APPENDIX

Appendix A

List of chemicals and materials used in the study

Chemicals	Sources
Ammonium ferric citrate (FAC)	BDH, England
Albumin Brovine (BSA)	Amresco, America
Butylated hydroxytoluene (BHT)	Sigma-Aldrich, USA
Calcium chloride ($CaCl_2.H_2O$)	Sigma-Aldrich, USA
Chloramin-T	Sigma-Aldrich, USA
Desferrioxamine (DFO)	Novatis, Switzerland
Dihydrogen phosphate potassium salt (KH_2PO_4)	Sigma-Aldrich, USA
1,2-Dimethyl-3-hydroxypyrid-4-one (L1)	Government Pharmaceutical Organization, Thailand
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich, USA
5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich, USA
Ethanol absolute	E. Merck, Germany
Glutathione	Sigma-Aldrich, USA
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich, USA
Hydroxyproline	Sigma-Aldrich, USA
Magnesium carbonate light	UNILAB, Australia
Methanol	E. Merck, Germany
β-Nicotimamide adenine dinucleotide phosphate (NADPH)	Sigma-Aldrich, USA
p-Dimethylamino benzaldehyde	E. Merck, Germany
3-[N-Morpholino]propanesulfonic acid (MOPS)	Sigma-Aldrich, USA
Penicillin-streptomycin	Gibco, USA
Phosphoric acid (85%)	E. Merck, Germany
Potassium chloride (KCl)	Sigma-Aldrich, USA
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich, USA

Sodium chloride (NaCl)	Sigma-Aldrich, USA
Sulfuric acid (96%)	LAB-SCAN ASIA, Australia
2-Thiobarbituric acid	Sigma-Aldrich, USA
Trichloroacetic acid	E. Merck, Germany

Appendix B

List of instruments and equipments used in this study

Instruments and equipments	Company
Analytical balance	Precisa, Germany
Carbon dioxide incubator ,	SHEL LAB Sheldon Manufacturing Inc., USA
Cellulose acetate membrane filter, pore size 0.45 μm	Sartorious AG, Germany
Centrifuge, refrigerated	Andreas Hettich, UK
Deionized water manufacturing machine, NANOPURE	Barnstead, USA
ELISA plate reader	BIOTEK Instrument,
ELISA plate, 96 well-flat buttom	Nunc™
Larminar Flow	ESCOpH meter
Refrigerator -20 °C	SHARP
Refrigerator -80 °C	Forma Scientific
Syringe Filter membrane (pore size 0.22 μm)	PALL
UV visible spectrophotometer	Shimadzu, Japan
Vortex mixture	Scientific Industries
Water bath	LabLine
6-well plate, 96-well plate	Nunclon

Appendix C

Preparation of reagents and buffers

1. Thiobarbituric acid-reactive substances (TBARS) assay reagents

1) Stock standard malonaldehyde solution (1 mM.)

Dilute 8.3 µl of 1,1,3,3-tetramethoxypropane (TMP) in 50 ml of 95% ethanol. Serial dilutions of standard solution were prepared by diluting the stock solution with 95% ethanol to reach required concentrations.

2) Thiobarbituric acid solution (0.6%, w/v)

Dissolve 0.3 g of thiobarbituric acid in 50 ml of deionized water and warm in water bath at 60 °C to dissolve.

3) Butylated hydroxytoluene solution (0.2%, w/v)

Dissolve 0.2 g of butylated hydroxytoluene (BHT) in 100 ml of ethanol.

2. Phosphate buffer saline (PBS) for cell culture

Dissolve sodium chloride 8.0 g, potassium chloride 0.2 g, disodium hydrogen phosphate 1.44 g, potassium dihydrogen phosphate 0.24 g in about 750 ml of deionized water, adjust pH to be 7.4 and make a final volume be 1000 ml with deionized water. This solution was sterilized by filtering through a sterile membrane (0.22 µm pore size) before use.

3. MTT dye solution

Dissolve 1.0 g of 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide (MTT dye) in 200 ml of PBS solution pH 7.4 (protected from light). The MTT solution was filtered through membrane (0.22 µm pore size).

4. DMEM incomplete media

Dissolve 13.5 g of DMEM (with glucose, L-glutamine, pyridoxine hydrochloride, 110 mg/ml sodium pyruvate, without sodium bicarbonate), 3.7 g of sodium bicarbonate and 10.0 ml penicillin-streptomycin (10000 units/ml) in deionized water, adjust pH to be 7.2, and finally adjust the final volume to 1,000 ml with deionized water. The mixture was filtered through a membrane (0.22 µm pore size, 43

mm in diameter) equipped on ultrafiltration apparatus (sterilized apparatus) in laminar flow.

5. DMEM complete medium (10 % fetal bovine serum)

20.0 ml of fetal bovine serum (inactivated at 56 °C for 30 minutes) was mixed with 286 µl of insulin, 200 µl of 1 mM dexamethazone and 180 ml of DMEM incomplete medium in laminar flow (sterilized technique).

6. Glutathione assay reagents

1) Stock standard glutathione solution (1 mM) Dissolve 3.1 mg of glutathione (reduced form) in 1 ml of deionized water. Working standard GSH solution was performed by diluting the stock solution with deionized water to reach required concentrations.

2) Assay buffer (5x) solution containing GSH 30 ml, 500 mM potassium phosphate, pH 7.0, 5 mM EDTA**.**

3). Glutathione reductase (400 U/ml) from bakers yeast in 3.6 M ammonium sulfate, pH 7.0, containing 0.1 mM dithiothreitol (DTT).

4) NADPH (25 mg vial) NADPH stock solution (40 mg/ml) was prepared by dissolving the contents of the vial of NADPH (25 mg) in 0.625 ml of water to give a 40 mg/ml solution. The solution was stored at –20 °C for at least 6 months.

5) Dimethyl sulfoxide (DMSO) solution

6) 5,5'-Dithiobis(2-nitrobenzoic acid) [DTNB] stock solution (1.5 mg/ml) Dissolve 8 mg of the DTNB with 5.33 ml of DMSO and stored in aliquots at –20 °C for at least 3 months.

7) 5-Sulfosalicylic acid (SSA) (2.5 g vial) 5% SSA solution was prepared by dissolving 2.5 g of SSA in 50 ml of water and kept at 4 °C.

8) Working mixture reagent. Add 228 µl of the diluted glutathione enzyme solution (6 U/ml) and 228 µl of DTNB stock solution (1.5 mg/ml) and mix well. This solution was freshly prepared before use.

7. Citrate buffer, pH 6 solution

Dissolve citric acid monohydrate 50 g, Sodium chloride 34 g, sodium acetate trihydrate 1.44 g in about 750 ml of deionized water. Add 12 ml of glacial acetic acid and adjust a final volume to 1000 ml with deionized water.

8. Ehrich's reagent

Dissolve 1 g of *p*-dimethylamino benzaldehyde in 20.0 ml of n-propanol, mix well, then add 6.6 ml of perchloric acid and adjust a final volume to 1000 ml with deionized water.

9. Chloramin-T solution

Dissolve 0.178 g of chloramin-T in 10.0 ml of deionized water, add 15 ml of n-propanol, mix well, and finally add 25 ml of citrate buffer. The solution is freshly prepared before use.

10. Standard hydroxyproline solution

Dissolve 2.0 mg of hydroxyproline in 1 ml of deionized water. Serial dilution of standard was prepared by diluting the stock solution with deionized water to reach required concentrations.

11. Colorimetric measurement of iron (TPTZ method)

Reagent formulae

1) Hydrochloric acid (HCl, analytical grade). For glass washing, an approximately 6 N HC1 may be made by diluting the concentrated reagent with an equal volume of distilled water.

2) 20% Trichloroacetic acid (TCA) solution. Twenty grams of the crystals are dissolved in d-d water in a 100-ml. volumetric flask.

3) TPTZ solution (0.004 M). Dissolve 124.8 mg of 2,4,6-tripyridyl-s-triazine (TPTZ) in 1.0 ml of 1.0 M HC1 and dilute to 100 ml. with deionized distilled water.

4) Hydroxylammonium chloride (hydroxylamine hydrochloride, NH_2OH. HC1) solution (10%) Either an iron-free prepared solution can be purchased, or the solution may be purified by adding 0.5 ml of 0.004 M TPTZ, 1 gram of sodium perchlorate, 2

ml of 50% ammonium acetate, and 10 ml of nitrobenzene to 100 ml of the hydroxylammonium chloride solution in a separatory funnel. Shake the mixture vigorously for 1 minute and stand until separation is complete. Draw off the lower nitrobenzene layer and discard.

5) 50% ammonium acetate solution (Analytical reagent grade material <0.0005% iron contamination) (50%).

6) Buffered chromogenic TPTZ solution. Mix ammonium acetate solution, hydroxylammonium chloride, and TPTZ solutions (2:1:1, v/v/v). This solution was prepared freshly for each day's use from the reagent stock solutions and kept separately in tightly sealed colored glass bottles.

12. Hematoxylin and Eosin (H&E) Staining

Reagent formulae

1) Lillie Mayer alum haematoxylin

Aluminium ammonium sulphate	200 g
Hematoxylin (CI 75290)	20 g
Ethanol	40 ml
Sodium iodate	4 g
Acetic acid	80 ml
Glycerol	1200 ml
Distilled water	2800 ml

To 1000 ml of the distilled water, add the aluminium ammonium sulphate. Place 4-liter volumetric flask on a heater/stirrer, turn on the heater and mix until the alum dissolves. Remove the flask from the heater/mixer, cool down the solution and add the remaining 1800 ml of distilled water to the solution. Add the hematoxylin powder to the alcohol and dissolve as much of the powder as possible by shaking for a few minutes. Pour the strong alcoholic solution of hematoxylin into the cooled alum solution and stir to ensure all the hematoxylin powder is dissolved preferably overnight. Add the sodium iodate, acetic acid, and finally the glycerol. Mix well, plug loosely and store. It is appropriate to make up a batch of the required amount, dependant upon the usage rate.

2) Acid alcohol (0.3%) solution

Commercial grade ethanol	2800 ml
Distilled water	1200 ml
Concentrated hydrochloric acid	12 ml

Add the acid to the water, then add the alcohol and mix thoroughly.

3) Scott's tap water substitute

Sodium hydrogen carbonate	10 g
Magnesium sulphate	100 g
Distilled water	5 liter

Dissolve the salts in the water. Store the stock solutions at room temperature.

4) Alcoholic acetified eosin/phloxine

1% eosin Y (CI 45380)	400 ml
1% aqueous phloxine (CI 45405) solution	40 ml
95% alcohol	3100 ml
Glacial acetic acid	16 ml

Mix the above reagents together and stir well.

13. Mason trichrome staining

Reagent formulae

1) Fixing agent: 10% formalin or Bouin's solution

2) Weigert's iron hematoxylin solution:

Stock Solution A:

Hematoxylin	1 g
95% Alcohol	100 ml

Stock Solution B:

29% Ferric chloride in water	4 ml
Distilled water	95 ml
Hydrochloric acid, concentrated	1ml

Weigert's iron hematoxylin working solution: Mix equal parts of stock solution A and solution B. This working solution is stable for 3 months.

3) Biebrich Scarlet-Acid Fuchsin Solution:

Biebrich scarlet, 1% aqueous solution	90 ml

Acid fuchsin, 1% aqueous solution	10 ml
Glacial acetic acid	1 ml

4) Phosphomolybdic-phosphotungstic acid solution:

5% Phosphomolybdic acid	25 ml
5% Phosphotungstic acid	25 ml

5) Aniline blue solution:

Aniline blue	2.5 g
Glacial acetic acid	2 ml
Distilled water	100 ml

5) 1% Acetic Acid Solution:

Acetic acid, glacial	1 ml
Distilled water	99 ml

14. Pearl's staining

Reagent formulae

1) 2% Hydrochloric acid solution

Hydrochloric acid, concentrated	2.0 ml
Distilled water	98.0 ml

2) 10% Triton X-100

Triton X-100	10.0 ml
Distilled water	90.0 ml

Add a few grains of thymol to prevent the growth of fungi.

3) 1% Potassium ferrocyanide solution

Potassium ferrocyanide	1.0 g
Distilled water	98.0 ml
10% Triton X-100	2.0 ml

4) Hydrochloric acid-potassium ferrocyanide solution

2% hydrochloric acid	20.0 ml
1% potassium ferrocyanide	20.0 ml

Prepare just before use and discard after use.

CURRICULUM VITAE

Name Miss Thidarat Saewong

Date of Birth September 10th, 1983

Place of Birth Phayao

Education:

March, 1998	Certificate of junior high school, Phayao Pitayacom School, Phayao, Thailand
March, 2001	Certificate of senior high school, Phayao Pitayacom School, Phayao, Thailand
March, 2004	Bachelor of Science (Medical Technology), Faculty of Associated Medical Science, Chiang Mai University