APPENDIX A

LIST OF CHEMICALS AND MATERIALS USED IN THIS STUDY

Name of chemical	Company
ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	Sigma, USA
Acetic acid	E.Merck, Germany
Alpha-tocopherol	Sigma, USA
Ammonium alum	E.Merck, Germany
Ascorbic acid	E.Merck, Germany
BHT (Butylated hydroxytoluene)	Sigma, USA
Bovine serum albumin	Sigma, USA
Chloral hydrate	E.Merck, Germany
Citric acid	E.Merck, Germany
Copper sulfate	E.Merck, Germany
Dibasic sodium phosphate	E.Merck, Germany
Dimethyl sulfoxide	E.Merck, Germany
Electra HR buffer	Helena
Ethanol	E.Merck, Germany
Fetal calf serum	Gibco, USA
Folin - Ciocalteau reagent	Sigma, USA
Glycerol	E.Merck, Germany
Haematoxylin	E.Merck, Germany
HEPES (N-2-hydroxythylpiperazine-N-2-ethanesulphonic acid)	Sigma, USA
Hydrochloric acide	E.Merck, Germany
Hydrogen peroxide solution	E.Merck, Germany
2-Mercaptoethanol	Gibco, USA

Name of chemical	Company
Methanol	Sigma, USA
Monobasic sodium phosphate	E.Merck, Germany
Myoglobin	Sigma, USA
Oil Red-O	E.Merck, Germany
Penicillin-streptomycin	Gibco, USA
Potassium bromide	E.Merck, Germany
Potassium chloride	E.Merck, Germany
Potassium dihydrogen phosphate	E.Merck, Germany
Potassium ferricyanide	E.Merck, Germany
Potassium permanganate	E.Merck, Germany
Potassium phosphate	E.Merck, Germany
RPMI 1640	Gibco, USA
2-Propanol	E.Merck, Germany
Sephadex G-15	Sigma, USA
Sephadex G-25	Sigma, USA
Sodium carbonate	E.Merck, Germany
Sodium chloride	E.Merck, Germany
Sodium hydrogen phosphate	E.Merck, Germany
Sodium hydroxide	E.Merck, Germany
Sodium iodate	E.Merck, Germany
Sodium potassium tartrate	E.Merck, Germany
Sudan black B	E.Merck, Germany
Tetraethoxypropane	E.Merck, Germany
Thiobarbituric acid	E.Merck, Germany
Titan III Lipo Plate	Helena
Trichloroacetic acid	E.Merck, Germany
Trolox (6-hydroxyl-2,5,7,8-tetramethylchlorman-2-carboxylic acid)	Sigma, USA
Trypan blue stain	E.Merck, Germany

APPENDIX B

LIST OF INSTRUMENTS USED IN THIS STUDY

Instrument	Company
Analytical balance	A&D Co., Ltd, Japan
Autoclave	Tomy autoclave
Automatic pipette	Gibco
Carbondioxide incubator	Forma Scientific
Centrifuge	Kokusan
Electrophoresis chamber	Helena
Freezer (-20^0 C)	Sanyo
Glassware	Pyrex
Hot air oven	Haraeus
Inverted microscope	Nikon
Larminar flow biological cabinet (MSC12)	Juan
Light microscope	Olympia Tokyo
Magnetic stirrer	Thermolyne Co.. USA
Microculture plate	Gibco
Pasture pipette	Pyrex
pH meter	BATCO, Thailand
Power supply	Helena
Screw capped tube	Pyrex
Serological pipette	Pyrex
T culture flask	Gibco
Ultracentrifuge	Backman
UV-spectrophotometer	Shimadzu Co., Japan
Vortex	Scientific industries
Water bath	GFL 108

APPENDIX C

REAGENT PREPARATION

1. Protein determination

Stock Solutions:

Solution A: 2% Na_2CO_3 in 0.1 M NaOH

Solution B: 1% $CuSO_4.5H_2O$

Solution C: 2% Sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$)

Lowry stock reagents: Freshly prepared before use by mixing

49 ml Solution A, 0.5 ml Solution B and 0.5 ml Solution C

Folin's Reagent:

Stock 2N Phenol reagent (Folin - Ciocalteau reagent)

Dilute 1:1 in deionized distilled water

Standard Bovine serum albumin (BSA):

The standard should be dissolved at concentration of 1 mg/ml in a buffer similar to unknown.

2. Phosphate buffered saline (PBS)

Sodium chloride	8 g
Disodium hydrogen phosphate	1.44 g
Potassium dihydrogen phosphate	0.24 g
Potassium chloride	0.2 g

Dissolve in 800 ml distilled water, adjust pH to 7.4 with HCl and make up to 1L with distilled water.

3. Sudan black B stain

Stock solution:

Sudan black B	1 g
Methanol 95 %	900 mL
Reflux	1 h

Adjust volume to 1000 ml with methanol 95 %

Mix well and stand for 10 minutes. Filter through Whatman No. 42. The filtrate can be used for 2 months.

Working solution: Freshly prepared before use.

Sudan black B stock solution	60 mL
10 % KOH	40 mL

Mix well

Rinsing solution:

Methanol 95 %	200 mL
Glacial acetic acid	200 mL
Distilled water	600 mL

4. Total antioxidant capacity

Stock 0.15 M phosphate buffer saline solution (PBS), pH 7.4:

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
Distilled water	900 mL

Adjust to pH 7.4 then top up to 1000 mL with distilled water

Working 5 mM PBS:

Dilute 0.15 M PBS 1:30 with distilled water

Stock 400 μM myoglobin solution:

Dissolve 0.068 g myoglobin in 5 mM PBS make up to 10 ml

Stock 740 μM Potassium ferricyanide:

Dissolve 0.02407 g potassium ferricyanide in distilled water and make up to 100 ml

Metmyoglobin (MetMb):

Add the stock myoglobin solution to an equal volume of freshly prepared potassium ferricyanide. After mixing, the solution was passed through an equilibrated Sephadex G-15 column for elimination of excess iron and the metmyoglobin fraction was collected. Absorbances were measured at 490, 560 and 580 nm, and subtracting the reading at 700 nm for background correction. The purity of the metmyoglobin prepared was estimated by applying the Whitburn equation. The accepted solution indicates by the methemoglobin fraction is more than 95 % of the total heme protein.

$$[\text{Met Mb}] = 146\,A_{490} - 108\,A_{560} + 2.1\,A_{580}$$

$$[\text{Ferryl Mb}] = -62\,A_{490} + 424\,A_{560} - 123\,A_{580}$$

$$[MbO_2] = 2.8\,A_{490} - 127\,A_{560} + 153\,A_{580}$$

where, Mb is myoglobin

5mM ABTS: 2,2 –azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)

Dissolve 0.02743 g ABTS in 5 mM PBS make up to 10 mL.

The reagent should be stored light protected at 4°C.

500 μM Hydrogen peroxide:

Hydrogen peroxide was titrated and adjusted to 500 μM. The reagent is stable for 10 day at 4^0 C.

2.5 mM Trolox:

Dissolve 0.031286 g Trolox in 1.5 mM PBS and make up to 50 ml, mix and sonicate for 0.5-1 hour until solution was clear. Store at -20°C in the dark.

5. Malondialdehyde (MDA) by TBARs

Trichloroacetic acid reagent (TCA):

Dissolve 100 g TCA in 100 mL of 0.6 M HCl. Store the reagent at room temperature and kept light protected.

Thiobarbituric acid reagent (TBA), 0.12 M:

Dissolve 17.298 g TBA in 100 mL of 0.26 M 2-amino-2-hydroxymethyl-1, 3-propanediol. Store the reagent at room temperature.

Normal saline solution (NSS) :

0.85 g NaCl is dissolved and adjusted to 100 mL with distilled water.

6. Culture medium

Incompleted medium

RPMI 1640	1	package 9.8 g.
HEPES	3.57	g.
$NaHCO_3$	2.0	g.
0.34% 2-mercaptoethanol	1.0	mL
Deionize distilled water	800	mL

Adjust pH to 7.2-7.4 and adjust volume to 1,000 mL then sterile by suction through filter membrane pore size 0.2 μm.

Completed medium

Incomplete RPMI 1640 medium	80 mL
Penicillin 1,000 units/mL / Streptomycin 1,000 µg /mL	1 mL
Fungizone 250 µg/mL	1 mL
Fetal calf serum	10 mL

Adjust volume to 100 mL by incompleted RPMI 1640 medium (stored at 4° C)

7. Trypan blue stain

Trypan blue 50 mg. Was dissolved and adjusted to 10 mL with 0.85% NaCl.

8. Mayer's hematoxylin stain

Hematoxylin	1.0 g
Potassium or ammonium alum	50 g
Sodium iodate	0.2 g
Citric acid	1.0 g
Chloral hydrate	50 g

Add the hematoxylin, ammonium alum and sodium iodate made up to 1L distilled water. Bring to boiling point and allow to cool overnight. Then add 1g citric acid and 50g chloral hydrate mix to dissolve. Cool and filter through Whatman No. 42.

9. Oil red O stain

Oil red O stock solution

(store at room temperature for up to 1 month)

Oil red O	300 mg
2-Propanol, 99%	100 mL

Mix well.

Oil red O working solution

Oil red O, stock solution	24 mL
Distilled water	16 mL

Mix well and let stand for 10 minutes.

Filter through Whatman No. 42.

The filtrate can be used for ~3 hours.

APPENDIX D

CALCULATION

1. Total antioxidant capacity

$$\% \text{ Inhibition of oxidation} = \frac{A_B - Au}{A_B} \times 100$$

Where A_B = absorbance of blank at 5 minutes

Au = absorbance of test at 5 minutes

2. LDL uptake

% Inhibition of LDL oxidation

$$= \frac{\text{LDL uptake (LDL oxidation with antioxidant) - LDL uptake (LDL oxidation)}}{\text{LDL uptake (native LDL) - LDL uptake (LDL oxidation)}} \times 100$$

2. MDA concentration

$$\text{MDA concentration } (\mu M) = \frac{A_{532} \times \text{sample dilution factor}}{1.52 \times 10^5}$$

% Inhibition of LDL oxidation

$$= \frac{\text{MDA (LDL oxidation) - MDA (LDL oxidation with antioxidant)}}{\text{MDA (LDL oxidation) - MDA (LDL)}} \times 100$$

3. Conjugated diene formation

$$\% \text{ Increase lag time} = \frac{\text{lag time (LDL oxidation with antioxidant) - lag time (LDL oxidation)}}{\text{lag time (LDL oxidation)}} \text{ x100}$$

4. Student's t-test: Comparison of two means is calculated:

$$t = \frac{|\bar{X}_A - \bar{X}_B|}{S_{AB}\sqrt{\frac{1}{n_A} + \frac{1}{n_B}}}$$

Where X_A and X_B are the means of the two populations and n_A and n_B are the sample sizes of each population. Finally, S_{AB} is the pooled standard deviation. To calculate this number we first calculate the pooled variance:

$$S_{AB} = \sqrt{\frac{(n_A - 1)\,S_A^2 + (n_B - 1)\,S_B^2}{n_A + n_B - 2}}$$

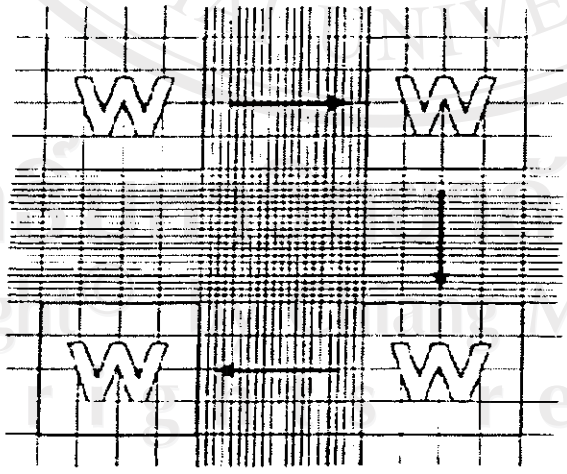
Where n_A and n_B are the sample sizes of the two groups, and S_A^2 and S_B^2 are the sample variances of the two groups. Take the square root of S_{AB}^2 to calculate S_B in the equation for t.

APPENDIX E

Cell counting and viability assay

Cell counting and viability assay

1. Clean the hemocytometer with ethanol.
2. Mount the coverglass over the ruled areas of the two chambers.
3. Prepare an aliquot of the cell sample for counting cells.
4. Add 90 µL 0.4% trypan blue in PBS to 10 µL cell suspendion in cell media. Total volume is thus 100 µL (and cell suspension has been diluted by 10-fold).
5. Mix the contents of the tube by gentle agitation by hand. Allow to stand for a few minutes but not longer than 10 minutes.
6. Fill the counting chambers with the mixtures.
7. Using a simple light microscope, count all unstained cells (viable cells) and stained cells (non-viable cells) in the four large corner squares in both slides counting chambers, if a high % of cells are stained (that is, dead), you may be in trouble.



W: Four large corner of squares count

8. Equation for calculating cell viability:

$$\% \text{ Viability} = \frac{\text{Number of Viable Cells}}{\text{Total number of cells count}}$$

9. Equation for calculating cells count/ml.

Total cell count

$$= \frac{\text{Total number of cells in 4 squares}}{\text{4 Number of squares count}} \times 10^{4} \times 10 \text{ (Dilution Factor)} \times \text{(Vol. of Cell suspension)}$$

VITA

Name	Mr.Ronnarong Kaewprasert
Date of birth	29 May 1967
Place of Birth	Chiang Rai
Education	Sukhothaivitayakom, Sukhothai
	March, 1977, Certificated of Matayom VI
	Mahidol University, Bangkok
	May, 1997, Bachelor of Sciences
Home	86/2 Moo 3, Tambon Konggajong
	Amphae Sawankalok, Sukhothai
Institution	Srisangwornsukhothai Hospital, Sukhothai
Position	Medical Technologist