CHAPTER V
RESULTS

5.1 Sample Preparation

The sample coloring was measured using C.I.E. LAB (L*, a*, b*) system. The C.I.E. LAB values of pandan leaves used in this experiment were 31.18 ± 1.25 of lightness (L*), -9.89 ± 0.25 of red/green chromaticity (a*) and 22.60 ± 1.12 of blue/yellow chromaticity (b*). The percentage of moisture and dry mass were then calculated for each sample set (Table 5.1).

Table 5.1 Percentage of moisture content of pandan leaves. The moisture contents of pandan leaves in experiment laboratory and product application using AOAC official method [105]

Method	Types	Regions	% Moisture content
Experiment laboratory	Freeze-dried leaves	Central	7.73 ± 0.09
		Northeast	6.72 ± 0.08
		North	6.71 ± 0.15
		South	7.91 ± 0.11
		East	6.54 ± 0.15
		West	7.74 ± 0.19
Product application	Fresh leaves	Central	82.96 ± 0.78
	Dried leaves	Central	3.57 ± 0.24

Each value was represented as mean ± SD (n = 3).

5.2 Optimum Extraction Condition

The extraction conditions of pandan leaves were optimized regarding the antioxidant and anti-AD properties. The extraction factors were varied, including extraction time (15, 30, 60, 120 and 240 minutes), solvent (0, 20, 40, 60, 80 and 100% v/v aqueous ethanol), extraction temperature (30, 50, 70 and 90°C) and solid-to-liquid ratio (1:20, 1:30, 1:40, 1:50 and 1:60 w/v). The extracts were then analyzed regarding

the biochemical properties against AD based on hypotheses of oxidative stress induction, cholinergic termination and β-amyloid formation.

5.2.1 Extraction conditions for antioxidant and anti-AD properties

The optimum extraction condition is currently of interest because high yields of bioactive compounds were industrially needed. Its benefits include time saving, cost effectiveness and labor reduction. Thus, the optimum extraction condition plays an important role in production process. There are many methodologies for analysis of antioxidant activities such as total radical trapping antioxidant parameter (TRAP), ORAC, DPPH radical scavenging and FRAP assays. This study used FRAP assay as a preliminary measurement to determine the optimized extraction condition, because this method is simple, fast and economically effective.

As results, it was found that solvent systems and solid-to-liquid ratios significantly affected FRAP values, in which 80% (v/v) aqueous ethanol and 1:60 (w/v) provided the highest antioxidant activities (Table 5.2). On the other hand, FRAP values of samples extracted from various extraction times and temperatures were insignificantly different (Table 5.2). Thus, only two factors, solvent system and solid-to-liquid ratio, were further investigated using RSM, while extraction time and extraction temperature were fixed at 15 minutes and 30°C, respectively.

Table 5.2 The FRAP values of pandan leaves extracted under various extraction conditions

Extraction conditions of pandan leaves				FRAP values
Time (minutes)	**Ethanol (% v/v)**	**Temperature (°C)**	**Solid-to-liquid ratio (% w/v)**	**(µmol TE/g dry weight)**
15	40	50	1:40	10.95 ± 0.44 [a]
30				11.52 ± 0.56 [a]
60				12.17 ± 0.94 [a]
120				11.34 ± 0.39 [a]
240				12.49 ± 0.51 [a]
15	0	50	1:40	9.63 ± 0.91 [e]
	20			10.58 ± 0.47 [d]
	40			13.42 ± 0.19 [c]
	60			17.23 ± 0.45 [b]
	80			19.36 ± 0.27 [a]
	100			9.54 ± 0.17 [e]
15	80	30	1:40	19.65 ± 1.17 [a]
		50		21.37 ± 0.68 [a]
		70		20.30 ± 0.49 [a]
		90		20.98 ± 0.66 [a]
15	80	30	1:20	16.02 ± 0.19 [d]
			1:30	18.20 ± 0.48 [c]
			1:40	20.20 ± 0.05 [b]
			1:50	20.74 ± 0.70 [b]
			1:60	23.16 ± 0.23 [a]

Each value was represented as mean ± SD (n = 3). The different superscript letters within same column in each tested conditions showed the significantly difference at p-value < 0.05 using one-way ANOVA followed by Tukey's-b *post hoc* test.
TE: Trolox equivalent

Besides, two cholinesterase enzymes, AChE and BChE, are indicators of AD occurrence. Cholinesterase inhibition was used as a preliminary measurement to determine the optimum extraction conditions for anti-cholinesterase agents. The extraction conditions including extraction times, solvent, extraction temperatures and solid-to-liquid ratios were varied. The results suggested that inhibitory activities were significantly different in samples extracted with various extraction solvents and solid-to-liquid ratios (Table 5.3). The highest percentage of inhibition was found in samples extracted with absolute ethanol and 1:60 w/v solid-to-liquid ratio. Moreover, the samples extracted with various extraction temperatures exhibited significantly lowest

inhibition under high temperature (90°C). The inhibitory activities were, however, insignificantly different under various extraction times.

Table 5.3 The percentage of inhibitory enzyme activity in pandan leaves extracted with various extraction conditions

Extraction conditions of pandan leaves				% Inhibition of enzyme activity	
Time (minutes)	**Ethanol (% v/v)**	**Temperature (°C)**	**Solid-to-liquid ratio (% w/v)**	**AChE**	**BChE**
15	40	50	1:40	62.63 ± 0.50 [a]	75.56 ± 1.35 [a]
30				63.28 ± 0.28 [a]	75.73 ± 1.38 [a]
60				61.54 ± 0.61 [a]	74.12 ± 2.59 [a]
120				63.61 ± 0.74 [a]	74.77 ± 1.17 [a]
240				62.95 ± 0.28 [a]	74.39 ± 1.54 [a]
15	0	50	1:40	21.28 ± 0.40 [a]	27.73 ± 0.26 [a]
	20			15.10 ± 0.53 [b]	15.41 ± 0.13 [b]
	40			12.17 ± 0.80 [c]	13.27 ± 0.80 [b]
	60			12.03 ± 0.89 [c]	13.01 ± 1.18 [b]
	80			10.48 ± 0.57 [c]	6.26 ± 0.30 [c]
	100			2.75 ± 1.77 [d]	2.39 ± 1.13 [d]
15	0	30	1:40	59.11 ± 1.95 [a]	57.60 ± 1.79 [a]
		50		59.19 ± 1.05 [a]	59.24 ± 1.82 [a]
		70		60.38 ± 0.53 [a]	58.63 ± 1.05 [a]
		90		53.57 ± 1.44 [b]	48.21 ± 1.92 [b]
15	0	30	1:20	84.01 ± 0.90 [a]	89.74 ± 0.68 [a]
			1:30	79.58 ± 0.88 [b]	85.17 ± 1.60 [b]
			1:40	73.59 ± 0.61 [c]	80.91 ± 1.90 [c]
			1:50	71.02 ± 1.24 [d]	75.25 ± 1.75 [d]
			1:60	67.58 ± 1.08 [e]	72.39 ± 1.36 [e]

Each value was represented as mean ± SD (n = 3). The different superscript letters within same column in each tested conditions showed the significantly difference at p-value < 0.05 using one-way ANOVA followed by Tukey's-b *post hoc* test.
The concentrations of pandan leaves extracts in enzyme assays including time, solvent, temperature and solid-to-liquid ratio were 10, 1, 2 and 10 g dry weight/L, respectively.

Since only one factor, solid-to-liquid ratio, was significantly affected anti-cholinesterase activities, further investigation in extraction of anti-cholinesterase agents using RSM was unnecessary. Therefore, the extraction conditions in this part of the experiments were included (1) 15 minutes of extraction time, (2) 0% v/v aqueous

ethanol of solvent system, (3) 30°C of extraction temperature and (4) 1:20 w/v solid-to-liquid ratio.

5.2.2 Experimental design using response surface methodology

RSM is used to determine the optimal extraction conditions in this experiment. The extraction conditions for antioxidants were focused on extraction time (X_1), solvent systems (X_2), extraction temperature (X_3) and solid-to-liquid ratios (X_4). Two factors including solvent systems and ratios were varied, while extraction time and extraction temperature were fixed at 15 minutes and 30°C, respectively.

The RSM extraction condition was created with central composite design by Minitab software. The conditions were set as coded levels (-1.5, -1, 0, +1 and +1.5), leading to interpretation of unfixed independent (uncoded) variables (65, 70, 80, 90 and 95% v/v aqueous ethanol of solvent system and 1:35, 1:40, 1:50, 1:60 and 1:65 w/v of solid-to-liquid ratios) (Table 5.4). According to these independent variables including varied and fixed factors, 14 extraction conditions were designed (Table 5.5). As results, it was showed that the highest FRAP value (29 µmol TE/g dry weight) was detected under the extraction conditions of 15 minutes of extraction times (X_1), 80% (v/v) aqueous ethanol of extraction solvent (X_2), 30°C of extraction temperature (X_3) and 1:65 w/v solid-to-liquid ratio (X_4).

Table 5.4 Coded and uncoded levels of independent variables used to found optimum extraction condition

Independent variables	Symbols	Coded levels and uncoded levels				
		-1.5	**-1**	**0**	**+1**	**+1.5**
Solvent systems (% aqueous ethanol)	X_2	65	70	80	90	95
Solid-to-liquid ratio (w/v)	X_4	1:35	1:40	1:50	1:60	1:65

Table 5.5 Experimental designs using Center Composite Design and values of the observed response (FRAP values)

Run no.	Variable (coded and uncoded level)				FRAP values (µmol TE per g dry weight)
	X_1	X_2	X_3	X_4	
	Extraction time (minutes)	Solvent systems extraction (%)	Extraction temperature (°C)	Solid-to-liquid ratio (w/v)	
1	15	65 (-1.5)	30	1:50 (0)	24.59 ± 0.37
2	15	70 (-1)	30	1:40 (-1)	24.30 ± 0.32
3	15	70 (-1)	30	1:60 (1)	28.25 ± 0.56
4	15	80 (0)	30	1:35 (-1.5)	25.04 ± 0.32
5	15	80 (0)	30	1:50 (0)	27.99 ± 0.38
6	15	80 (0)	30	1:50 (0)	28.19 ± 0.36
7	15	80 (0)	30	1:50 (0)	27.44 ± 0.38
8	15	80 (0)	30	1:50 (0)	28.08 ± 0.67
9	15	80 (0)	30	1:50 (0)	28.34 ± 0.81
10	15	80 (0)	30	1:50 (0)	27.76 ± 0.02
11	15	80 (0)	30	1:65 (1.5)	29.00 ± 0.34
12	15	90 (1)	30	1:40 (-1)	23.31 ± 0.25
13	15	90 (1)	30	1:60 (1)	24.87 ± 0.27
14	15	95 (1.5)	30	1:50 (0)	20.94 ± 0.24

The FRAP values were expressed as mean ± SD (n=3).

The Minitab software then integrated the response values from Table 5.5 and interpreted into Table 5.6. The result showed that the regression equation was significantly exhibited all variable factors (p<0.05), suggesting that all factors including X_2, X_4, X_2X_2, X_4X_4 and X_2X_4 had potential effect on FRAP value. By applying multiple regression analysis on the experimental data, the relationship between factors of interest and the response can be written as a second-order polynomial equation:

$Y = 27.99 - 1.68\ X_2 + 1.96\ X_4 - 5.08\ X_2^2 - 0.83\ X_4^2 - 1.20\ X_2X_4$ ***equation (1)***

The analysis of variance (ANOVA) showed degree of freedom, sum of square, f-value and p-value, which calculated from the Minitab software. As results, the lack of fit used to check fitness and adequacy of the regression model. The p-value of the lack-of-fit was 0.32, which is not significantly difference (p>0.05). It is indicated that the model can adequately fit the experiment data. The satisfactory coefficient of determination (R^2) is defined as ratio of the explained variation to the total variation. This value provides a correlation measure for testing the goodness-of-fit of the regression equation. R^2 was found to be 0.96 in this experiment, suggesting a

high degree of correlation between the observed and predicted values, therefore the model is suitable.

Table 5.6 Analysis of variance (ANOVA) for the regression equation

SD	DF	SS	F-value	S
Model	5	228.54	221.83	*
X_2	1	34.03	165.18	*
X_4	1	46.27	224.55	*
X_2^2	1	140.16	693.45	*
X_4^2	1	3.78	18.32	*
X_2X_4	1	4.30	20.88	*
Lack of fit	3	0.74	1.22	

SD: Source of deviation; DF: Degree of freedom; SS: sum of squares; S: Significant (*: p-value < 0.05)

The three-dimensional response surface plot and two-dimensional contour plot indicated that increasing solid-to-liquid ratios could enhance FRAP values to reach its maximum point at a certain level (Figure 5.1). However, increasing ethanol concentration exhibited initial enhancement of FRAP values, which were then decreased when ethanol concentration continued to elevate (Figure 5.1). The extraction condition was optimized at 15 minutes of extraction time, 75% (v/v) aqueous ethanol of solvent system, 30°C of extraction temperature and 1:65 w/v solid-to-liquid ratio. To confirm these results, three triplicate tests were performed under optimized conditions, and the FRAP value was found to be 29.00 μmol TE/g dry weight. The prediction of FRAP value using equation (1) was 29.54 μmol TE/g dry weight, which clearly showed that the designed model fitted perfectly with experimental data. Moreover, the desirable value was 1.00, suggesting that the setting method was favorable for all responses values. Therefore, these optimum extraction conditions could be further employed for extraction of antioxidants from pandan leaves.

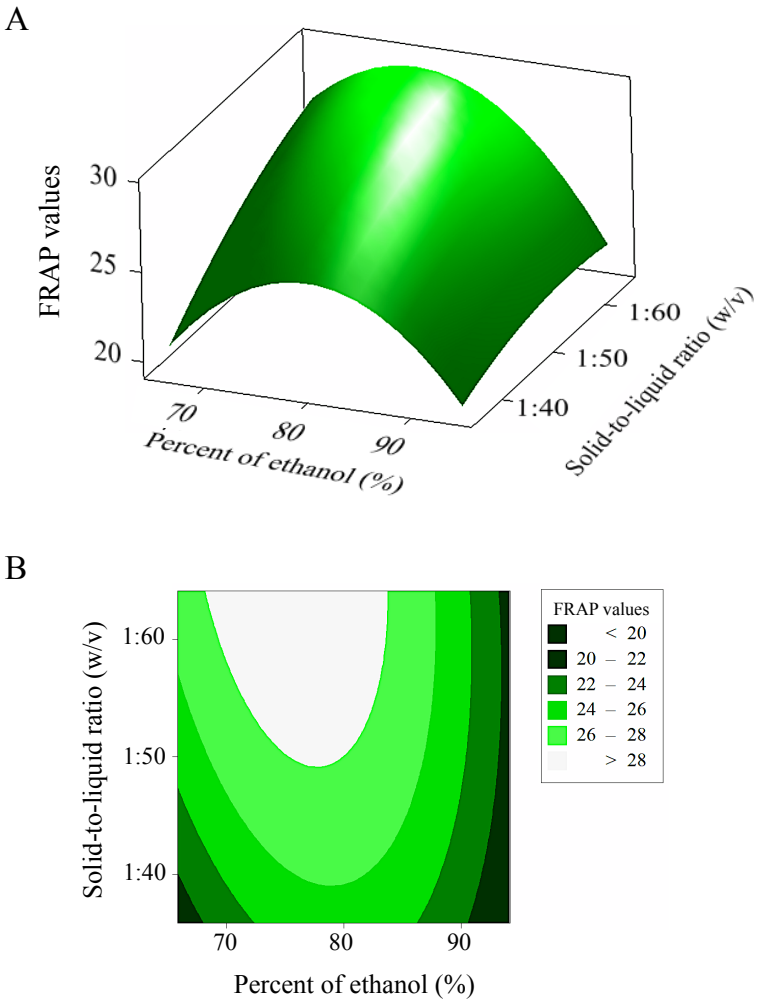

Figure 5.1 (A) Three-dimensional response surface plot and (B) two-dimensional contour plot of independent variables interaction. The interaction of solvent system and solid-to-liquid ratio for extraction of antioxidants from pandan leaves was presented as FRAP values.

5.3 Antioxidant and Anti-Alzheimer's Activities of Pandan Leaves Extracts

The samples of pandan leaves were analyzed regarding the biochemical properties against AD based on hypotheses of oxidative stress induction, cholinergic termination and β-amyloid formation. The optimum extraction conditions, 15 minutes of extraction time, 75% (v/v) aqueous ethanol of extraction solvent, 30°C of extraction temperature and 1:64 w/v solid-to-liquid ratio, were used to determine antioxidant

activities, including TPCs, FRAP, DPPH radical scavenging and ORAC assays. Likewise, the extraction conditions of 15 minutes of extraction time, 0% (v/v) aqueous ethanol of solvent system, 30°C of extraction temperature and 1:20 w/v solid-to-liquid ratio was used to determine cholinesterase inhibitory activities. Finally, the optimal extraction conditions of both hypotheses were used to analyze anti-BACE1 activities.

5.3.1 Variations of particle sizes and cultivated locations

Freeze-dried samples of pandan leaves were blended with the cyclotex sample mill. The blended samples were then separated according to their sizes. The particle sizes were divided into 4 groups, including group 1 (particle size >0.42 mm), group 2 of (particle sizes of 0.42-0.177mm), group 3 (particle sizes of 0.177-0.125 mm) and group 4 (particle sizes <0.125 mm). All samples were extracted under optimum extractions conditions and then analyzed antioxidant activities and enzymatic assays. As results, it was found that samples with small particle sizes (group 3 and group 4) exhibited significantly higher TPCs and antioxidant activities than the ones with large particle sizes (group 1 and group 2) as shown in Table 5.7. The highest TPC was 9.79 mg GAE/g dry weight, while the highest antioxidant activities were 27.00 μmol TE/g dry weight, 12.40 μmol TE/g dry weight and 538.53 μmol TE/g dry weight as being detected by FRAP, DPPH radical scavenging and ORAC assays, respectively. Besides, it was found that the samples (10 g dry weight/L) in group 2, 3 and 4 exhibited significantly higher anti-cholinesterase activities (AChE and BChE) than the sample in group 1 (Table 5.7). The results showed that the highest AChE and BChE inhibitory activities were 52.29% and 61.26% inhibition, respectively.

On the other hand, the different cultivated locations of pandan leaves were obtained form 6 regions of Thailand, including Central (Nakhon Pathom), Northeast (Udon Thani), North (Phrae), South (Songkhla), East (Chon Buri) and West (Ratchaburi). The samples were sieved, and the samples with particle size less than 0.177 mm (group 3 and group 4) were chosen. The samples form different cultivated locations were analyzed regarding their antioxidant activities. The results suggested that pandan leaves from the South provided the highest antioxidant activities (TPCs of 10.32 mg GAE/g dry weight, FRAP values of 33.32 μmol TE/g dry weight, DPPH

radical scavenging values of 13.17 μmol TE/g dry weight and ORAC values of 548.23 μmol TE/g dry weight) (Table 5.8).

Likewise, anti-cholinesterase activity was analyzed using pandan leaves from different cultivated locations. The results indicated that the sample from the North provided the highest inhibitory activities (84.71%, 97.17% and 97.57% inhibition for AChE and BChE respectively). Besides, the half maximal inhibitory concentration (IC_{50}) of pandan leaves from different cultivated locations confirmed that the sample from the North provided the highest cholinesterase inhibition (IC_{50} of 0.86 and 0.76 g dry weight/L for anti-AChE and anti-BChE activities, respectively) as shown in Table 5.9. Moreover, the samples from South and North regions, which provided the highest TPCs, antioxidant activities and anti-cholinesterase, were chosen for analysis of anti-BACE1 activities. The samples (1 g dry weight/L of South region and 2 g dry weight/L of North region) from the South and the North exhibited 18.25% and 97.57% BACE1 inhibition, respectively (Table 5.8 and Table 5.9).

Table 5.7 The different particle sizes of pandan leaves extracts analyzed with total phenolic contents (TPCs), antioxidant activities (FRAP, DPPH and ORAC methods) and enzymatic assays (AChE and BChE)

Groups	Particle sizes (mm)	Percentage of amount (%)	Folin-Ciocalteu assay (mg GAE/g dry weight)	Antioxidant activities (µmol TE/g dry weight))			% Inhibition	
				FRAP	DPPH	ORAC	AChE	BChE
1	x > 0.42	5.84	6.94 ± 0.17 [c]	19.93 ± 0.65 [c]	9.89 ± 0.52 [b]	345.55 ± 1.80 [b]	48.05 ± 1.65 [b]	53.28 ± 0.64 [b]
2	0.42 > x > 0.18	32.86	7.96 ± 0.41 [b]	22.40 ± 0.85 [b]	10.13 ± 0.10 [b]	367.31 ± 15.52 [b]	51.28 ± 2.47 [a]	58.47 ± 1.79 [a]
3	0.18 > x > 0.13	21.84	9.79 ± 0.16 [a]	27.00 ± 1.36 [a]	12.40 ± 0.03 [a]	480.10 ± 12.62 [a]	52.29 ± 3.61 [a]	59.39 ± 1.17 [a]
4	x < 0.13	39.46	9.77 ± 0.31 [a]	26.88 ± 0.82 [a]	11.47 ± 0.45 [a]	538.53 ± 38.96 [a]	52.09 ± 1.31 [a]	61.26 ± 1.49 [a]

Each value was represented as mean ± SD (n = 3). Mean within a column in each tested conditions were shown with difference superscript letters, which were significantly different ($p < 0.05$) using one-way ANOVA followed by Tukey's-b *post hoc* test.
The concentration of pandan leaves extracts in enzyme assays was 10 g dry weight/L.
x: Particle sizes of sample, GAE: Gallic acid equivalent, TE: Trolox equivalent

Table 5.8 Total phenolic contents (TPCs), antioxidant activities and BACE1 inhibitory enzyme in different cultivated location of pandan leaves extracts

Cultivated locations	Folin-Ciocalteu assay (mg GAE/g dry weight)	Antioxidant activities (µmol TE/g dry weight))			BACE1 % Inhibition
		FRAP	DPPH	ORAC	
Central (Nakhon Pathom)	9.60 ± 0.31 [b]	28.01 ± 0.27 [b]	9.95 ± 0.17 [c]	503.53 ± 19.24 [ab]	NA
Northeast (Udon Thani)	7.61 ± 0.02 [e]	19.83 ± 0.09 [e]	9.10 ± 0.09 [cd]	296.87 ± 10.17 [c]	NA
North (Phrae)	6.20 ± 0.02 [f]	15.32 ± 0.17 [f]	8.78 ± 0.19 [d]	267.86 ± 32.83 [c]	NA
South (Songkhla)	10.32 ± 0.35 [a]	33.32 ± 1.08 [a]	13.17 ± 0.73 [a]	548.23 ± 52.71 [a]	18.25 ± 2.47
East (Chon Buri)	9.07 ± 0.02 [c]	25.36 ± 0.58 [c]	12.12 ± 0.12 [b]	434.31 ± 42.58 [b]	NA
West (Ratchaburi)	8.16 ± 0.20 [d]	22.04 ± 0.15 [d]	13.14 ± 0.59 [ab]	405.80 ± 39.21 [b]	NA

Each value was represented as mean ± SD (n = 3). Mean within a column in each tested conditions was shown with difference superscript letters, which were significantly different ($p < 0.05$) using one-way ANOVA followed by Tukey's-b *post hoc* test.
GAE: Gallic acid equivalent, TE: Trolox equivalent
The concentration of pandan leaves extracts in BACE1 assay was 1 g dry weight/L.
NA: Not assessable

Table 5.9 The enzyme assays including AChE, BChE and BACE1 inhibitory enzyme activities and IC_{50} of AChE and BChE in different cultivated locations of pandan leaves extracts

Cultivated locations	Enzymes assays				
	AChE		BChE		BACE1
	% Inhibition	IC_{50} (g dry weight/L)	% Inhibition	IC_{50} (g dry weight/L)	% Inhibition
Central (Nakhon Pathom)	73.30 ± 0.53 ^{c}	1.15 ± 0.04 ^{b}	91.93 ± 2.16 ^{c}	0.99 ± 0.08 ^{ab}	NA
Northeast (Udon Thani)	78.67 ± 0.49 ^{b}	1.12 ± 0.05 ^{b}	93.73 ± 1.42 ^{bc}	1.47 ± 0.13 ^{c}	NA
North (Phrae)	84.71 ± 0.96 ^{a}	0.76 ± 0.02 ^{a}	97.17 ± 0.65 ^{a}	0.86 ± 0.07 ^{ab}	97.57 ± 1.65
South (Songkhla)	79.15 ± 1.09 ^{b}	1.12 ± 0.04 ^{b}	95.68 ± 1.48 ^{ab}	0.80 ± 0.02 ^{a}	NA
East (Chon Buri)	78.59 ± 0.58 ^{b}	1.83 ± 0.10 ^{d}	93.80 ± 2.39 ^{bc}	1.01 ± 0.10 ^{ab}	NA
West (Ratchaburi)	77.79 ± 0.85 ^{b}	1.32 ± 0.05 ^{c}	91.68 ± 2.69 ^{c}	1.10 ± 0.13 ^{b}	NA

Each value was represented as mean ± SD (n = 3). Mean within a column in each tested conditions was shown with difference superscript letters, which were significantly different ($p < 0.05$) using one-way ANOVA followed by Tukey's-b *post hoc* test.
The concentration of pandan leaves extracts in enzyme assays (AChE, BChE and BACE1) were 10, 10 and 2 g dry weight/L, respectively.
NA: Not assessable

5.3.2 Product applications of pandan leaves

Dried and fresh pandan leaves were prepared for tea and juice, respectively. The samples were extracted with DI and RO water to investigate the effect of water types on antioxidant and anti-cholinesterase activities. As results, tea from pandan leaves extracted with DI water provided the highest TPCs and antioxidant activities (TPCs of 6.59 mg GAE/g dry weight, FRAP values of 13.19 μmol TE/g dry weight, DPPH radical scavenging values of 17.48 μmol TE/g dry weight and ORAC values of 256.33 μmol TE/g dry weight), followed by tea extracted with RO water, juice extracted with DI water and juice extracted with RO water, respectively (Table 5.10). Besides, pandan tea extracted with DI water also exhibited the highest percentage of AChE and BChE inhibition (62.56% and 57.56% inhibition, respectively). Juice from pandan leaves extracted with RO water; however, exhibited the lowest inhibition of both enzymes.

Table 5.10 The pandan leaves tea and pandan leaves juice were analyzed with total phenolic contents (TPCs), antioxidant activities (FRAP, DPPH and ORAC methods) and enzyme assays (AChE and BchE)

Types	Solvent extracts	Folin-Ciocalteu assay (mg GAE/g dry weight)	Antioxidant activities (µmol TE/g dry weight))			% Inhibition	
			FRAP	DPPH	ORAC	AChE	BChE
Pandan leave tea	DI	6.59 ± 0.45 [a]	13.19 ± 0.86 [a]	17.48 ± 0.74 [a]	256.33 ± 17.76 [a]	62.56 ± 1.54 [a]	57.56 ± 1.49 [a]
	RO	5.55 ± 0.41 [b]	10.45 ± 0.64 [b]	7.24 ± 0.52 [b]	244.21 ± 21.52 [a]	61.38 ± 2.40 [a]	43.42 ± 1.48 [b]
Pandan leave juice	DI	1.63 ± 0.02 [c]	4.50 ± 0.13 [c]	4.34 ± 0.17 [c]	70.19 ± 4.88 [b]	60.82 ± 1.02 [a]	56.33 ± 1.76 [a]
	RO	1.21 ± 0.02 [c]	3.06 ± 0.15 [d]	1.14 ± 0.06 [d]	69.08 ± 3.79 [b]	24.27 ± 3.20 [b]	24.58 ± 2.49 [c]

Each value was represented as mean ± SD (n = 3). Mean within a column in each tested conditions was shown with difference superscript letters, which were significantly different ($p < 0.05$) using one-way ANOVA followed by Tukey's-b *post hoc* test.
GAE: Gallic acid equivalent, TE: Trolox equivalent
The concentration of pandan leaves extracts in enzymatic assays were 2 g dry weight/L.

5.4 Phytochemicals of Pandan Leaves

Pandan leaves were investigated regarding their phytochemicals (volatile compounds, phenolic acids, flavonoids and carotenoids). Phenolic acids, flavonoids and carotenoids were detected using HPLC analysis, while volatile compounds were detected by GC-MS analysis.

5.4.1 Volatile compounds

Based on our results, the volatile compounds of pandan leaves exhibited approximately 46.33% peak area, while other compounds were mainly from contaminants by environment and instruments (Table 5.11). Pandan leave exhibited twenty-two volatile compounds including isopropenyl methyl ketone, isoxazole, fluoroacetamide, hexanal, 5-amino-1-ethylpyrazole, benzaldehyde, 1-octen-3-ol, 3-methyl-2(5H)-furanone, octanol, decanal, 2-acetyl-1H-pyrrole, 1-octanal, nonanal, 4-aminophenol, dodecane, cis-pinane, 2-chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl) pyrimidine, ethyl-4-nitrobenzoate, tetradecane, 1-propylpentachlorotriphosphazene, phytol and 7-chloro-2,3-dihydro-3-(4-N,N-dimethylamino benzylidene)-5-phenyl-1H-1,4-benzodiazepin-2-one. The major volatile compound was nonanal, which showed 12.91 % peak area. Some volatile compounds in pandan leave including hexanal, benzaldehyde, 1-octanal, nonanal, dodecane and phytol were reported in the previous studies [87, 89, 90]. However, the principle volatile compound in pandan leave, 2-AP, was not found in this study.

Table 5.11 Volatile compounds of pandan fresh leaves

No.	Volatile compounds	RT	peak area ± SD (1,000,000)	percentage of total
1	Isopropenyl methyl ketone	2.10	6.08 ± 1.43	0.54
2	Isoxazole	2.16	9.64 ± 1.54	0.85
3	Fluoroacetamide	2.33	75.27 ± 13.44	6.48
4	Hexanal*	3.34	4.92 ± 1.28	0.46
5	5-Amino-1-ethylpyrazole	5.82	63.64 ± 0.12	5.53
6	Benzaldehyde*	6.81	23.17 ± 0.27	2.02
7	1-Octen-3-ol	7.26	12.52 ± 0.71	1.10
8	3-Methyl-2(5H)-furanone*	7.37	13.99 ± 1.07	1.22
9	Octanol	7.92	59.85 ± 0.57	5.20
10	2-Acetyl-1H-pyrrole	9.58	6.01 ± 0.34	0.48
11	1-Octanal*	9.75	18.74 ± 0.60	1.63
12	Nonanal*	10.70	148.28 ± 4.46	12.91
13	4-Aminophenol	11.15	6.65 ± 0.37	0.53
14	Dodecane*	13.19	6.04 ± 0.13	0.53
15	Decanal	13.36	5.95 ± 1.18	0.52
16	2-Chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl)pyrimidine	14.54	13.70 ± 2.21	1.18
17	Ethyl-4-nitrobenzoate	15.55	14.49 ± 1.85	1.27
18	Tetradecane	18.06	7.71 ± 1.80	0.66
19	1-Propylpentachlorotriphosphazene	18.23	9.46 ± 0.88	0.84
20	7-Chloro-2,3-dihydro-3-(4-N,N-dimethylaminobenzylidene)-5-phenyl-1H-1,4-benzodiazepin-2-one	21.76	8.79 ± 0.40	0.82
21	cis-Pinane	27.02	6.79 ± 0.48	0.64
22	Phytol*	31.70	10.48 ± 1.42	0.92
	% of identified volatiles			46.33
	% of unidentified volatiles and contaminant form using DVB/CAR/PDMS fiber			53.67
	Total			100

* The volatile compounds in pandan leaves that were reported in the previous studies [87, 89, 90]
Each value was represented as mean ± SD (n = 2).

5.4.2 Phenolic acids and flavonoids

Phenolic acids of pandan leaves consisted of caffeic acid, *p*-coumaric acid and sinapic acid as being analyzed by HPLC. Among 6 regions of cultivated locations, the sample from the South contained the highest concentration of caffeic acid (12.28 μg/g dry weight) and synaptic acid (3.28 μg/g dry weight), while the sample from the East possessed the highest concentration of *p*-coumaric acid (51.16 μg/g dry weight) (Table 5.12). The samples from all regions of Thailand exhibited high total phenolic acids (52.09-59.34 μg/g dry weight) in exception of the North region (29.10 μg/g dry

weight).

Flavonoid contents from pandan leaves extracts were quercetin and kaempferol. The Central region possessed the highest concentration of both flavonoids (44.91 μg/g dry weight of quercetin and 38.33 μg/g dry weight of kaempferol). The lowest quercetin was exhibited in the West region (9.42 μg/g dry weight), while the lowest kaempferol was presented in the Northeast and North regions (1.61 μg/g dry weight of the Northeast and 4.08 μg/g dry weight of the North).

5.4.3 Carotenoids

Carotenoid contents in pandan leaves consisted of lutein, α-carotene and β-carotene (Table 5.13). Lutein was expressed in high concentration in almost all regions of Thailand (633-664 μg/g dry weight), except for the Central regions (545 μg/g dry weight). The highest quantity of α-carotene (263 μg/g dry weight) was significantly presented in the Northeast region. Besides, pandan leaves in the East contained the highest concentration of β-carotene (343.01μg/g dry weight). On the other hand, the lowest concentration of lutein and α-carotene were presented in the Central (545.14 μg/g dry weight of lutein and 171.64 μg/g dry weight of α-carotene), while β-carotene was exposed in low concentration in the Central, the North and the South (280.31, 273.73 and 271.21 μg/g dry weight, respectively) regions.

Table 5.12 Phenolic acid and flavonoid contents from pandan leaves extracts

Cultivated locations	Phenolic acids (µg/g dry weight)			Total phenolic acids	Flavonoids (µg/g dry weight)		Total flavonoids
	Caffeic acid	*p*-Courmaric acid	Sinapic acid		Quercetin	Kaempferol	
Central (Nakhon Pathom)	2.46 ± 0.03 ^{e}	49.31 ± 3.04 ^{ab}	0.32 ± 0.01 ^{f}	52.09 ± 3.08 ^{a}	44.91± 1.68 ^{a}	38.33 ± 2.24 ^{a}	83.23 ± 3.92 ^{a}
Northeast (Udon Thani)	3.16 ± 0.03 ^{d}	47.98 ± 1.15 ^{ab}	2.25 ± 0.10 ^{b}	53.38 ± 1.22 ^{a}	ND	1.66 ± 0.07 ^{d}	1.66 ± 0.07 ^{e}
North (Phrae)	1.28 ± 0.03 ^{f}	26.47 ± 0.48 ^{c}	1.35 ± 0.02 ^{d}	29.10 ± 0.44 ^{b}	13.17 ± 0.23 ^{c}	4.08 ± 0.16 ^{d}	17.25 ± 0.07 ^{d}
South (Songkhla)	12.28 ± 0.21 ^{a}	43.78 ± 0.34 ^{b}	3.28 ± 0.09 ^{a}	59.34 ± 0.64 ^{a}	12.14 ± 0.03 ^{c}	14.12 ± 0.64 ^{c}	26.26 ± 0.67 ^{c}
East (Chon Buri)	6.36 ± 0.23 ^{b}	51.16 ± 2.91 ^{a}	1.79 ± 0.05 ^{c}	59.30 ± 3.20 ^{a}	20.46 ± 0.24 ^{b}	20.84 ± 0.02 ^{b}	41.30 ± 0.44 ^{b}
West (Ratchaburi)	5.50 ± 0.05 ^{c}	46.08 ± 0.05 ^{ab}	1.43 ± 0.01 ^{d}	53.01± 0.56 ^{a}	9.42 ± 0.25 ^{d}	16.80 ± 1.09 ^{c}	26.22 ± 1.34 ^{c}

Each value was represented as mean ± SD (n = 2). Mean within a column in each tested conditions were shown with difference superscript letters, which were significantly different ($p < 0.05$) using one-way ANOVA followed by Tukey's-b *post hoc* test.
ND: not detected

Table 5.13 Carotenoid contents from pandan leaves extracts

Cultivated locations	Carotenoids contents (µg/g dry weight)			Total carotenoids
	Lutein	α-carotene	β-carotene	
Central (Nakhon Pathom)	545.14 ± 15.29 [b]	171.64 ± 3.01 [d]	280.31 ± 5.70 [c]	997.08 ± 24.00 [e]
Northeast (Udon Thani)	664.56 ± 1.37 [a]	263.17 ± 0.24 [a]	312.34 ± 2.21 [b]	1240.08 ± 3.82 [a]
North (Phrae)	662.58 ± 32.57 [a]	226.42 ± 5.31 [b]	273.73 ± 4.08 [c]	1162.74 ± 41.96 [b]
South (Songkhla)	635.40 ± 19.37 [a]	156.77 ± 3.29 [e]	271.21 ± 7.03 [c]	1063.38 ± 29.70 [d]
East (Chon Buri)	644.55 ± 5.02 [a]	229.32 ± 0.21 [b]	343.01 ± 7.74 [a]	1216.89 ± 12.97 [a]
West (Ratchaburi)	633.79 ± 2.13 [a]	181.76 ± 2.37 [c]	292.82 ± 9.12 [bc]	1108.38 ± 13.62 [c]

Each value was represented as mean ± SD (n = 2). Mean within a column in each tested conditions was shown with difference superscript letters, which were significantly different ($p < 0.05$) using one-way ANOVA followed by Tukey's-b *post hoc* test.