CHAPTER IV

MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

The chemicals including 2,2’–azobis (2–amidiopropane) dihydrochloride (AAPH), fluorescein sodium salt, 6–hydroxy–2,5,8–tetramethychroman–2–carboxylic acid (Trolox), 1,1–diphenyl–2–picrylhydrazyl (DPPH), 2,4,6–tripyridyl–*s*–triazine (TPTZ), ferric chloride hexahydrate ($FeCl_3.6H_2O$), Folin–Ciocalteu’s phenol reagent, gallic acid monohydrate, sodium bicarbonate (Na_2CO_3), pyridine, benzene sulfonyl chloride, 5–5’–dithiobis (2–nitrobenzoic acid) (DTNB), *N*–phenacyl–4,5–dimethyyhiazolium bromide (DMPTB), D–glucose, ethylenediaminetetra acetic acid (EDTA), triton X–100, potassium chloride (KCl), *p*–nitrophenyl–α–D–maltopentaoside (PNPG–5), *p*–nitrophenyl–α–D–glucopyranoside (pNPG), acetylthiocholine (ATCh), butyrylthiocholine choride (BTCh), magnesium chloride ($MgCl_2$), trifluoroacetic acid (TFA) and hippuryl–histidyl–leucine (HHL) purchased from Sigma–Aldrich (*St. Louis, MO*, USA).

Standards including chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, galic acid, syringic acid, vanillic acid, *p*-coumaric acid, sinapic acid, *t*-cinnamic acid, naringenin, ferulic acid, myricetin, luteolin, isorhamnetin, quercetin, hesperitin, kaemferol and apigenin were purchased from Sigma–Aldrich (*St. Louis, MO*, USA).

Enzymes and proteins used in this research including *Candida rugosa* lipase (Type 8, ≥700 unit/mg), porcine pancreatic α–amylase (Type 7, ≥10 unit/mg), *Saccharomyces cerevisiae* α–glucosidase (Type 1, ≥10 unit/mg), *Eleectrophorus electricus* acetylcholinesterase (AChE, 200–1000 units/mg protein), equine serum butyrylcholinesterase (BChE, ≥10 units/mg protein), rabbit lung angiotensin–converting enzyme (≥2 unit/mg), bovine serum albumin (BSA, ≥98% agarose gel electrophoresis) and BACE1 (β−secretase) FRET (Fluorescence resonance energy transfer) Assay Kit were received from Sigma–Aldrich (St. Louis, MO, USA).

Solvents including ethanol, methanol and acetronitrile received from RCI Labscan (Bangkok, Thailand). Tertiarybutylhydroquinone (tBHQ) and ascorbic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and hydrocroloc acid (HCl) were received from Merck (New Jersey, USA).

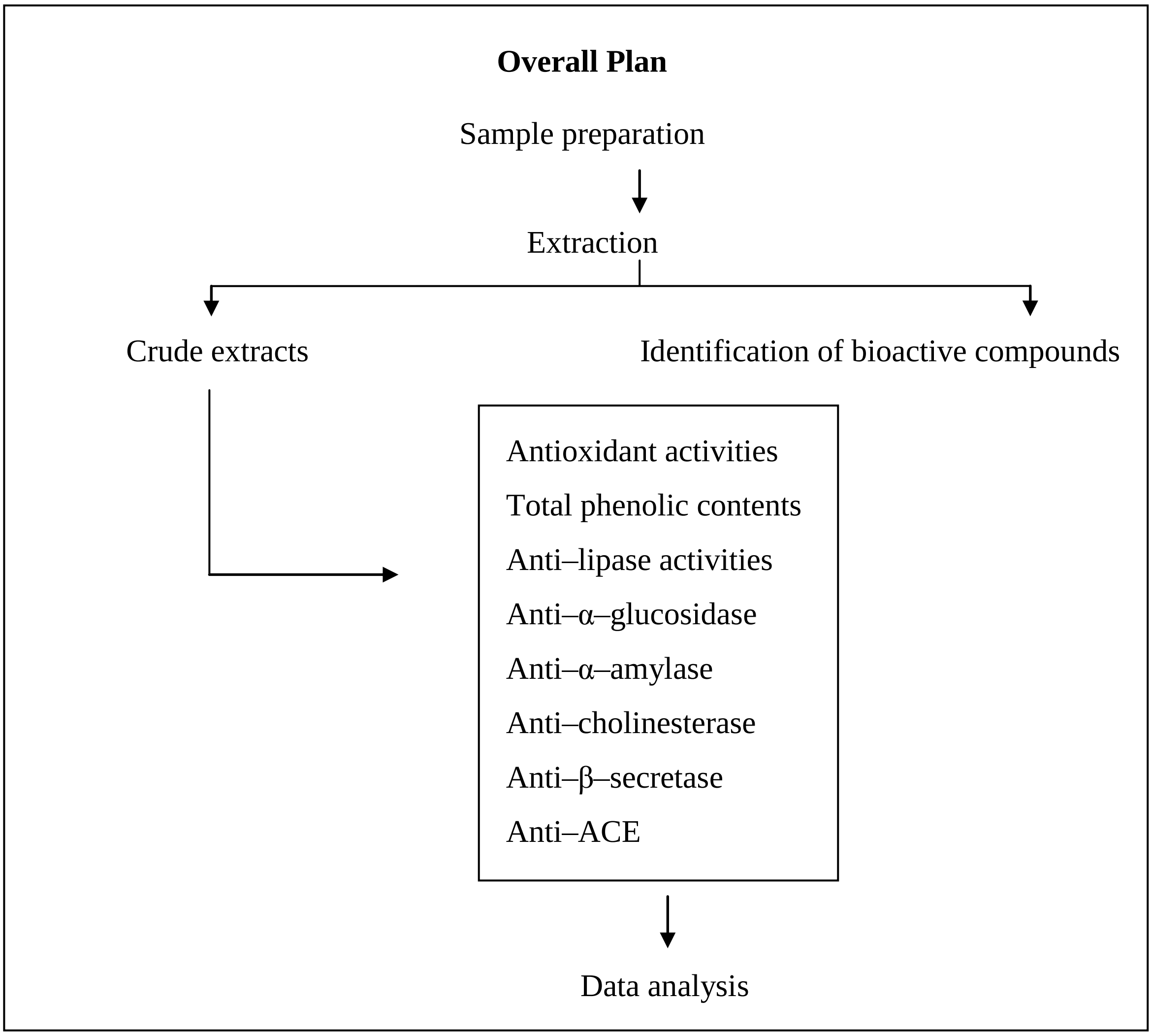
4.1.2 Legumes

All legumes including mung bean, black bean, red kidney bean, white bean, peanut and soybean were purchased from local market under the brand names Khaothong (Thai Food Industry (1964) Co., Ltd, Bangkok, Thailand) and Raitip (Thai cereals world (1957) Co., Ltd, Bangkok, Thailand) (Table 4.1).

Table 4.1 List of common and scientific name of investigated legumes including mung bean, black bean, red kidney bean, white bean, soybean and peanut.

Common name	Scientific name
Mung bean	*Vigna radiata* (L.) R. Wilczek
Black bean	*Phaseolus mungo* Linn
Red kidney bean	*Phasecolus vulgaris* L.
White bean	*Phasecolus vulgaris* L.
Soy bean	*Glycine max* (L.) Merrill
Peanut	*Arachis hypogaea* L.

4.2 Experimental Procedures



Overall Plan

4.2.1 Sample preparation

All legumes including mung bean, black bean, red kidney bean, white bean, soybean and peanut were analyzed as raw whole legumes, cooked whole legumes and raw seed coats. The colors of legumes were analyzed by a HunterLab ColorFlex EZ spectrophotometer (Reston, Virginia, USA) using C.I.E. LAB (L*, a*, b*) system. The cooked legumes were prepared by boiling legumes (100 g per 200 mL water) at 96–98 °C for 20 minutes. The raw seed coats of legumes were prepared by soaking legumes at room temperature (25 °C) water (100 g per 300 mL water) for 2 hours before removing seed coats. All samples were freeze–dried before being ground into fine powder by a grinder. The samples were stored at –20 °C for further analysis.

The moisture content of fresh and freeze–dried samples was determined using *Association of Analytical Communities* (AOAC, 2005). Air oven was regulated to the temperature of 105 ± 2 °C. The samples (approx. 2 g) were distributed and placed in a low, covered aluminum dishes (≥50 mm diameter and 40 mm deep). The samples were incubated in the oven for 2 hours before cooling in a desiccators. After that, the dish was returned to hot air oven and weighted every 30 minutes. The different weight between each time interval must be less than 0.005 g. The percentage of moisture content and the percentage of dry mass were calculated using the following equation:

$$\text{\% Moisture (w/w)} = \left[\frac{b-c}{W}\right] x\ 100,$$

$$\text{\% Dry matter} = 100 - \text{\% moisture content},$$

where b is a weigh of sample before drying, c is a weight of sample after drying and W is a weigh of sample.

4.2.2 Extraction of crude extracts

The extraction of legumes was performed according to the procedure of Nepote *et al.*, 2005 [108]. Legume powder (1 g) was extracted with 70% (v/v) aqueous ethanol (20 mL) for 2 hours at 30 °C with shaking at 100 rpm. After that, the samples were filtered through Whatman No.1 filter paper and stored at –20 °C for further analysis (APPENDICES A and B).

4.2.3 Identification of bioactive compounds

The identification of flavonoids and phenolics were performed according to the procedure of Merken and Beecher, 2000 [109] with some modifications as follows (APPENDIX C). Legume powder was extracted with 62.5% (v/v) aqueous methanol containing 0.5 g/L tBHQ and 6 N HCl at 80 °C for 2 hours. After cooling the extract, 1% (v/v) ascorbic acid was added to the mixture. The mixture was then transferred and adjusted the volume with methanol to 50 mL. The mixture was filtered using a 0.2 µm PTFE syringe filter, and the filtrate was analyzed using high performance liquid chromatography (HPLC).

The HPLC analysis was performed using Agilent 1100 series (Agilent Technologies, USA) with a photodiode array detector and 5 µm Zorbax Eclipase

XDB–C18 column (4.6 mm x 150 mm, Agilent Technologies, USA). The gradient mobile phase composed of Milli–Q water containing 0.05% (v/v) TFA, methanol containing 0.05% (v/v) TFA, and acetronitrile containing 0.05% (v/v) TFA with a constant flow rate of 0.6 mL/min. Chromatograms were monitored at 338 nm and evaluated using ChemStation (Agilent Technologies, USA). Flavonoids (quercetin, kaempferol, isorhamnetin, myricetin, apigenin, luteolin, naringenin and hesperitin) and phenolics (ferulic acid, chlorgenic acid, and caffeic acid) were used as standards.

4.2.4 Determination of antioxidant capacity

The samples were analyzed for antioxidant activities using 1–diphenyl–2–picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay and oxygen radical antioxidant capacity (ORAC) assay (APPENDICES C and D).

DPPH radical scavenging assay

The assay was performed according to the method of Fukumoto and Mazza, 2000 [110] with some modifications as follows. The samples were mixed with DPPH solution (135 µM in 95% (v/v) aqueous ethanol), an indicator of free radical scavenging activity, in a 96–well flat–bottom microplate before being incubated in dark at room temperature (25 °C) for 30 minutes. The reaction was monitored at a wavelength of 520 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT) and Gen5 data analysis software. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the equation:

$$\% \text{ Radical scavenging activity} = 100 \text{ x } (1-(\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}})),$$

where $\text{Abs}_{\text{sample}}$ is the absorbance at 520 nm of the sample and DPPH reagent, while $\text{Abs}_{\text{control}}$ is the absorbance at 520 nm of 95% (v/v) aqueous ethanol and DPPH reagent. Trolox solution (0.08, 0.16, 0.32, 0.64 or 1.28 mM) was used as a standard. The free radical scavenging activities were present as Trolox equivalent (TE) per 100 gram dry weight.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was determined according to the method of Benzie and Strain, 1996 [111] with some modifications. The FRAP reagent containing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM in 40 mM HCl) and $FeCl_3{\bullet}6H_2O$ solution (20 mM) in a ratio of 10:1:1 was warmed at 37 °C before use. The assay was performed in the 96 well flat–bottom microplate. The samples (20 µL) were mixed with FRAP reagent (150 µL) and incubated at room temperature (25 °C) for 8 minutes. The reaction was monitored using the plate reader at a wavelength of 600 nm. Trolox solution (6.25, 12.5, 25, 50, 100, 250, 500 or 1000 µM) were used as a standard. The FRAP values were determined using a standard curve of Trolox solution and expressed in µmol TE per 1 g dry weight.

Oxygen radical antioxidant capacity (ORAC) assay

The ORAC assay was determined according to the method of Ou *et al.*, 2001 [112] with some modifications as follows. The assay was performed in a 96–well black plate for fluorescence measurement. Sodium fluorescein was used as a fluorescent probe. The loss of fluorescence is an indication of the extent of damage from its reaction with the peroxyl radical induced by AAPH. The samples were mixed with fluorescein (40 nM) solution and incubated for 15 min at 37 °C. After the incubation, AAPH (153 nM), a peroxyl radical generator, was added to the reaction mixture rapidly to initiate the reaction. The fluorescence intensity was monitored for 90 minutes in the microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The results were calculated based on the differences in areas under the sodium fluorescein decay curve (AUC). The AUC was 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 initiation fluorescence reading at 0 min, and f_i is the fluorescence reading at i min. Trolox (3.125, 6.25, 12.5, 25, 50 or 100 µM) was used as a standard. The results were expressed as TE per 1 g dry weight.

4.2.5 Determination of total phenolic content

Total phenolic content was determined according to the method of Folin–Ciocalteu method, which was adapted from Ainsworth and Gillespie, 2007 (APPENDIX C) [113]. The samples were mixed with 10% (v/v) Folin–Ciocalteu reagent (50 µL). After 5 minutes of incubation, saturated sodium bicarbonate (7.5% (w/v), 200 µL) was added, and the reaction was mixed well. The mixture was incubated at room temperature (25 °C) in dark room for 2 hours. The TPC was measured at a wavelength of 765 nm using the microplate reader. Gallic acid (0–200 µg/ml) was used as a standard. The TPC was expressed in gallic acid equivalents (GAE) per 1 g dry weight.

4.2.6 Determination of lipase inhibitory activity

The determination of lipase inhibitory activity was adapted from the method of Choi *et al.*, 2003 (APPENDICES C and E) [114]. This assay was investigated by a colorimetric couple assay utilizing the reaction of sulfhydryl moiety, a product from lipase reaction and a substrate DMPTB, and DTNB as an indicator. The reaction consisted of lipase (0.5µg) in 50 mM Tris (pH 8.0) containing 0.1% (w/v) BSA, DMPTB (0.05 mM) in 50 mM Tris (pH 7.2) containing 10 mM KCl and 1 mM EDTA, DTNB (0.8 mM) in 50 mM Tris (pH 7.2) containing 10% (v/v) Triton X–100, 10 mM KCl and 1 mM EDTA and extracted samples. The lipase activity was monitored at 412 nm using the microplate reader. The results calculated as a percentage of inhibition using the equation;

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

where A is an initial velocity of the control reaction with enzyme (control), a is an initial velocity of the control reaction without enzyme (control blank), B is an initial velocity of the enzyme reaction with extract (sample) and b is an initial velocity of the reaction with extract but without enzyme (sample blank).

4.2.7 Determination of α–amylase inhibitory activity

The α–amylase inhibitory assay was performed according to the method of Funke and Melzig, 2006 (APPENDICES C and F) [115]. This colorimetric assay was investigated by the reaction of α–amylase and PNPG–5, a substrate–indicator complex. The assay was consisted of α–amylase (4 mg), PNPG–5 (6.25 mM) and legume extracts in KPB (50 mM, pH 7.0) containing 200 mM KCl. The reaction was monitored at a wavelength of 405 nm using the microplate reader. The results were calculated as the percentage of inhibitory activity as above.

4.2.8 Determination of α–glucosidase inhibitory activity

The α–glucosidase inhibition was performed according to the method of You *et al.*, 2011 (APPENDICES C and G) [60]. This colorimetric assay was investigated by the reaction of α–glucosidase and pNPG, a substrate–indicator complex. The assay was consisted of α–glucosidase (0.1 U/mL), pNPG (0.5 mM) and legume extracts in KPB (50 mM, pH 7.0). The reaction was monitored at a wavelength of 405 nm using the microplate reader. The results were calculated as the percentage of inhibitory activity as above.

4.2.9 Determination of cholinesterase inhibitory activity

The analysis of AChE and BChE assays were adapted from the method of Jung *et al*., 2009 (APPENDICES C and H) [102]. These assays were investigated by a colorimetric couple assay utilizing the reaction of sulfhydryl moiety, a product from cholinesterase reaction and thiocholine substrates, and DTNB as the indicator. The AChE assay was consisted of AChE (10 ng), ATCh (0.08 mM), DTNB (0.8 mM) and extracted samples in KPB (50 mM, pH 7.0). Likewise, the BChE assay was consisted of BChE (50 ng), BTCh (0.1 mM), DTNB (0.8 mM) and extracted samples in KPB (50 mM, pH 7.0) containing $MgCl_2$ (1 mM). The cholinesterase activity was monitored at 412 nm using the microplate reader. The inhibitory activity was calculated as percentage of inhibition as above.

4.2.10 Determination of β–secretase inhibitory activity

The BACE1 inhibitory activity was measured using a BACE1 FRET Assay Kit (APPENDICES C and I), which was consisted of BACE1 (β−secretase), BACE1 substrate (Rh–EVNLDAEFK–Quencher in 50 mM ammonium bicarbonate), BACE1 stop solution (2.5 M sodium acetate), BACE1 assay buffer (50 nM sodium acetate) and BACE1 product standard (Rh–EVNL in 50 mM ammonium bicarbonate). All reactions were performed in a 96–well microplate and monitored at an excitation wavelength of 545 nm and an emission wavelength of 585 nm using the microplate reader. The inhibitory activity was reported as percentage of inhibition as above.

4.2.11 Determination of ACE inhibitory activity

High–throughput ACE inhibitory assay was determined according to the method of Schwager *et al*., 2006 (APPENDICES C and J) [116]. The ACE inhibitory activity was determined by the colorimetric couple assays, utilizing the reaction of L–His–L–Leu (HL) from ACE reaction with *o*–phthaldialdehye and β–mercaptoethanol. The assay was consisted of ACE (5 mU/mL), HHL (0.1 mM), β–mercaptoethanol (6% v/v), *o*–phthaldialdehye (1.5 mg/mL) and extract in assay buffer (50 mM KPB, pH 7.0). The reaction was monitored with 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 as above.

4.3 Statistical Analysis

All data were expressed as mean of triplicate experiments ± standard deviation (SD). One way analysis of variance (ANOVA) and Tukey’s multiple comparison tests were performed to determine the significant differences at $p<0.05$. The significant differences of the effect of cooking were determined by independent t–test group with at $p<0.05$. All statistical analyses were carried out using SPSS statistical analysis (version 16 for Windows, SPSS Inc., Chicago, USA).