CHAPTER IV
MATERIALS AND METHODS

4.1 Materials and Equipments

4.1.1 Chemicals and reagents

Chemicals and reagents including fluorescein sodium salt, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), magnesium chloride ($MgCl_2$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium bicarbonate (Na_2CO_3), dimethyl sulfoxide (DMSO), 5,5'-azobis (2-amidinopropane) dihydrochloride (AAPH), Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCh), butyrylthiocholine chloride (BTCh), tertiary butylhydroquinone (tBNQ), ascorbic acid, triethylamine, ammonium acetate, were received from Sigma-Aldrich (St. Louis, MO, USA). Di-potassium hydrogen phosphate (K_2HPO_4), trifluoroacetic acid (TFA), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), and sodium chloride (NaCl) were obtained from Merck (Darmstadt, Hessen, Germany). Hydrochloric acid (HCl) was supplied from RCI Labscan (Bangkok, Thailand).

Standards including 6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid (trolox), gallic acid monohydrate, quercetin, kaempferol, isorhamnetin, myricetin, apigenin, luteolin, hesperetin, naringenin, caffeic acid, chlorogenic acid, syringic acid, vanillic acid, *p*-coumaric acid, sinapic acid, ferulic acid, cinnamic acid, 4-hydroxybenzoic acid, capsanthin, β-cryptoxanthin, lycopene, and β-carotene were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lutein, zeaxanthin and α-carotene were purchased from Carotenature (Luchsingen, KG, Switzerland).

Enzymes consist of *Electrophorus electricus* acetylcholinesterase (AChE, 200-1000 units/mg protein), equine serum butyrylcholinesterase (BChE, ≥10 units/mg protein) and BACE1 (β-secretase) FRET (Fluorescence resonance energy transfer) assay kit were received from Sigma-Aldrich (St. Louis, MO, USA).

Solvents including ethanol, methanol, ethyl acetate, acetonitrile (CH_3CN), dichloromethane (CH_2Cl_2) and hexane were supplied from RCI Labscan (Bangkok, Thailand).

4.1.2 Equipments

The color of pandan fresh leaves was analyzed using a ColorFlex EZ spectrophotometer from Hunter Associates Laboratory, Inc. (Reston, VA, USA). ALyovac GT2-S-type freeze dryer (lyophilizer) was purchased from GEA freeze-drying equipment (Köln, NW, Germany) for freeze-drying sample. A cyclotex sample mill (series 1903 with 200–240V and 50/60 Hz) was received from FOSS (Höganäs, Skåne, Sweden) for blending samples into small particles. A large volume centrifugation was performed using a ROTINA 38R Centrifuge from Hettich Lab Technology (Tuttlingen, Germany). A small volume sample (microcentrifuge scale) was centrifuged using a tabletop Spectrafuge 16M microcentrifuge from Labnet International, Inc. (Edison, NJ, USA).

The enzymatic assay was performed on a Synergy HT 96-well UV-visible spectrophotometer using Gen5 data analysis software from BioTek Instruments, Inc. (Winooski, VT, USA).

High performance liquid chromatography (HPLC) was consisted of an Agilent 1100 series HPLC with a photodiode array detector and an Eclipase XDB-C18 guard column (4.6 mm x 12.5 mm, 5 μm), which were received from Agilent Technologies (Santa Clara, CA, USA). The column for analyses of phenolic acids and flavonoids was a ZorbaxEclipase XDB-C18 column (4.6 mm x 150 mm, 5 μm) from Agilent Technologies (Santa Clara, CA, USA), while the column for carotenoids analysis was an isocratic reverse phase column, Vydac 201TP54-C18 (4.6 mm x 250 mm, 5 μm) from Grace Davison Discovery Science (Columbia, MD, USA). The PTFE syringe filters (0.2 μm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quantity and quality of chromatogram was analyzed by ChemStation (Agilent Technologies, Santa Clara, CA, USA). Water used in all HPLC experiments was Milli-Q water (18.2 MΩ-cm conductivity). An Evaporator was performed using a N-1200B series rotary evaporator with OSB-2100 water bath were received from EYELA (Tokyo, Japan).

Identification of volatile compounds was performed using a gas chromatography-mass spectrometry (GC-MS). It was consisted of an Agilent 7890A gas chromatograph equipped with a 5975C mass spectrometry inert XL MSD and triple- axis detector form Agilent Technologies (Santa Clara, CA, USA), which were supplied by the Salaya Central Instrument Faculty (SCIF), Mahidol University. Volatile compounds were absorbed on a divinylbenzene/carboxen/polydimethyl-siloxane (DVB/CAR/PDMS) fiber from Sigma-Aldrich (St. Louis, MO, USA) and separated on a HP-5MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm) from Agilent Technologies (Santa Clara, CA, USA). The compounds were identified using NIST library in mass spectrometry from Scientific Instrument Services, Inc. (Ringoes, NJ, USA).

Water extraction of pandan leave tea and pandan leave juice were using with hot plate from Fisher Scientific Isotemp Digital Hotplate Stirrer 11-100-49SH NIB (Grand Blanc, MI, USA) for prepared water extraction. Temperature was measured using DIGICON DP-74SD four-channel thermometer by Sang Chai Meter Co., Ltd. (Bangkok, Thailand). The tea sample was dried in a heating chamber with mechanical convection FED 15 from BINDER, North America (Bohemia, NY, USA). After that, it was blended with Philips blender type HR 2115 600W with 200–240V and 50/60 Hz (Jakarta, Indonesia). Finally, the water activity (aW) of tea sample was analyzed by MS 1 portable water activity meter from Novasina AG (Lachen, Switzerland).

4.1.3 Plant materials

Fresh leaves (50-60 cm length x 3- 4 cm width) of pandan were collected in October, 2013 from 6 regions of Thailand including of Center (Nakhon Pathom), Northwest (Udon Thani), North (Phrae), South (Songkhla), East (Chon Buri) and West (Ratchaburi) regions (Figure 4.1).

Figure 4.1 Color and size of pandan leaves used in this study for analyses of TPCs, antioxidant activities and anti-AD properties. The samples were selected as greenery leaves without their basal leave. The young leave and senescence leave were separated.

4.2 Methods

This research was presented in two sections. Firstly, pandan leaves were studied regarding their *in vitro* biological properties, including TPCs, antioxidant activities (FRAP, DPPH and ORAC) and anti-AD properties (anti-cholinesterase and anti β-secretase). Bioactive compounds were also analyzed and identified using HPLC analyses, GC-MS and HPLC. Secondly, pandan leaves juice and tea were developed and examined *in vitro* biological properties, similarly to the previous part (Figure 4.2).

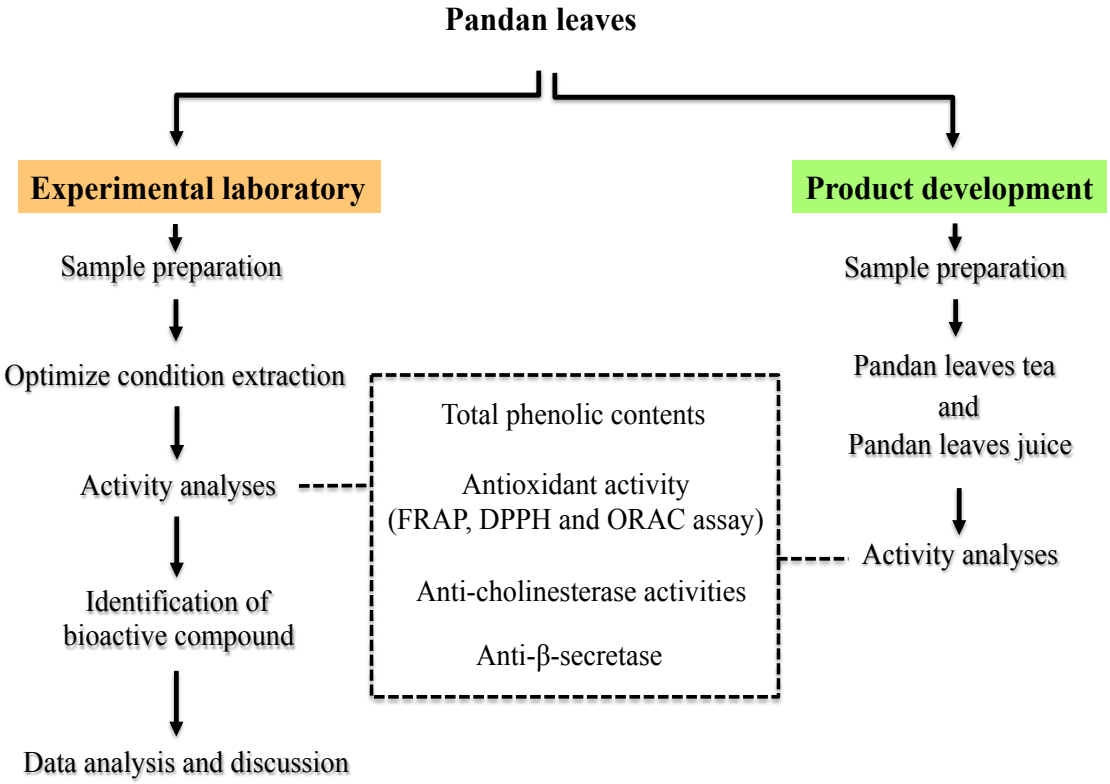

Figure 4.2 The flow chart of overall experiments for laboratory analyses and product development of pandan leaves. The pandan leaves were studied in experimental laboratory and product application. Antioxidant capacities, TPCs, anti-AD properties and bioactive compounds were analyzed.

4.2.1 Sample preparation

Pandan leaves were washed with deionized water (DI) and cut into small pieces (3-4 cm x 0.5 cm). The sample coloring was measured by ColorFlex EZ spectrophotometer (Hunter Associates Laboratory) using C.I.E. LAB (L*, a*, b*) system. Samples were freeze-dried using the freeze-dryer for approx. 40-50 hours and then ground into fine powder using the cyclotex sample mill. All samples were kept in vacuum bag (aluminum foil bag) and stored at -20°C for further analysis.

4.2.2 Moisture content determination

The moisture content of the freeze-dried samples was analyzed by Association of Official Analytical Chemists (AOAC) official method [105]. Hot air

oven was regulated to the temperature of 105 ± 2°C. The samples (approx. 2 g) were spread in a low, covered aluminum dishes (≥50 mm diameter and 40 mm deep) until evenly distributed. The samples were then incubated in the hot air oven for 2 hours before cooling in a desiccator for 1 hour. The dish was reheated in the hot air oven for another 30 minutes, cooled in the desiccator for 30 minutes and weighed again. The procedures were repeated until the difference between present and previous weight of the dish was less than 0.005 g.

$$\% \text{ Moisture content (w/w)} = 100 \times \frac{\text{weight loss on drying (g)}}{\text{weight test portion}}$$

$$\% \text{ Dry matter} = 100 - \% \text{ Moisture content (w/w)}$$

4.2.3 Determination of optimized extraction conditions

The extraction conditions were investigated according to the method of Liu *et al*., 2010 [106] and some adaptations. The experimental factors were consisted of extraction time (15, 30, 60, 120 and 240 minutes, X_1), concentration of ethanol (0, 20, 40, 60, 80 and 100% v/v, X_2) extraction temperature (30, 50, 70 and 90°C, X_3) and solid–to–liquid ratio (1:20, 1:30, 1:40, 1:50 and 1:60 w/v, X_4). Each condition was designed by varying level of factor and fixing residual factors to roughly investigate the optimized level of each condition. The experiments were created using response surface methodology (RSM) with central composite design on a computer program, Minitab® statistical software (version 15.0, Minintab Inc., PA, USA). The supernatant was collected by centrifugation at 2,810 g for 10 minutes, followed by filtration using Whatman No.1 filter paper.

4.2.4 Determination of total phenolic compounds

Total phenolic compounds were determined according to the method of Folin–Ciocalteu method [107]. The samples (25 µL) 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 then incubated at room temperature (25°C) in dark room for 2 hours. The TPCs were measured at a wavelength of 765 nm using the microplate reader. Gallic

acid (10, 20, 40, 60, 80, 100 and 200 µg/mL) was used as the standard. The TPCs were expressed in gallic acid equivalents (GAE) per 1 g dry weight of sample.

4.2.5 Determination of antioxidant capacities

Antioxidant activities were determined using ferric reducing antioxidant power (FRAP), 1,1–diphenyl–2–picrylhydrazyl–radical scavenging (DPPH) radical scavenging and oxygen radical antioxidant capacity (ORAC) assays.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was determined according to the method of Benzie and Strain, 1996 [108] with some modifications. The FRAP reagent containing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM in 40 mM HCl) and $FeCl_3 \cdot 6H_2O$ solution (20 mM) in a ratio of 10:1:1 were warmed at 37°C before use. 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 microplate reader at a wavelength of 595 nm. Trolox (7.8125, 15.625, 31.25, 62.5, 125 and 250 µM), a water-soluble analogue of vitamin E, was used as the standard. The FRAP values were determined using a standard curve of trolox and expressed as trolox equivalence (TE) per 1 g dry weight of sample.

2,2- diphenyl-1-picry hydrazyl radical (DPPH) scavenging assay

The DPPH assay was performed according to the method of Fukumoto and Mazza, 2000 [109] with some modifications as follows. The samples (22 µL) were mixed with DPPH (150 µM) in 95% (v/v) aqueous ethanol and incubated in dark at room temperature (25°C) for 30 minutes. The reaction was determined by measuring the absorbance at 520 nm using the microplate reader. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the equation:

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

where Abs_{sample} is the absorbance at 520 nm of the sample and DPPH reagent, and $Abs_{control}$ is the absorbance at 520 nm of 95% (v/v) aqueous ethanol and DPPH reagent. Trolox (0.04, 0.08, 0.16, 0.32, 0.64 and 1.28 mM) was used as the standard.

The results were determined using a standard curve of trolox and expressed in TE per 1 g dry weight of sample.

Oxygen radical antioxidant capacity (ORAC) assay

The ORAC assay was determined according to the method of Ou *et al.*, 2001 [110] 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 (25 µL) were mixed with fluorescein (40 nM) solution and incubated for 15 minutes at 37°C. After the incubation, AAPH (153 nM), a peroxyl radical generator, was added to the reaction mixture rapidly to start 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) as follows:

$$AUC = (0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_i/f_0) \times CT,$$

where f_0 is the initial fluorescence reading at 0 minute, f_i is the fluorescence reading at time i minutes and CT is cycle time in minutes. Trolox (3.125, 6.25, 12.5, 25, 50 and100 µM) was used as the standard. The results were determined using a standard curve of trolox and expressed as TE per 1 g dry weight of sample.

4.2.6 Determination of cholinesterase inhibitory activity

The determination of cholinesterase consisted of two colorimetric assays of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Both assays were employed to determine the termination of physiological role of cholinergic synapses, one of the hypotheses for AD development.

Acetylcholinesterase inhibitory activity

The analysis of acetylcholinesterase activity was adapted from the method of Jung *et al.*, 2009 [22]. Enzyme assay was consisted of AChE (10 ng), ACh (0.08 mM) and DTNB (0.8 mM) in KPB (50 mM, pH 7.0). Enzyme inhibitory activity was spectrophotometrically measured at a wavelength of 412 nm using the 96-well

microplate reader. The inhibitory activity of plant extracts was calculated as percentage of inhibition using the equation:

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

where *A* is an initial velocity of the reaction with enzyme, *a* is an initial velocity of the reaction without enzyme, *B* is an initial velocity of the enzyme reaction with extract, and *b* is an initial velocity of the reaction with extract but without enzyme.

Butyrylcholinesterase inhibitory activity

The analysis of butyrylcholinesterase activity was adapted from the method of Jung *et al.*, 2009 [22]. Enzyme assay was consisted of BChE (50 ng) in KPB (50 mM, pH 7.0) containing $MgCl_2$ (1 mM), BCh (0.1 mM) in KPB (50 mM, pH 7.0) and DTNB (0.8 mM). Enzyme inhibitory activity was spectrophotometrically measured at a wavelength of 412 nm using the 96-well microplate reader. The inhibitory activity of plant extracts was calculated as percentage of inhibition as above.

4.2.7 Determination of β-secretase inhibitory activity

The BACE1 inhibitory activity was measured using a BACE1 (β−secretase) FRET assay kit [111], which consists of BACE1 (β−secretase) enzyme, 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 the 96–well microplate and monitored at excitation wavelength of 545 nm and emission wavelength of 585 nm using the microplate reader. The inhibitory activity was reported as percentage of inhibition as above.

4.2.8 Identification of volatile and bioactive compounds

Bioactive compounds including volatile compounds, carotenoids, phenolic acids and flavonoids of pandan leaves were analyzed. Volatile compounds were analyzed using gas chromatography-mass spectrometry (GC-MS), while phenolic

acids flavonoids and carotenoids, were determined using high performance liquid chromatography (HPLC).

Volatile compounds

Volatile compounds were analyzed, according to the procedure of Azar *et al.*, 2011 [112]. Fresh leaves of pandan were washed with DI water and cut into small piece (0.5 cm x 2-3 cm). The sample (1.5 g) in a vial was incubated in air bath at 70°C for 30 minutes. The volatile compounds were absorbed on DVB/CAR/PDMS fiber for 20 minutes and separated on a HP-5MS capillary column (30 m x 0.25 mm with a phase thickness of 0.25 μm). The oven temperature was set at 60°C for 3 minutes. The ramp rate was 6°C per minutes. Once it reached 250°C, the temperature was held constant for 3 minutes. The carrier gas, helium (99.999 %), was set with a flow rate of1 mL per minutes. The temperature insplit/splitless injector was set at 250°C. The mass spectrometer was operated in the electron-impact mode (EI) at 70 eV. The compounds were identified using NIST library in mass spectrometry.

Phenolic acids and Flavonoids

Sample extraction was performed using the method of Judprasong et al., 2013 [113]. Freeze-dried samples (0.5 g) were extracted with 62.5% (v/v) aqueous methanol (40 mL) containing 0.5g/L tBHQ. To the extractant, 6 N HCl (10 mL) was added, and the samples were shaken in a water bath at 90°C for 2 hours and then cooled in ice for 5 minutes. Then, 1% (v/v) ascorbic acid (100 μL) was added to the mixture and mixed well. The volume was made up to 50 mL by adding methanol. The extract was then sonicated in ultrasonic bath for 5 minutes and then filtered through 0.2 μm PTFE syringe filter (~1-2 mL). The HPLC analysis was utilized by the ZorbaxEclipase XDB-C18 column with a constant flow rate of 0.6 mL/min at ambient temperature. The gradient mobile phase consisted of Milli-Q water containing 0.05% (w/w) TFA (Solvent A), methanol containing 0.05% (w/w) TFA (Solvent B) and acetronitrile containing 0.05% (w/w) TFA (Solvent C). Samples were kept in the autosampler at 4°C until injection (10 μL). The contents of phenolic acids and flavonoids were detected at 325 and 338 nm and calculated using ChemStation. Phenolic acids (gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, syringic acid,

vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid and *t*-cinnamic acid) and flavonoids (quercetin, kaempferol, isorhamnetin, myricetin, apigenin, luteolin, naringenin, and hesperetin) and were used as standards to identify phenolic acids and flavonoids of sample by comparing retention time and particular spectral fingerprint. Concentration of phenolic acids and flavonoids were expressed as μg/g of sample (dry weight).

Carotenoids

The determination of carotenoid content was performed using the method of Judprasong *et al*., 2013 [113]. Freeze-dried samples (0.2 g) were extracted by boiling in 2N ethanolic potassium hydroxide (KOH) (50 mL) with 10% (w/v) ascorbic acid for 30 minutes. Hexane (70 mL) was then added to extracted sample, and the mixture was shaken for 2 min at room temperature. The solvent was removed by evaporation, and residue was re-dissolved in a mobile phase solvent (acetronitrile:methanol:dichloromethane (80:11:9 v/v/v) with 0.01% (v/v) triethylamine and 0.01% (w/v) ammonium acetate) (3 mL). The extract was filtrated through 0.2 μm PTFE syringe filter before being analyzed by HPLC. The HPLC was utilized by the Vydac 201TP54-C18 column with a constant flow rate of 0.7 mL/min at ambient temperature. The quality and quantity of chromatogram were analyzed at 450 nm by ChemStation. Carotenoids including capsantin, lutein, zeathantin, β-cryptoxanthin, lycopene, α-carotene, trans-β-carotene and cis-β-carotene were used as standards. The concentration of carotenoids was showed as μg/g of sample (dry weight).

4.2.9 Determination of pandan leave tea and pandan leave juice

Tea from pandan leave was prepared using fresh pandan leaves, which were cut into small pieces (2-3 cm x 0.5 cm). The samples were dried in a hot air oven at 100°C for 30 minutes. The dry samples were ground into fine powder by a blender and kept in a tea bag (1 g dried weight/bag, particle size of >0.42 mm). Two types of water were chosen for extraction, DI water and reverse osmosis water (RO), which was received from Singha® drinking water (100 mL). The tea was extracted at 95°C for 10 minutes before analysis [114, 115]. The extractant was filtered through

Whatman No. 1 filter paper. TPCs, antioxidant and anti-cholinesterase activities were then analyzed.

Likewise, juice from pandan leave was prepared using fresh pandan leaves, which were cut into small piece (2-3 cm x 0.5 cm). The sample was boiled for 10 minutes with DI and RO water. The juice was filtered through Whatman No. 1 filter paper. TPCs, antioxidant and anti-cholinesterase activities were then analyzed.

4.3 Statistical Analysis

All experiments were carried out in triplicate. The data was 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 on SPSS software were performed to determine the significant differences between values. Significance of difference was defined at $p < 0.05$.