CHAPTER IV
MATERIALS AND METHODS

4.1. Materials

4.1.1 Chemicals

All chemicals, drugs, buffers, salts, enzymes and proteins were received from Sigma–Aldrich (St. Louis, MO, USA). Lists of chemicals and drugs were fluorescein sodium salt, 2,2'–azobis(2–amidinopropane) dihydrochloride (AAPH), sodium azide, 6–hydroxy–2,5,8–tetramethylchroman–2–carboxylic acid (Trolox), 1,1–diphenyl–2–picrylhydrazyl (DPPH), 2, 4, 6–tripyridyl–*s*–triazine (TPTZ), methylglyoxal (MG), ferric chloride hexahydrate ($FeCl_3 \bullet 6H_2O$), Folin–Ciocalteu's phenol reagent, gallic acid monohydrate, 5–5'–dithiobis (2–nitrobenzoic acid) (DTNB), hippuryl–histidyl–leucine (HHL), *o*-phthaldialdehyde, N–phenacyl–4,5–dimethylthiazolium bromide (DMPTB), Triton X–100, D–glucose, ethylenediaminetetraacetic acid (EDTA), orlistat (≥98%, solid), lisinopril (≥98%). Buffers and salts used were di-potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), hydrochloric acid (HCl) and tris(hydroxymethyl) aminomethane ($NH_2C(CH_2OH)_3$). Enzymes and proteins were supplied as *Candida rugosa* lipase (type VII, ≥700 unit/mg), rabbit lung angiotensin-converting enzyme (≥2.0 units/mg, modified Warburg-Christian) and bovine serum albumin (BSA, ≥98% agarose gel electrophoresis). Solvents, absolute ethanol and absolute methanol, were received from RCI Labscan (Bangkok, Thailand).

4.1.2 Plant materials

Fifteen Thai herbal teas and five conventional teas from *C. sinensis* used in this study were listed in Table 4.1. All samples were carefully checked for quality and approved by Thailand Food and Drug Administration (FDA). All dried herbs were

non–contaminated with other herbs or substances and were purchased from local market in Nakorn Pathom province, Thailand (more detailed in Appendix B).

Table 4.1 List of conventional and herbal teas used for tea extracts in this study

English common name	Abbreviation	Thai common name	Scientific name
Conventional tea			
Green tea	GT	Cha–kheaw	*Camellia sinensis*
White tea	WT	Cha–khaw	*Camellia sinensis*
Oolong tea	OT	Cha–oolong	*Camellia sinensis*
Black tea	BT	Cha–dum	*Camellia sinensis*
Pu–erh tea	PT	Cha–mak	*Camellia sinensis*
Thai herbal tea			
1) Fruit			
Bael fruit	BF	Ma–toom	*Aegle marmelos* (L.) Corr.
Indian gooseberry	IG	Ma–kham–pom	*Phyllanthus emblica* L.
Bitter cucumber	BC	Ma–ra–kee–nok	*Momordica charantia* Linn.
2) Flower			
Rosella	RS	Kra–jieb	*Hibiscus sabdariffa* L.
Safflower	SF	Kum–phoi	*Carthamu stinctorius* L.
Chrysanthemum	CS	Kek–huay	*Chrysanthemum indicum* L.
3) Leaf			
White mulberry	MB	Mone	*Morus alba* L.
Asiatic pennywort	AP	Bua–bok	*Centella asiatica* (L.) Urban
Pandanus	PD	Toey–hawm	*Pandanus amaryllifolius* Roxb.
Jiaogulan	JL	Pan–ja–khan	*Gynostemma pentaphyllum* (Thunb.) Mak.
Stevia	ST	Ya–wan	*Stevia rebaudiana* Bertoni
Cat’s whisker	CW	Ya–nuat–maeo	*Orthosiphon aristatus* (Blume) Miq

Table 4.1 List of conventional and herbal teas used for tea extracts in this study (cont.)

English common name	Abbreviation	Thai common name	Scientific name
Thai herbal tea			
4) Stem and leaf			
Lemon grass	LG	Ta–krai	*Cymbopogon citrates* (DC.) Stapf
Jewel vine	JV	Thao–wan–priang	*Derris scandens* Benth.
5) Root			
Ginger	GG	Khing	*Zingiber officinale* Roscoe

4.2. Experimental Procedure

4.2.1 Preparation of tea extracts

Tea extracts were prepared by mixing sample (1 g of dried basis) in extracted solvent (60 mL). The mixture was sonicated for 10 minutes before being shaken in water bath shaker at 100 rpm. Then, the extracts was filtered through Whatman No. 1 filter paper and stored at 4 °C before use. In order to optimize extraction conditions, tea extracts were prepared by varying solvent systems using aqueous ethanol (0, 30, 50, 70 and 90% (v/v) ethanol in dH_2O) with 2 hours extraction time in 30 °C water bath shaker.

In addition, tea infusions were performed according to the method of Deetae *et al*., 2012 [10], in which samples (1 g of dried basis) were extracted with dH_2O (100 mL) under 95 °C for 5 minutes. The extracts were then filtered through Whatman No. 1 filter paper and stored at 4 °C. All extractions were prepared in triplicate.

4.2.2 Determination of antioxidant capacity

Antioxidant activity was determined using oxygen radical antioxidant capacity (ORAC), DPPH (1,1–diphenyl–2–picrylhydrazyl)–radical scavenging and

ferric reducing antioxidant power (FRAP) assays (more details mechanisms in Appendix A and reagent preparation in Appendix C).

Oxygen radical antioxidant capacity (ORAC) assay

The ORAC assay was determined according to the method of Ou *et al.*, 2001 [101] with some modifications as follows. The extracted samples were mixed with fluorescein solution (30 nM) in a 96–well black plate before being incubated for 15 minutes at 37 °C. After the incubation, AAPH (19.125 nM), a peroxyl radical generator, was added to the reaction mixture and immediately started the reaction. The fluorescence intensity was monitored for 90 min using a microplate reader (Synergy HT multi–detection microplate reader, Bio–Tek Instruments, Inc., Winooski, VT), with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Loss of fluorescence indicates an extended damage of the reaction as the peroxyl radical being induced by AAPH. Trolox, a water–soluble analogue of vitamin E (3.125, 6.25, 12.5, 25, 50 or 100 μM), was used as a control standard, and phosphate buffer (75 mM, pH 7.4) was used as a control blank. The results were calculated based on the differences in areas under the sodium fluorescein decay curve (AUC), and were expressed as μmol Trolox equivalence (TE) per 1 g sample. The AUC can be calculated as,

$$\text{AUC} = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots. + (0.5)f_{90}/f_0,$$

where f_0 is the initial fluorescence reading at 0 min and $f_{1, 2,\ldots, 90}$ is the fluorescence reading at 1, 2,…, 90 min.

DPPH–radical scavenging assay

This assay was performed according to the method of Fukumoto and Mazza, 2000 [102] with some modifications. The samples were mixed with DPPH solution (135 μM in 95% (v/v) ethanol), an indicator of free radical scavenging activity, in a 96–well flat–bottom microplate before being incubated in dark at room temperature for 30 minutes. The reaction was determined using the microplate reader at a wavelength of 520 nm. Trolox solution (0.08, 0.16, 0.32, 0.64 or 1.28 mM) was

used as a control standard, and ethanol solution (95% v/v) was used as a control blank. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the equation,

$$\% \text{ radical scavenging activity} = 100 \times \{1-[Abs_{sample}/Abs_{control}]\},$$

where Abs_{sample} is the absorbance of the DPPH solution with tea extracts and $Abs_{control}$ is the absorbance of the DPPH solution only.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was determined according to the method of Benzie and Strain, 1996 [103] with some modifications. The FRAP reagent containing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM) in HCl (40 mM) and $FeCl_3 \cdot 6H_2O$ solution (20 mM) in a ratio of 10:1:1 was warmed at 37°C before use. The samples (20 µL) were mixed with FRAP reagent (150 µL) in the 96–well flat–bottom microplate and incubated at room temperature for 8 minutes. The reaction was monitored using the microplate reader at a wavelength of 600 nm. Trolox solution (7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 µM) was used as a control standard and deionized water was used as a control blank. The FRAP values for the samples was then determined using a standard curve of Trolox solution and expressed in µmol TE per 1 g sample.

4.2.3 Determination of total phenolics content

Total phenolics content was determined according to the procedure of Folin–Ciocalteu, which was adapted from Ainsworth and Gillespie, 2007 [104]. The samples were mixed with 10% (v/v) Folin–Ciocalteu reagent in the 96–well flat–bottom microplate. After 5 minutes incubation, saturated sodium bicarbonate (7.5% (w/v), 200 µL) was added, and the reaction was mixed well. The mixture solution was then incubated in dark at room temperature for 2 hours. The absorbance at 765 nm was measured using the microplate reader. Gallic acid (10, 20, 40, 60, 80, 100 and 200 µg/mL) was used as a control standard, and deionized water was used as a control blank. The total phenolics content in the samples was expressed in milligram of gallic acid equivalents per 1 gram sample (mg GAE/g sample).

4.2.4 Determination of anti–glycation activity

The anti–glycation was determined according to the method of Vinson and Howard, 1996 [105] with some modifications. The samples were incubated with BSA (10 mg/mL), D–glucose (250 mM) or MG (1 mM) in phosphate buffer (100 mM, pH 7.4) containing 0.02% (w/v) sodium azide at 37°C for three weeks (more detailed reagent preparation in Appendix C). The total AGEs formation was then measured using the microplate reader with the excitation and emission wavelengths of 330 and 410 nm, respectively. The inhibitory activity against AGEs formation was calculated using the equation;

$$\% \text{ inhibition} = \left(1 - \frac{B-b}{A-a}\right) \times 100,$$

where *A* and *a* were the fluorescence intensity of the deionized water control with glucose (or MG) and without glucose (or MG), respectively, while *B* and *b* were the fluorescence intensity of a sample incubated with glucose (or MG) and without glucose (or MG), respectively.

4.2.5 Determination of lipase inhibitory activity

High throughput analysis of lipase activity was adapted from the method of Choi *et al.*, 2003 [106]. The assay based on the reaction between 5,5'- dithiobis (2-nitro benzoic acid) (DTNB) and the enzymatic hydrolysis product of 2,3-dimercapto-1-propanol tributyrate (DMPTB). The assay consisted of extracted sample, *Candida rugosa* lipase (0.1 µg in 50 mM Tris (pH 8.0) containing 0.1% (w/v) BSA), DMPTB (50 µM in buffer containing 50 mM Tris (pH 7.2), 10 mM KCl, 1 mM EDTA and 10% (v/v) Triton X-100) and DTNB (0.8 mM) (more detailed reagent preparation in Appendix C). The enzyme reaction was monitored at a wavelength of 412 nm using the microplate reader. The results were calculated as a percentage of inhibitory activity using the equation;

$$\% \text{ inhibition} = \left(1 - \frac{B-b}{A-a}\right) \times 100,$$

where *A* was the absorbance of the reaction mixture using the enzyme with substrate, *a* was the absorbance of the reaction mixture using the substrate only, *B* was the absorbance of the reaction mixture using the enzyme with substrate with tea extracts, and *b* was the absorbance of the reaction mixture using the tea extracts only.

4.2.6 Determination of ACE inhibitory activity

High–throughput fluorometric assay for ACE was determined according to the method of Schwager *et al.*, 2006 [107] with some modifications. The assay based on the reaction of a substrate, hippuryl–histidyl–leucine (HHL), and a fluorescent adduct of the enzyme-catalyzed product, histidyl–leucine (HL). The enzyme assay consisted of the extracted sample, HHL (0.57 mM in potassium phosphate buffer, pH 8.3) and ACE (153 nM) in the 96–well black microplate. The reaction was incubated at 37°C for 30 minute before being quenched with NaOH (0.17 M). The product was then interacted with an indicator, *o*-phthaldialdehyde (20 mg/mL). The mixture was incubated for 10 minutes at room temperature before adding HCl (0.25 M) to stop reaction (more detailed reagent preparation in Appendix C). The enzyme reaction was monitored at an excitation wavelength of 360 nm and an emission wavelength of 485 nm using the microplate reader. The results were calculated as a percentage of inhibitory activity using the equation;

$$\% \text{ inhibition} = \left(1 - \frac{B-b}{A-a}\right) \text{x } 100,$$

where *A* was the absorbance of the reaction mixture using the enzyme with substrate, *a* was the absorbance of the reaction mixture using the substrate only, *B* was the absorbance of the reaction mixture using the enzyme with substrate with tea extracts, and *b* was the absorbance of the reaction mixture using the tea extracts only.

4.3 Statistical Analysis

All experiments were carried out in triplicate. The data's were expressed as mean ± standard deviation (SD). All analyses for enzyme reactions were determined using a GraphPad Prism software version 5.1 (GraphPad Software, Inc., La Jolla, CA). One way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed to determine the significant differences between values. Significance of difference was defined at $p<0.05$. Pearson's correlation coefficient was calculated using Microsoft Excel 2007.