueous extract of *Artocarpus lakoocha* heartwood (10.02 g) was dissolved in a small amount of methanol, triturated with 126.5 g of silica gel 60 (No. 7734) and dried under reduced pressure. It was then fractionated by quick column chromatography. The method was slightly modified from Sritularak, 1998. The details were as follows:

Adsorbent	:	Silica gel 60 (No. 7734), particle size 0.063-0.200 mm
Packing method	:	dry packing
Sample loading	:	The sample was dissolved in a small amount of methanol, mixed with a small quantity of adsorbent, triturated, dried as above and then placed gently on top of the column
Detection	:	Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 365 nm.

Elution was performed in a polarity gradient manner with mixtures of chloroform and methanol as the solvents. The ratios and volumes of solvents used in this column chromatography are summarized in Table 3.

Table 3 The ratios and volumes of solvents for quick column chromatography of methanol extract of *Artocarpus lakoocha*

Fraction	Ratio of $CHCl_3$:MeOH	Volume of solvent (ml)
1-2	95:5	400
3-25	90:10	4600

The eluants were examined by thin layer chromatographic method (TLC) using the condition below:

Adsorbent	:	Alumina sheet silica gel 60 F_{254}
Layer thickness	:	0.2 mm

Distance	:	5 cm
Temperature	:	Ambient temperature
Mobile phase	:	15% methanol in chloroform
Detection	:	UV light at wavelength of 254 and 365 nm

Fractions with similar chromatographic pattern were pooled (fraction 10-13, 69.13% yield). The fractions that have fewer impurities were further evaporated and separated using gel filtration chromatographic method as described in detail below:

Gel filter	:	Sephadex LH 20
Packing method	:	100 g of gel filter was suspended in the eluent (mobile phase) and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly.
Mobile phase	:	Methanol (flow rate 2.90 ml/min)
Sample loading	:	The sample was dissolved in a small volume of eluent and applied on top of the column.

The fractions of 20 ml were collected and examined by TLC method. The TLC chromatogram of the fractions that showed a single spot under UV light at 254 nm (R_f 0.24) were pooled and evaporated under reduced pressure using rotary evaporator (Figure 8) to give a compound as a pale-yellow crystal. This compound was later identified as 2, 4, 3′, 5′-tetrahydroxystilbene or oxyresveratrol using nuclear magnetic resonance (NMR) spectra.

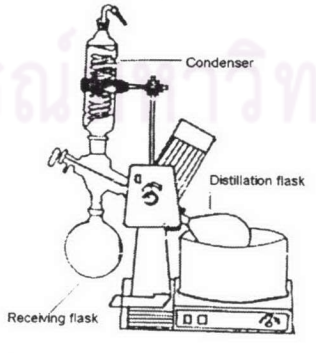


Figure 8. Rotary evaporator

Part 2. Stability Evaluation of Aqueous Extract of *Artocarpus lakoocha* Heartwood (Puag-Haad) Solutions

The objective of the present study was to investigate the physical and biochemical stability of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) solutions. Propylene glycol in water (20% v/v) was selected as the solvent since it was shown to provide adequate solubility for Puag-Haad and other reference testing substances like antityrosinase agents and various antioxidants (Pengrungrangwong, 2001). Test samples were kept at ambient temperature and dark condition with and without antioxidants for 24 weeks. At initial time (week 0) and every 4 weeks the physical appearance and the pH values of the samples were investigated. Biochemical stability of the test samples at various times was also determined by tyrosinase inhibitory activity.

1. Samples preparation

Solutions of Puag-Haad at 0.25% w/v concentration dissolved in 20% v/v propylene glycol in water were chosen for stability test. Degradation of Puag-Haad solution occurs probably via oxidation, which results in brownish color and can be stabilized by proper use of antioxidants (Pengrungrangwong, 2001). The antioxidants (0.1% w/v sodium metabisulfite, 0.01% w/v butylated hydroxyanisole (BHA) and 0.4% w/v Rovisome®) were chosen as representative antioxidants for their different mechanisms of action. Thus, the test samples consisted of 0.25% w/v Puag-Haad, with and without the three antioxidants, as well as their combination using 20% v/v propylene glycol in water as a solvent. A freshly prepared 0.25% w/v solution licorice extract was used as the reference during each assay to validate the accuracy of the tyrosinase inhibitory activity test. The freshly prepared Puag-Haad solution was also used to serve as control sample in every period. The test samples were as follows:

P	=	0.25% w/v Puag-Haad
P + A1	=	0.25% w/v Puag-Haad + 0.1% w/v sodium metabisulfite
P + A2	=	0.25% w/v Puag-Haad + 0.01% w/v BHA
P + A3	=	0.25% w/v Puag-Haad + 0.4% w/v Rovisome®

P + A4 = 0.25% w/v Puag-Haad + 0.1% w/v sodium metabisulfite + 0.01% w/v BHA

P + A5 = 0.25% w/v Puag-Haad + 0.1% w/v sodium metabisulfite + 0.01% w/v BHA + 0.4% w/v Rovisome®

The test samples were kept at room temperature in tightly closed glass vials encased in a box to protect from light for up to 24 weeks at ambient temperature. At the start and every 4 weeks of the study period, their physical properties (color/clarity and pH value) and biochemical property (tyrosinase inhibitory activity) were investigated.

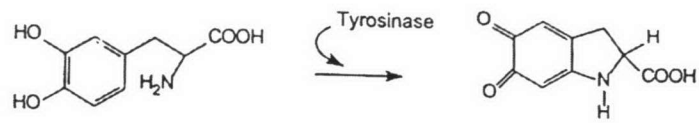
2. Physical stability test

For the physical stability test the samples were investigated for pH value, clarity, and color of the solution. The color of each vial was visually compared with the freshly prepared solution of 0.25% Puag-Haad. The degree of discoloration was measured using the following simple numerical scoring system:

Score	Degree of discoloration
0	Normal (pale yellow), no change
+1	Slightly (light yellow) changed
+2	Noticeably (light brown) charged
+3	Markedly (brown) charged
+4	Seriously deteriorated (dark brown)
+5	Almost or completely deteriorated (intense deep brown)

3. Biochemical stability test

The biochemical stability of the test samples was determined by tyrosinase inhibitory activity. Tyrosinase inhibitory activity was determined by the DOPAchrome method using L-DOPA as a substrate. DOPAchrome is one of the intermediate substances in the melanin biosynthesis. The red color of DOPAchrome can be detected by visible light. In this experiment a microplate reader (Bio-Rad, model 450) with 492 nm interference filter was used for detection.



DOPA DOPAchrome

The potential tyrosinase inhibitory activity of the test substance would show minimal DOPAchrome absorption. This method was modified from the methods of Sritularak (1998) and Shin et al. (1998).

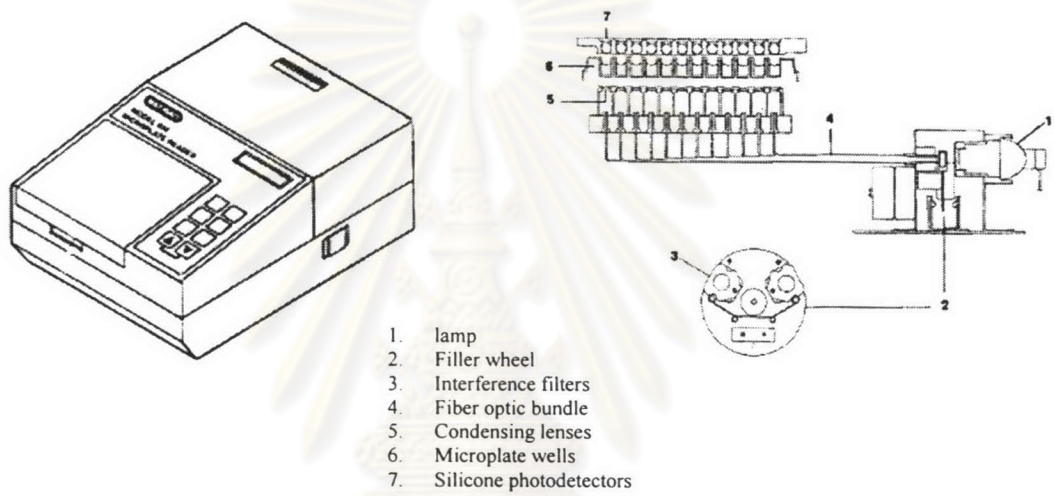


Figure 9. Microplate Reader, Model 450

3.1 Instrument

Microplate reader

3.2 Reagents

3.2.1 Phosphate buffer pH 6.8 (20mM)

3.2.2 L-Dopa (0.85 mM)

3.2.3 Tyrosinase (480 units/ml)

3.2.4 Test solution

3.3 Preparation of the reaction mixtures

3.3.1 L-DOPA (0.85 mM)

0.8 mg of L-DOPA was dissolved in 5 ml of 20 mM phosphate buffer (pH 6.8).

3.3.2 Tyrosinase (480 units/ml)

0.606 mg of tyrosinase (3960 units/mg) was dissolved in 5 ml of 20 mM phosphate buffer (pH 6.8).

3.3.3 Test solution

All the test samples were diluted 50 times with 20% v/v propylene glycol in water prior to analysis for tyrosinase inhibitory activity.

3.4 Measurement of activity

The reaction mixture (total volume of 200 µl each) was measured in four wells designated as A, B, C and D. In each well, the substance was added in the order of mixing, as shown below:

A (control)	40 µl of mushroom tyrosinase solution (480 units/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of solvent (20% v/v propylene glycol in water)
B (blank of A)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of solvent
C (test)	40 µl of mushroom tyrosinase solution (480 units/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test solution in solvent
D (blank of C)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test solution in solvent

After each well was mixed and pre-incubated at ambient temperature for further 10 minutes, 40 µl of 0.85 mM L-DOPA was added and incubated at ambient temperature for 10 minutes. The absorbance of each well was measured at 492 nm with the microplate reader (Figure 9) after incubation.

3.5 Calculation of the percent inhibition of tyrosinase enzyme

The percent inhibition of tyrosinase reaction was calculated according to the following equation.

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

Where A: The difference in optical density before and after incubation at 492 nm without test sample (only enzyme and substrate)

B: The difference in optical density before and after incubation at 492 nm without test sample and enzyme (blank of A)

C: The difference in optical density before and after incubation at 492 nm with test sample (enzyme plus substrate and test substance)

D: The difference in optical density before and after incubation at 492 nm with test sample, but without enzyme (blank of C)

3.6 Calculation of the percent tyrosinase inhibitory activity relative to the initial value

The % relative tyrosinase inhibitory activity of each test sample was calculated using the following equation:

$$\% \text{ relative inhibitory activity} = \left(\frac{A}{B}\right) x\ 100\%$$

Where A: = % tyrosinase inhibitory activity at storage testing time (wk)

B: = % tyrosinase inhibitory activity at initial time (o wk)

Thus, the percentage of tyrosinase inhibitory activity remaining at any particular time-point was expressed in relative to the initial inhibitory activity at time zero.

Part 3. Determination of Antioxidant and Free Radical Scavenging Activities of *Artocarpus lakoocha* Heartwood Extract (Puag-Haad)

1. Determination of DPPH free radical scavenging activity

Scavenging activity has been determined by measuring the remaining concentration of DPPH radical which was used as the substrate. The violet color of DPPH radical can be detected by a UV- visible spectrophotometer at 517 nm. The presence of any potential DPPH radical scavenger would decrease the absorbance of DPPH in the reaction mixture. This method was modified from the method of Haraguchi et al. (1998).

1.1 Instrument

UV- visible spectrophotometer

1.2 Reagents

1.2.1 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (150 μM)

1.2.2 Absolute Ethanol AR grade

1.2.3 Test solution

1.3 Preparation of the reaction mixture

1.3.1 Preparation of DPPH solution (150 μM)

5.91 mg of DPPH (MW 394.3) was dissolved in 100 ml of absolute ethanol and the solution was subsequently stirred for 15 min.

1.3.2 Preparation of the test solution

The test samples (oxyresveratrol and Puag-Haad) were prepared as an ethanolic solution with initial concentration of 200 μg/ml. For IC_{50} analysis, serial dilution was performed to give eight concentrations (0, 1, 2, 5, 10, 20, 40 and 100 μg/ml). Assays were carried out in triplicate. The test sample (1 ml) was added to the DPPH solution (1 ml) to make the total volume of 2.0 ml. The final concentration (Table 4) was calculated by the formula below.

$$N_1V_1 = N_2V_2$$

N_1 = Beginning concentration (μg/ml)
V_1 = Beginning volume (ml)
N_2 = Final concentration (μg/ml)
V_2 = Beginning volume (ml)

Table 4 The initial and final concentration (μg/ml) of the test sample

Initial concentration (μg/ml)	100.0	40.0	20.0	10.0	5.0	2.0	1.0
Final concentration (μg/ml)	50.0	20.0	10.0	5.0	2.5	1.0	0.5

1.4 Measurement of activity

One ml of the test sample was added to 1 ml of DPPH solution. The reaction mixture was incubated at room temperature for 30 min and then the absorbance of each sample was measure at 517 nm. The absolute ethanol was used instead of test sample to serve as control (0 μg/ml). L-ascorbic acid, epigallocatechin gallate (EGCG), pine bark extract, and water soluble form of vitamin E (Trolox®) were used as reference antioxidants.

DPPH• (Purple)

DPPH-H (Pale yellow)

DPPH• = 1, 1-diphenyl-2-picrylhydrazyl free radical

AH = antioxidant

Figure 10. Structure of DPPH and reaction with an antioxidant

1.5 Calculation of percentage of free radical scavenging activity and IC_{50}

The percentage of scavenging activity was calculated as follows.

$$\% \text{ DPPH reduction} = \frac{[A - B]}{A} \times 100$$

A = The absorbance of control solution at 517 nm.

B = The absorbance of reaction mixture at 517 nm.

After the % DPPH reduction of the test sample in each concentration was calculated, a plot of sample concentration versus % DPPH reduction was constructed. The IC_{50} of each test sample was then obtained from the graph. By definition, IC_{50} was the concentration at which 50% DPPH reduction was obtained.

1.6 Statistic analysis

Regression of the initial portion of the concentration versus % DPPH reduction curve was made to obtain IC_{50} for each of the test and reference antioxidants. Statistical comparison of IC_{50} values was then made using ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate.

2. Superoxide anion scavenging activity (NBT test)

Riboflavin photo-oxidation is used for the determination of superoxide anion scavenging. In this method, the photo-excitation of riboflavin lead to the generation of riboflavin radical which then auto oxidizes and generates superoxide anions (Geetha et al., 2004). Then, superoxide anion reduced NBT, resulting in the formation of blue formazan (Dasgupta and De, 2004).

The assay was based on the capacity of the samples to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) into formazan by superoxide anion in the presence of riboflavin-light-NBT system (Banerjee, Dasgupta, and De, 2005). The production of blue formazan was followed by measuring the absorbance at 560 nm after a

30 min illumination from a fluorescent lamp. This method was modified from the method of Dasgupta and De (2004) and Banerjee et al. (2005).

2.1 Instrument

UV- visible spectrophotometer

2.2 Reagents

2.2.1 Methionine (130 mM)

2.2.2 Riboflavin (200 μM)

2.2.3 EDTA (1 mM)

2.2.4 NBT (750 μM)

2.2.5 Phosphate buffer pH 7.4 (50 mM)

2.2.6 Test solution

2.3 Preparation of the reaction mixture

2.3.1 Preparation of the methionine solution (130 mM)

1.94 g of methionine (MW 149.21) was dissolved in 100 ml of phosphate buffer and stirred until a clear solution was obtained.

2.3.2 Preparation of riboflavin solution (200 μM)

7.53 mg of riboflavin (MW 376.38) was dissolved in 100 ml of phosphate buffer and stirred until a clear solution was obtained.

2.3.3 Preparation of EDTA solution (1 mM)

37.22 mg of EDTA (MW 372.24) was dissolved in 100 ml of phosphate buffer and stirred until a clear solution was obtained.

2.3.4 Preparation of NBT solution (750 μM)

0.61 mg of NBT (MW 817.64) was dissolved in 1 ml of phosphate buffer and stirred until a clear solution was obtained.

2.3.5 Preparation of test solution

The test samples (oxyresveratrol and Puag-Haad) were prepared in phosphate buffer with initial concentration of 1 mg/ml. For IC_{50} analysis, the solution was added in different volumes to the reaction mixture to give six final concentrations of 0, 5, 10, 25, 50 and 100 µg/ml. Assays were carried out in triplicate.

2.4 Measurement of activity

In a test tube, 100 µl methionine (130 mM) and 100 µl riboflavin (200 µM) and 100 µl EDTA (1 mM) and 100 µl NBT (750 µM) were placed. Then the test solution and phosphate buffer were added to give a total volume of 1.0 ml. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 30 min illumination from a fluorescent lamp (0.36 mW/cm^2). The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with corresponding reaction mixtures were kept in the dark and served as respective blanks for the individual samples. The sample without test solution (0 µg/ml) was used as a control. L-ascorbic acid, epigallocatechin gallate (EGCG), pine bark extract, and water soluble form of vitamin E (Trolox®) were used as reference antioxidants.

2.5 Calculation of percentage of free radical scavenging activity and IC_{50}

The percentage of scavenging activity was calculated as follows.

$$\% \text{ superoxide anion scavenging activity} = \frac{[(A-B)-(C-D)]}{(A-B)} x\ 100$$

Where A: The absorbance of control solution at 560 nm

B: The absorbance of control solution in dark at 560 nm (blank of A)

C: The absorbance of test solution at 560 nm

D: The absorbance of test solution in dark at 560 nm (blank of C)

After the % superoxide anion scavenging activity of the test sample in each concentration was calculated, the data were plotted against the concentration for each

substance. The IC_{50} of each substance was then obtained from the graph by regression analysis of the initial portion of the graph.

2.6 Statistic analysis

Statistical comparison of the IC_{50} values was made using ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate.

3. Hydroxyl radical scavenging activity ($ABTS/H_2O_2/FeCl_3$ method: Fenton reaction)

Hydrogen peroxide can readily react with transition-metal catalysts to generate the hydroxyl radical ($OH^{\cdot}$) This so-called iron-catalyzed Haber-Weiss reaction, was first proposed as one of several possible reactions in 1934 (Halliwell et al., 1989). In this reaction, the extent of hydroxyl radical was quantitated by measuring changes in the absorbance of 2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS), an intermediate radical which interacts with hydroxyl radical giving a green color solution that can be measured at 416 nm. This method was slightly modified from the method of Pumthong (1999).

3.1 Instrument

UV- visible spectrophotometer

3.2 Reagents

3.2.1 ABTS (500 μM)

3.2.2 H_2O_2 (20 mM)

3.2.3 $FeCl_3 \cdot 6H_2O$ (0.33 mg/ml)

3.2.4 Phosphate buffer pH 7.4 (0.05 mM)

3.2.5 Test solution

3.3 Preparation of the reaction mixture

3.3.1 Preparation of the ABTS solution (500 μM)

27 mg of ABTS (MW 548.68) was dissolved in 100 ml of phosphate buffer and stirred until a clear solution was obtained.

3.3.2 Preparation of H_2O_2 (20 mM)

113 µl of 30% w/v of H_2O_2 was added with phosphate buffer to complete 50 ml.

3.3.3 $FeCl_3 \cdot 6H_2O$ (0.33 mg/ml)

3.30 mg of $FeCl_3.6H_2O$ (MW 270.30) was dissolved in 10 ml of phosphate buffer and stirred until a clear solution was obtained.

3.3.4 Preparation of test solution

The test samples (oxyresveratrol and Puag-Haad) were prepared in phosphate buffer with initial concentration of 1 mg/ml. For IC_{50} analysis, the solution was added in different volumes to the reaction mixture to give six final concentrations of 0, 5, 10, 20, 50 and 100 µg/ml. Assays were carried out in triplicate.

3.4 Measurement of activity

The reaction mixture containing ABTS solution (500 µM) 2.0 ml, H_2O_2 (20 mM) 50 µl, the test solution and phosphate buffer solution, with a final volume of 2.57 ml were mixed and the reaction was started by the addition of 0.33 mg/ml of $FeCl_3 \cdot 6H_2O$ 20 µl. After allowing the mixture to stand at room temperature for 5 min, the absorbance of ABTS radical was determined colorimetrically at 416 nm. The sample without test solution (0 µg/ml) was used as control. L-ascorbic acid, epigallocatechin gallate (EGCG), pine bark extract, and water soluble form of vitamin E (Trolox®) were used as reference antioxidants

3.5 Calculation of percentage of free radical scavenging activity and IC_{50}

The percentage of scavenging activity was calculated as follows.

$$\% \text{ hydroxyl radical scavenging activity} = \frac{[(A-B)-(C-D)]}{(A-B)} x\ 100$$

Where A: The absorbance of control solution at 416 nm

B: The absorbance of control solution without $FeCl_3 \cdot 6H_2O$ at 416 nm (blank of A)

C: The absorbance of test solution at 416 nm

D: The absorbance of test solution without $FeCl_3 \cdot 6H_2O$ at 416 nm (blank of C)

After the % hydroxyl radical scavenging activity of the test sample in each concentration was calculated. A graph showing concentration versus % inhibition of the free radical was plotted for each antioxidant. The IC_{50} of each test sample was then obtained from the graph by means of regression analysis of the initial portion of the graph.

3.6 Statistic analysis

Statistical comparison of the IC_{50} values was made using ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate.

4. UV-induced lipid peroxidation (Hemolysis test)

The reaction was based on the principal that the lipid component of the red blood cell (RBC) membrane can be oxidized by singlet oxygen, which is a product resulted from the degradation of hematoporphyrin, a photosensitizer, under UV exposure. This will lead to membrane lipid peroxidation with a subsequent hemolysis. Thus, any antioxidant that can prevent lipid peroxidation or acts as a singlet oxygen scavenger would be able to inhibit red blood cell hemolysis. The method was modified by Kawashima et al. (2003).

4.1 Instrument

UV-visible spectrophotometer

4.2 Reagents

4.2.1 Hematoporphyrin (208.33 μM)

4.2.2 Sheep erythrocytes

4.2.3 Phosphate buffer saline solution pH 7.4

4.2.4 Test solution

4.3 Preparation of the reactive mixture

4.3.1 Preparation of hematoporphyrin (208 μM)

5 mg of hematoporphyrin (50% purity, MW 598.71) was dissolved in 20 ml of phosphate buffer saline solution and stirred until a clear solution was obtained.

4.3.2 Preparation of RBC pellets

The blood sample from sheep was centrifuged to obtain RBC pellets. Then, the pellets were rinsed with phosphate buffered saline (PBS) at a volume ten times that of the pellets and then centrifuged at 3000 rpm for 10 min to separate the pellets. The procedure was repeated 2 more times.

4.3.3 Preparation of test solution

Primary solutions of Puag-Haad and reference antioxidants (750 μg/ml) were prepared in PBS. For the antihemolytic activity, the primary solution was added in different volumes to the reaction mixture to give 4 final concentrations of 0, 200, 400 and 600 μg/ml. Assays were carried out in triplicate.

4.4 Measurement of antihemolytic activity

4.4.1 The reaction mixture was prepared by mixing 0.3 ml of RBC pellets and 1.5 ml of hematoporphyrin (208 μM) with appropriate volumes of the antioxidant solution (Puag-Haad and other reference antioxidants) and PBS to give a final volume of 30 ml. The final concentration of the reaction mixture was 1% v/v RBC, 10 μM hematoporphyrin. The composition of each component in the reaction mixture is as shown below:

Conc. of antioxidants	Reaction mixture				Blank solution			
(μg/ml)	0	200	400	600	0	200	400	600
RBC pellets	0.3 ml	0.3 ml	0.3 ml	0.3 ml	-	-	-	-
208 μM Hematoporphyrin	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml
Antioxidants (750μg/ml)	0 ml	8 ml	16 ml	24 ml	0 ml	8 ml	16 ml	24 ml
PBS qs.	30 ml	30 ml	30 ml	30 ml	30 ml	30 ml	30 ml	30 ml

4.4.2 The reaction mixture was transferred to a transparent glass beaker placed above a magnetic stirrer and subsequently exposed to UVB radiation using two UVB lamps (Philips TL20W/12), which emitted a continuous spectrum between 280 and 320 nm with a peak emission at 312 nm. The intensity of the emitted UVB light at the site of cell irradiation (10 cm from the lamp source) was 1.033 mW/cm^2. The UVB dose was equivalent to 5.58 J/cm^2 for 90-min exposure time, as measured by a radiometer with a UVB probe. Exposure was conducted at an ambient temperature under constant stirring.

4.4.3 Following the start of UVB exposure 2.0 ml of the reaction mixture was sampled immediately before (time 0) and at 90 min after exposure and split into two 1.0-ml portions. The first portion was diluted with 2.0 ml PBS whereas the latter was diluted with 2.0 ml distilled water to induce complete hemolysis.

4.4.4 The two diluted portions were then centrifuged at 3000 rpm for 10 min and the absorbance of their respective supernatants was measured spectrophotometrically at 540 nm.

4.4.5 The extent of hemolysis for each antioxidant was calculated by the following formula:

$$\% \text{ Hemolysis} = \left(\frac{A-B}{C-D}\right) \text{x } 100\ \%$$

Whereas A = Absorbance of the first portion (PBS added)

B = Blank absorbance of A (reaction mixture without RBC treated under the same conditions)

C = Absorbance of the latter portion (water added to induce complete hemolysis)

D = Blank absorbance of C (reaction mixture without RBC treated under the same conditions)

4.4.6 Steps 4.4.1 – 4.4.5 were repeated by varying the final concentration of each antioxidant in the reaction mixture. The reaction mixture which contained no antioxidant (zero concentration) was designated as control. Puag-Haad was evaluated in comparison with other antioxidants including oxyresveratrol , l-ascorbic acid, epigallocatechin gallate (EGCG), pine bark extract, and water soluble form of vitamin E (Trolox®).

4.4.7 The extent of singlet oxygen scavenging activity of each antioxidant was expressed in terms of % hemolysis relative to control (% relative hemolysis) according to the following formula:

$$\% \text{ Relative hemolysis} = \frac{A}{B} \times 100\ \%$$

Where A = Percent hemolysis in the presence of antioxidant

B = Percent hemolysis of control (no antioxidant)

4.4.8 The extent of % hemolysis and % relative hemolysis was compared, using ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate.