APPENDIX

Appendix A

List of chemicals and materials used in the study

1. Chemicals

All chemicals used in this study were analytical grades and listed as following:

Chemicals	Companies
Absolute Ethanol	Merck
ABTS (2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid	Fluka
Acetone	Carlo Erba
Chloroform	BDH
Cyclohexane	Scharlau
DPPH (1, 1-Diphenyl-2-picryl-hydrazyl)	Sigma
DL-alpha-tocopherol (Vitamine E)	Fluka
Ethyl acetate	J.T.Baker
Gallic acid	Sigma
Glacial acetic acid	Scharlau
Hexamine	Unilab
Hexane	Lab-Scan
Hydrochloric acid	BDH
Iron (III) chloride	BDH

Chemicals	Companies
Iron (II) sulfate heptahydra	Scharlau
Methanol	Merck
Potassium chloride	Scharlau
Potassium dihydrogen phosphate	Scharlau
Phosphoric acid	Scharlau
Quercetin	Acros
Sodium acetate-3-hydrate	Fisher
Sodium carbonate anhydru	Fluka
Sodium hydroxide	Scharlau
Sulfuric acid	Merck
Sodium tungstate	J.T.Bake
Sodium carbonate anhydrous	Merck
Sodium Molibdate	Fluka
TMM (Tetramethylmurexide)	Sigma
TPTZ (2, 4, 6-Tris (2-pyridyl)-s-triazine)	Sigma
Trolox (6-hydroxy-2,5,7,8-tetramethyl chlorman-2-carboxylic acid)	Fluka

2. Instruments

Instruments	Companies
UV/VIS Spectrophometer Jasco model 7800	Jasco
pH meter	Metrohm
Microcentrifuge	Beckman
Ultrasonic bath	Branson
Electronic Analytical Balance	Sartorius
Elisa plate, 96 well-flat bottom	Nunc™
Vortex mixer	Scientific industries
Automatic micropipette	Gilson
Filter paper	Whatman
Incubator	Tabai ESPEC
Hot air oven	Memmert
Water bath	Heto, Labline
Freezer	Sanyo Reflux

Appendix B

List and figure of 30 Thai indigenous plants used in the study

1. *Phyllanthus emblica* Linn.

2. *Musa sapientum* Linn.

3. *Terminalia chebula* Retz.

4. *Artocarpus heterophyllus* Lamk.

5. *Morinda citrifolia* Linn.

6. *Gymnema inodorum* Decne.

7. *Sesbania grandiflora* (L.) Desv.

8. *Solanum torvum* Sw.

9. *Kaempferia parviflora* Wall.

10. *Momordica charantia* Linn.

11. *Lycopersicon esculentum* Mill.

12. *Houttuynia cordata* Thunb.

13. *Luffa acutangula* (Linn.) Roxb.

14. *Capsicum frutescens* Linn.

15. *Piper retrofractum* Vahl

16. *Allium sativum* Linn.

1

17. *Zingiber officinale* Linn. Adrak

18. *Costus speciosus* Koen. J.E. Smith

19. *Aegle marmelos* correa (L.)

20. *Coccinia grandis* (L.) Voigt. Syn.

21. *Eryngium foetidum* Linn.

22. *Ipomcea aquatica* Forsk.

23. *Acacia penneata* Linn.

24. *Coriandrum sativum* Linn.

25. *Piper samentosum* Roxb.

26. *Apium graveolens* Lin.

27. *Ocimum basilicum* Linn.

28. *Pisum sativum* Linn.

29. *Abelmoschus esculentus* Linn.

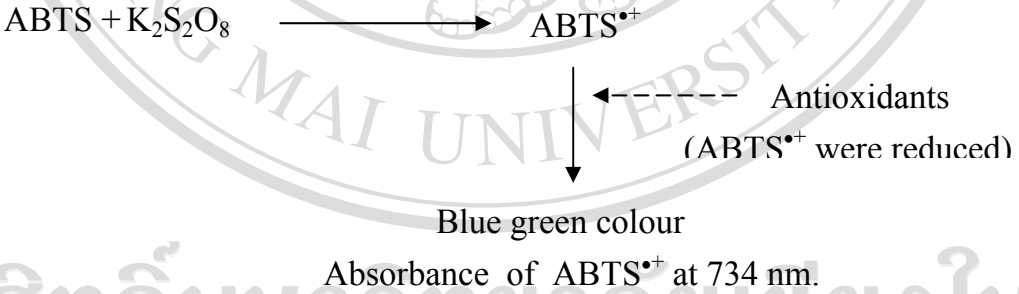
30. *Glycine max* Merr.

Appendix C

ABTS free radical cation decolorization assay

Principle of the Method

The ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation decolorization test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. The experiments were carried out using an improved ABTS decolorization assay. It is applicable for both lipophilic and hydrophilic compounds. $ABTS^{\bullet+}$ was generated by oxidation of ABTS with potassium persulfate as shown in the following scheme:



$$\% \text{ Inhibition} = \left[\frac{\text{Absorbance negative control} - \text{Absorbance sample}}{\text{Absorbance negative control}} \right] \times 100$$

Procedure See chapter III

TEAC assay reagent preparation

Trolox standard stock solution

Trolox stock standard solution 25 mM was prepared in absolute ethanol. Serial dilution of standard was prepaed by diluted stock standard with 95% absolute ethanol to reached required concentration.

Stock ABTS solution (7 mM)

Dissolved ABTS in deionized water, and kept at 4 ^{o}C.

Stock $K_2S_2O_8$ solution (2.45 mM)

Dissolved $K_2S_2O_8$ in deionized water, kept at 4 ^{o}C.

Working ABTS

Mixed stock of ABTS with $K_2S_2O_8$ (ratio 1: 0.5 mole/mole), stored 12-16 hours in tempature 4 ^{o}C.

The protocol of ABTS free radical cation decolorisation assay

$ABTS^{\bullet+}$ working solution

+

Sample or Standard solution

↓

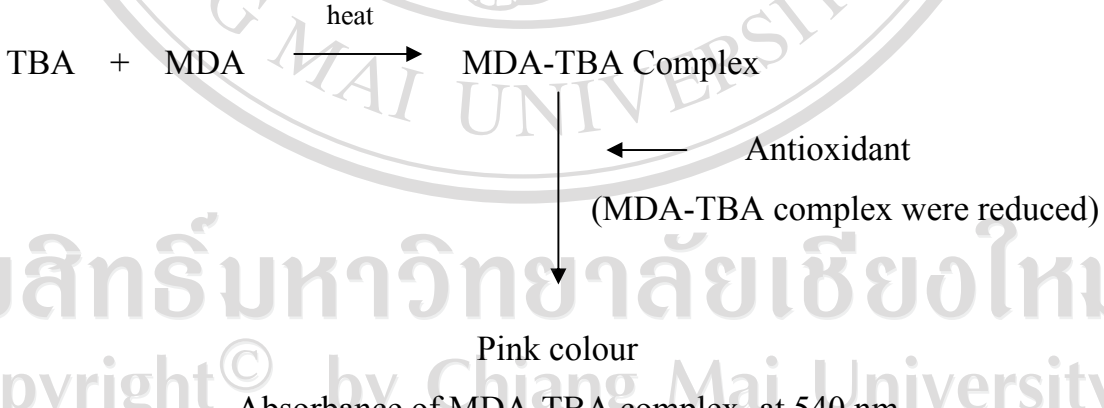
$ABTS^{\bullet+}$ λ_{max} 734 nm

Appendix D

Thiobarbituric acid-reactive substance assay (TBARS)

Principle of the Method

MDA, is a decomposition product of lipid hydroperoxide. The absorbance of the test solution was measured at 540 nm. The amount of TBARS was calculated as malonaldehyde (MDA) equivalent using 1,1,3,3-tetramethoxypropane as standard. The experiments were carried out the assessment of antioxidant activity using an improved MDA decolorization assay. TBARS free radical was generated by oxidation of TBA with MDA as shown in the following scheme:



$$\text{MDA } (\mu M) = \frac{\text{Absorbance unknown } \mathbf{x} \text{ Standard concentration}}{\text{Absorbance Standard}}$$

Chemical reagent preparation

Standard malonaldehyde

Stock standard 1 mM was prepared by dissolved 1,1,3,3-tetramethoxypropane (TMP) in 95% ethanol**.** Serial dilution of standard was prepared by diluted stock standard with 95% ethanol to reached required concentration.

Thiobarituric acid solution

Dissolved 0.3 g of thiobarbituric acid (0.6 %, w/v) in 50 ml of deionized water.

Butylated hydroxytoluene solution.

Dissolved 0.2 g of butylated hydroxytoluene (0.2%, w/v) in100 ml of ethanol.

Appendix E

Red blood cells oxidative stress by flow cytometry assay

Principle of the Method

In principle 2', 7'-dichlorofluorscin diacetate (DCFH-DA) can simply diffuse into the cells and be hydrolyzed by esterase enzyme in viable cells to produce 2', 7'-dichlorofluorscin diacetate (DCFH-DA), firstly remains in a reduced form. This compound will be subsequently oxidized by existing free radicals and transform to oxidized form, dichlorofluorscin (DCF) as shown below. Finally, DCF exhibits the green fluorescence illumination when exposed to UV light or laser beam. The valve of fluorescence intensity (FI) is correlated with oxidative stress and directly proportional to the amount of free radicals in the cells.

λexcite/λemis : 483/530

DCFH-DA solution

Dissolved DCFH-DA 0.01 g in methanol 1 ml. Stored in dark container, -4^{o}C.

Appendix F

Superoxide anion and Nitric oxide scavenging assays

Superoxide anion scavenging assay

Principle of the Method

Plasma superoxide anion radical is a product by the complex oxidation reactions of plasma sample which added the PBS buffer (mixture in EDTA, NBT and NADH) containing PMS. Absorbance of the test solution was measured at 560 nm. The amount of O_2^- was calculated reduction of NBT. The experiments were carried out assessment of the antioxidant activity using an improved NBT decolorization assay as shown in the following scheme:

Plasma sample + PMS + mixture PBS (added EDTA+NBT+NADH)

↓ At temparature 37˚C, incubate 30 min

Plasma O_2^- complex

↓ ← Antioxidant (NBT were reduced)

Blue-black colour

Absorbance of NBT reduction at 560 nm

Nitric oxide scavenging assay

Principle of the Method

Plasma nitric oxide radical is a product by nitrite/nitrate oxidation reactions. Plasma nitrate was oxidized to be nitrite radical in iron-haem reaction of haemoglobin by Nitrate reductsae as shown below. Absorbance of the test solution was measured at 540 nm. The amount of Nitrite/Nitrate radical was measured based on the Griess reaction. The experiments were carried out assessment of the antioxidant activity using an improved radical decolorization assay as shown in the following scheme:

Plama sample + Nitrate reductase + NADPH in PBS buffer (pH 7.4)

↓ Incubated 30 mins, at temparature 37 ˚C

Deproteination

↓ Incubated overnight

Incubated 10 mins with Griess reagent, at 4 ^{0}C

↓

Pink colour

Absorbance measured at 540 nm

Protein precipitant solution

Placed in a 1-L volumetric flask 33.3 ml of thioglycolic acid, 98.4 ml of trichloroacetic acid solution, a 400 ml of deionised water, swirled to dissolve. Slowly added 2 mol of HCl, added deionised to volume and stored in a dark brown bottle.

Appendix G

Glucose Determination

Principle of the Method

Plasma glucose was determined by the glucose oxidase method using the commercial diagnostic kit. Glucose present in the plasma is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid with the liberation of hydrogen peroxide, which is converted to water and oxygen by the enzyme peroxidase (POD). 0-Dianisidine, a chromogenic oxygen acceptor, takes up the oxygen and forms a red coloured chomogen which can be measured at 500 nm

$$\beta\text{-D-Glucose} + O_2 + 2H_2O \xrightarrow{\text{GOD}} \text{D-Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{phenol} \xrightarrow{\text{POD}} \underset{\text{(Red color)}}{\text{Quinoneimine}} + 4H_2O$$

Absorbance at 500 nm

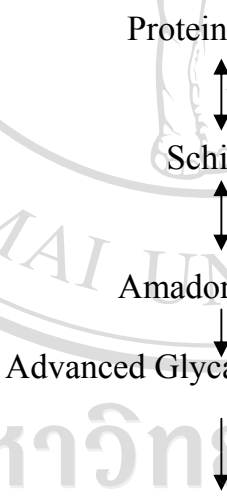
$$\text{Glucose (mg/dl)} = \frac{\text{Absorbance unknown}}{\text{Absorbance Standard}} \times \text{Standard concentration}$$

Appendix H

Glycation of protein analysis

Principle of the Method

Glycated protein was measured in from of the amadori product from BSA reacting with both glucose and fructose. The irreversibly formed advanced glycation end products do not return to normal when hyperglycemia is corrected and continue to accumulate over the lifetime of the protein. The experiments were carried out assessment of the inhibition of glycation from plant extract as shown in the following scheme:



AGEs Assay

(Advanced glycation endproducts Assay)

I. AGEs Assay

1. BSA
2. sample (plant extract)
3. Glucose (25 mM)
3. Fructose (25 mM)

II. Solotion preparation

1. BSA stock solution
   - BSA + Phosphate Buffer Solution (PBS)
     ⟶ Adjust to final conc., Buffer ; pH = 7.2
   - KH_2PO_4 MW =136.09 + NaA (Sodium azide) Adjust pH = 7.2
   - Na_2HPO_4 8.90 gm
2. 25 mM glucose solution + PBS ⟶ Adjust to 25 mM by PBS
3. 25 mM Fructose solution + PBS ⟶ Adjust to 25 mM by PBS
4. Sample in PBS

III. Standard preparation Aminoquanidine (AG) MW = 110.5; in PBS

VI. Control

1. BSA
2. PBS (= sample)
3. Glucose (25 mM)
4. Fructose (25 mM)

CURRICULUM VITAE

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LIST OF PUBLICATIONS

Journal:

1. Kusirisin1, W.; Srichairatanakool, S.; Lerttrakarnnon, P.; Lailerd, N.; Suttajit, M.; Jaikang, C. & Chaiyasut, C. (2009). Antioxidative Activity, Polyphenolic Content and Anti-Glycation Effect of Some Thai Medicinal Plants Traditionally Used in Diabetic Patients. *MedicinalChemistry*, 5, 139-147.

2. *Winthana Kusirisin*, Chaivat Chaiyasut, Somdet Srichairatanakool, Narissara Lailerd, Peerasak Lerttrakarnnon, Churdsak Jaikang. (2007). *Antioxidant activity of indigenous plants on diabetic oxidative stress: potential of polyphenolic compounds in vitro. Chiang Mai Medical Bullutin*, 46(Suppl.3), 34.

3. Winthana Kusirisin, Chaiyavat Chaiyasut, Somdet Srichairatanakool, Peerasak Lerttrakarnnon, Narissara Lailerd, Churdsak Jaikang and Maitree Suttajit. (2008). Free radical scavenging and anti-glycation of some Thai medicinal plants: Effect of antioxidant capacity on diabetic oxidative stress. *Naresuan Phayao Journal*, 1 (Suppl1), 183.

Poster presentations:

1. *Winthana Kusirisin*, Chaivat Chaiyasut, Somdet Srichairatanakool, Narissara Lailerd, Peerasak Lerttrakarnnon, Churdsak Jaikang. *Antioxidant activity of indigenous plants on diabetic oxidative stress: potential of polyphenolic compounds in vitro.* Mahidol meeting day, Faculty of Medicine, Chiang Mai University, 24 September 2007. Proceeding page 34.

2. *W. Kusirisin*, C. Chaiyasut, S. Srichairatanakool, N. Lailerd, P. Lerttrakarnnon. *Effect of antioxidative activity in five indigenous plants on diabetic oxidative stress: potential of polyphenolic compounds.* The 2nd Thailand Congress of Nutrition. Bitec Exhibition & Convention Hall center, Bangkok, 3rd -5th October 2007. Proceeding page 219.

3. W. Kusirisin, C. Chaiyasut, S. Srichairatanakool, N. Lailerd, P. Lerttrakarnnon. *Protective Effect of biologically fermented Thai plants beverages against diabetic oxidative stress.* The 3rd Thailand Congress of Nutrition. Bitec Exhibition & Convention Hall center, Bangkok, 1st - 3rd October 2008. Proceeding page 149.

4. Winthana Kusirisin & Chaiyavat Chaiyasut. *Antioxidant activity and phytochemical of Solnum torvum on diabetic oxidative stress.* The 10th

anniversary meeting: Nutrition Eduacation, Graduate School Chiang Mai, University, 7th -8 th November 2008. Proceeding page 53.

5. Winthana Kusirisin, Chaiyavat Chaiyasut, Somdet Srichairatanakool, Peerasak Lerttrakarnnon, Narissara Lailerd. *Protective Effect of biologically fermented Thai plants beverages against diabetic oxidative stress*. The 1st SFRR-Thai Meeting and Workshop on Advance of Free Radicals, Oxidative Stress and Their Evaluation Methods. Amora hotel, Chiang Mai, 15th-16th December 2008. Proceeding poster 12.

6. Winthana Kusirisin, Chaiyavat Chaiyasut, Somdet Srichairatanakool, Peerasak Lerttrakarnnon, Narissara Lailerd, Churdsak Jaikang and Maitree Suttajit. *Free radical scavenging and anti-glycation of some Thai medicinal plants: Effect of antioxidant capacity on diabetic oxidative stress.* The 2nd Internation conference on Natural product for Health and Beauty. Naresuan University, 17th -19 th December 2008. Proceeding page 183.

Oral presentations:

1. Winthana Kusirisin, Chaivat Chaiyasut, Somdet Srichairatanakool, Narissara Lailerd, Peerasak Lerttrakarnnon, Churdsak Jaikang. *Effect of biologically fermented plants on diabetic oxidative stress.* Oral presentation and proceeding in NSTDA and food & drug committee Symposium, Fortune Hotel, Bangkok. Thailand. May 25, 2007.

2. Winthana Kusirisin, Chaivat Chaiyasut, Peerasak Lerttrakarnnon, Somdet Srichairatanakool, Narissara Lailerd. *Effect of Thai indigenous plants and product on diabetic oxidative stress.* Oral presentation in Research Seminar, Deparment of Family Medicine, Faculty of Medicine, Mai, University. February 28, 2008.

RESEARCH AWARD

สมาคมโภชนาการแห่งประเทศไทยในพระราชูปถัมภ์สมเด็จพระเทพรัตนราชสุดาฯ สยามบรมราชกุมารี
Nutrition Association of Thailand under the Patronage of Her Royal Highness Princess Maha Chakri Sirindhorn

หนังสือรับรอง

สมาคมโภชนาการแห่งประเทศไทย ในพระราชูปถัมภ์ สมเด็จพระเทพรัตนราชสุดา ฯ สยามบรมราชกุมารี ขอรับรองว่า **นางวินธนา คูศิริสิน** บุคลากรในสังกัด มหาวิทยาลัยเชียงใหม่ ได้รับรางวัลโปสเตอร์ดีเด่น

จากผลงานวิจัยด้าน ห้องปฏิบัติการ : โมเลกุล เรื่อง **Effect of antioxidant activity in five indigenous plants on diabetic oxidative stress: Potential of polyphenolic compounds** ในงานประชุมวิชาการโภชนาการแห่งชาติ ครั้งที่ ๒ วันที่ ๓-๕ ตุลาคม พ.ศ.๒๕๕๐ ณ ศูนย์นิทรรศการและการประชุมไบเทค บางนา กรุงเทพมหานคร

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