CHAPTER VI
DISCUSSION

6.1 Optimum Extraction Conditions

The methods for pandan leaves extraction have been proposed in many previous studies. In this study, pandan leaves were extracted with independent variables including time, solvent, temperature and solid-to-liquid ratio. The aim of this study was to investigate the effectiveness of independent variables to provide optimum recovery yield of antioxidants and anti-AD agents. The variables levels were obtained from previous studies [106]. The extraction time was varied as 15, 30, 60, 120 and 240 minutes. Aqueous ethanol (0, 20, 40, 60, 80 and 100% v/v) was chosen as extraction solvent, since it is non-hazard and wildly used in extraction systems. The extraction temperature was started at 30°C and finished at 90°C. The solid-to-liquid ratio (1:20, 1:30, 1:40, 1:50 and 1:60 w/v) was a proportion of sample per extraction solvent. These independent variables are also affected by various internal and external factors such as plant material, extraction method and environment.

6.1.1 Extraction conditions for antioxidant and anti-AD properties

The extraction of antioxidants and anti-AD agents were optimized under dissimilar conditions. The extraction condition for antioxidants was optimized at 15 minutes of extraction time, 80% (v/v) aqueous ethanol of extraction solvent, 30°C of extraction temperature and 1:60 w/v of solid-to-liquid ratio. It was found that antioxidant activities as being measured by FRAP assay were insignificant different under all investigated extraction times and extraction temperatures. Thus, these two variables were fixed at 15 minutes and 30°C, respectively, due to time and economic efficiency. Besides, 80% (v/v) aqueous ethanol showed the significant highest antioxidant activity, suggesting that antioxidants in pandan are mostly soluble in this suitable solvent. In previously reported that the bioactive compounds in pandan leaves were phenolics, flavonoids, carotenoid and alkaloids, which likely dissolved in solvent with higher polarity than water but lower than absolutely ethanol [116]. Interestingly, the optimized antioxidant activity was observed with high solid-to-liquid ratio (1 g of pandan powder per 60 mL solvent), suggesting that large volume of extraction solvent is required for solubilization of antioxidants. This matter can be explained in term of the increased driving force for mass transfer or increased solvent-sample interface area [117].

On the other hand, the extraction condition of anti-AD (AChE and BChE) agents were optimized at 15 minutes of extraction time, 0% (v/v) aqueous ethanol, 30°C of extraction temperature and 1:20 w/v solid-to-liquid ratio. Insignificant difference in ChE inhibitions was observed under various extraction times. Likewise, similar ChE inhibitions were detected with samples extracted under 30-70°C of extraction temperature. However, once the temperature reached 90°C, ChE inhibition was declined. It was previously suggested that high temperature might decrease the amount of ChEIs isolated from natural sources [118, 119]. Thus, the extraction time of 15 minutes and the extraction temperature at 30°C were, again, chosen due to time and economic efficiency. Moreover, the highest cholinesterase inhibition was obtained in samples extracted with low polarity solvent (0% v/v aqueous ethanol). It is possible that anti-ChE agents in pandan leaves are mostly dissolved in pure water. Interestingly, the highest ChE inhibition was observed in sample with low quantity of solid-to-liquid ratio (1:20 w/v), suggested that high concentration of sample was provided high anti-ChE agents. Besides, low volume of solvent (20 mL/g dry weight) is sufficient enough to isolate anti-ChE agents from pandan leaves.

These preliminary investigations of antioxidant activities and anti-ChE properties were set as a baseline for optimizing extraction consitions. These experiments, however, did not include independent variables and interactions between factors, which may be important for improvement of experimental results. Therefore, RSM was required for further investigation on antioxidant activities and enzymatic assays.

6.1.2 Experimental design using response surface methodology

RSM is used to design and analyze experimental data. It integrates the experimental design and raw data in order to interpret results, including efficient factor, effect of interaction factors, lack-of-fit and percentage of variance (R^2) [120]. In this study, RSM was used to find the optimum extraction conditions of antioxidant activity and enzymatic assays [106]. The experiments with fixed variables (time and temperature) and varied variables (solvent and solid-to-liquid ratio) were created using central-composite design by Minitab software. Experimental matrix presented fourteen extraction conditions, which were employed to analyze FRAP values. As results, the optimized antioxidant activity was achieved at 15 minutes of extraction time, 30°C of extraction temperature, 75% (v/v) aqueous ethanol of extraction solvent and 1:65 w/v solid-to-liquid ratio. These results were corresponded to prior researches [106], which reported that the extraction time was not included in the experimental design due to its insignificant present in the results. Thus, extraction time was set as the shortest time period (15 minutes in our case). Besides, the extraction temperature and solvent were corresponded with RSM results in the previous study [121]. It was suggested that sample extracted with low-medium temperature (40°C) and aqueous ethanol with high polarity index (80% v/v) exhibited the highest TPCs and antioxidant activity [121]. Moreover, sample extracted with high solid-to-liquid ratio exhibited high TPCs and antioxidant activity, the results that were corresponded to the previous study [122]. Another study also suggested that extraction of total flavonoids, total phenolics and antioxidant capacity from pandan leaves was optimized under 78.8% (v/v) aqueous methanol, 69.5°C of extraction temperature and 32.4 mL/g solid-to-liquid ratio [95]. These extraction conditions, however, possess a weak point against product development perspective, since their solvent extraction was hazard. In our case, DI and ethanol were used due to their safe-to-consume property, which can be further promotes as functional food. The results of RSM presented as regression equation, which affected the independent variables in linear, quadratic and interaction terms. The response variable, FRAP value, was depended on level of independent variables, including solvent and solid-to-liquid ratios by regression analysis.

The analysis of variance (ANOVA) for this experiment was significantly presented in linear, quadratic and interaction terms. It is described that the relationship between response variable (FRAP value) and independent variables (solvent and solid-to-liquid ratios) is not simply a linear one. A regression model shows lack-of-fit, which is a parameter to judge the fitness and adequacy of the model. It is calculated from experimental replication. Besides, the lack-of-fit represents pure error, which is used for calculation of variability of the response variable within each treatment. In this study, the lack-of-fit showed insignificant difference for this model, suggesting that the model was adequately fit with the data. The accuracy of models (R^2) was 0.96, the high value of correlation between the response variable and predicted values. Therefore, this model was appropriated to investigate extraction conditions for optimizing TPCs and antioxidant activity.

Although RSM plays a key role on optimization of extraction condition for antioxidant activities and enzymatic assays, extraction condition of enzymatic assay was observed in extreme level of independent variables (0% v/v aqueous ethanol of solvent extraction and 1:20 w/v solid-to-liquid ratio). Moreover, the extraction time and extraction temperature were fixed at 15 minutes and 30°C, respectively. Therefore, the optimum extraction conditions from these preliminary results could be applied directly for the investigation of cholinesterase assay.

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

The main topics for AD occurrence including loss of cholinergic neurotransmitter in the synaptic neuron, formation and accumulation of aggregated protein in neuron, induction of neurofibrillary tangles in the brain and oxidative stress are proposed. These factors can induce brain damage and neurodegenerative [22]. The AD synthetic drugs are used worldwide, even though more side effects such as diarrhea, nausea, dizziness, vomiting and headaches are discovered [123].

The natural therapies are currently of interest due to lower side effect and consumption as daily diet. Pandan leaves contain many bioactive compounds including phenolics, flavonoids, carotenoids, vitamin E and alkaloids [5, 124]. These plants provide several health benefits and prevent some diseases such as heart disease, fever, headache and diabetes [4, 91]; however, its association with AD properties has not been reported. Therefore, the aims of this study were to determine anti-AD properties regarding hypotheses of oxidative stress induction, cholinergic termination and β-amyloid formation.

In this study, various particle sizes and different cultivated locations of pandan leave were found to be significant factors that affected antioxidant and anti-AD properties. In addition, natural drinking products including tea and juice of pandan leaves were also investigated regarding their remained antioxidant and anti-AD activities as to promote them as functional foods.

6.2.1 Variations of particle sizes and cultivated locations

Freeze-dried samples of pandan leaves were blended by the cyclotex sample mill. The blended samples were then separated according to their sizes. The particle sizes were divided into 4 groups, including group1 (particle size >0.42 mm), group 2 (particle sizes of 0.42-0.18 mm), group 3 (particle sizes of 0.18-0.13 mm) and group 4 (particle sizes <0.13 mm). All samples were extracted under optimum conditions and analyzed antioxidant activities and enzymatic assays. As results, the sample with smaller particles presented higher values of TPCs and antioxidant activities than the ones with larger particles. Besides, it was found that the samples with 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. The results were corresponded with previous study, which suggested that an increase in surface area of sample would promote the release of bioactive compounds into solvent [125, 126].

On the other hand, different cultivated locations of pandan were also analyzed regarding antioxidant activities and anti-AD properties. The highest TPCs and antioxidant activities were present in the sample from the South, while the lowest results were observed in the sample from the North. Conversely, the highest enzyme inhibition and the lowest IC_{50} concentration were observed in the sample from the North. These data suggested that the different cultivated locations significantly impacted on quantity and quality of antioxidant agents, flavonoids, phenolics and other bioactive compounds. This matter was associated with several factors such as soil quality, humidity, temperature attitude, climate, sun light exposure and diversity of natural vegetation area [127]. In prior study, TPCs and antioxidant activities profile of pandan in Malaysia indicated that the different cultivated locations provided different TPCs and antioxidant activities. The sample from the North of Malaysia (Bachok) provided the highest phenolics (gallic acid) and flavonoid (catechin and kaempferol) contents. Moreover, it was also provided the highest antioxidant activity (DPPH and FRAP assays). Conversely, the sample from the South of Malaysia (Pontian) provided the lowest TPCs, total flavonoids and antioxidant activities [124].

The representative extraction conditions for pandan leaves in South (antioxidant condition) and North region (anti-AD condition) were employed for analysis of BACE1 inhibitory activity. It found that the pandan leaves from the North that extracted with optimized extraction condition for anti-ChE activities exhibited higher anti-BACE1 activity than that of the sample from the South that extracted with extraction condition optimizing for antioxidant activities. The extraction conditions may affect quantity and types of bioactive compounds in pandan leaves that are released into extraction solvent. Besides, ethanol (80% v/v aqueous ethanol) may interfere with enzyme stability, thus reducing enzyme activity in BACE1 reaction. Our results were corresponded to the previous study in pandan leaves, which showed that water extracted sample provided higher enzyme inhibition (maltase and sucrase activity) than that of ethanolic extraction [91].

The cholinesterase inhibitors (ChEIs) are widely used for AD patients including physostigmine (esterine), tacrine, donepezil, galantamine and rivastigmine [123]. Their functions were neurotransmitter (ACh) enhancers through reduction of cholinesterase activity [70]. Galantamine drug is used as a control to compare efficiency with plant materials. The IC_{50} of galantamine against AChE and BChE were reported to be 0.028 µM (0.008 mg/L) and 3.26 µM (0.936 mg/L) [128], respectively, while the IC_{50} of pandan leave against AChE and BChE were 1.22 and 1.03 g dry weight/L. It was observed that the IC_{50} of galantamine in both enzyme assays were significantly less than those of pandan leaves extract, suggesting that the drug is a more powerful anti-ChE agent. Thus, high dose of pandan leave extract is required to reach the same requirement for AD treatment as those of the drug.

6.2.2 Product applications of pandan leaves

Natural drinking products developed in this study were tea and juice from pandan leaves. Antioxidant activities, TPCs and anti-AD activities of both products were investigated and compared regarding the effect of extraction solvent (DI and RO water). The results indicated that the tea extracted with DI water exhibited higher TPCs and antioxidant activities than those of the tea extracted with RO water. Similar results were observed with pandan juice, in which the sample extracted with DI water exhibited the higher TPCs and antioxidant activities than those of the juice extracted with RO water. These results were in good agreement with prior study, which suggested that DI water can influence the release of bioactive compounds from raw material rather than RO water [129]. The water quality can be defined as different quantity of ions (such as Ca^{2+} and Mg^{2+}) presented in the water. These metals can promote bioactive compound releasing into solvent [129]. Besides, DI water is slightly acidic, thus reducing viscosity of organic molecules in raw material and supporting the release of bioactive compounds into solvent [130].

Interestingly, tea samples provided higher TPCs and antioxidant activities than juice extracted by both DI and RO water. These results indicated that tea process (heated at 100°C for 30 minutes) can maintain higher level of TPCs and antioxidant activities than those of juice (boiled at 100°C for 10 minutes). Even though longer heating time was applied to tea process, the particle size of tea (>0.42 mm) is much less than those of juice (2-3 cm x 0.5 cm), which, in turn, has more effect toward TPCs and antioxidant activities.

Likewise, the tea extracted with DI water exhibited higher anti-ChE activities than those of the tea extracted with RO water. Similar results were observed with pandan juice, in which the sample extracted with DI water exhibited the higher anti-ChE activities than those of the juice extracted with RO water. These results suggested that pH and metals in DI water may influence the release of anti-ChE agents better than those of RO water. Besides, tea samples provided higher anti-ChE activities than juice extracted by both DI and RO water. Since these results were similar to the ones with antioxidant activities and TPCs, it is possible to hypothesize that some antioxidants may have bi-functional properties i.e., being antioxidants and anti-ChE agents. Some phenolics with antioxidant activities were reported to exhibit cholinesterase inhibitory activities. For examples, myricetin, quercetin and luteolin exhibited anti-human recombinant AChE (K_i of 37.8, 38.3 and 65.8 μM, respectively) and anti-human plasma BChE (K_i of 71.0, 68.0 and 166.1 μM, respectively) [131, 132]. Besides, commercial ascorbic acid (60 mg/kg intraperitoneal injection) significantly inhibited AChE activity with 17.13% inhibition in brains of mice [133].

6.3 Phytochemical of *Pandanus amaryllifolius* Roxb. Leaves

Pandan leaves contains many bioactive compounds including phenolics, flavonoids, carotenoid, α-tocopherol, tocotrienols, essential oils, non-specific lipid transfer proteins, fatty acids and alkaloid [5, 124, 133, 134, 135, 136]. These phytochemicals were reported to provide many potential health benefits such as being antioxidants that prevent chronic diseases and non-chronic diseases [3, 4, 8, 9, 91, 92]. Interestingly, different cultivated locations of pandan were significantly affected the levels of phytochemicals (phenolics and carotenoids). Thus, the aim of this study was to analyze the phytochemicals associated with AD hypotheses.

6.3.1 Volatile compounds

Pandan is volatile plant, which is used for food scent and aromatherapy. Interestingly, it has special odor, which come from combination of its volatile compounds. Therefore, pandan leave was analyzed using GC-MS for characterization of its volatile compounds, which might be relevant to its ability to promote antioxidant activity and anti-AD property. Fresh pandan leaves were incubated bath at 70°C in a vial to release the volatile compounds, which were subsequently absorbed into SPME fiber coated with DVB/CAR/PDMS. Then, the compounds were distinguished by GC-MS and identified by matching the mass spectra to database in NIST library.

As results, twenty-two volatile compounds in pandan leave including isopropenyl methyl ketone, isoxazole, fluoroacetamide, hexanal, 5-amino-1-ethylpyrazole, benzaldehyde, 1-octen-3-ol, 3-methyl-2(5H)-furanone, octanol, 2-acetyl-1H-pyrrole, 1-octanal, nonanal, 4-aminophenol, dodecane, decanal, 2-chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl)pyrimidine, ethyl-4-nitrobenzoate, tetradecane, 1-propylpentachlorotriphosphazene, 7-chloro-2,3-dihydro-3-(4-N,N-dimethylamino benzylidene)-5-phenyl-1H-1,4-benzodiazepin-2-one, cis-pinane and phytol were identified. Some volatile compounds were previously reported in prior studies, including hexanal, benzaldehyde, 1-octanal, nonanal, dodecane and phytol [87, 89, 90], while some were newly discovered in this study, including isopropenyl methyl ketone, isoxazole, fluoroacetamide, 5-amino-1-ethylpyrazole, 1-octen-3-ol, 3-methyl-2(5H)-furanone, octanol, 2-acetyl-1H-pyrrole, 4-aminophenol, decanal, 2-chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl) pyrimidine, ethyl-4-nitrobenzoate, cis-pinane, tetradecane, 1-propylpentachlorotriphosphazene and 7-chloro-2,3-dihydro-3-(4-N,N-dimethylaminobenzylidene)-5-phenyl-1H-1,4-benzodiaz-epin-2-one. These results suggested that the quantity and quality of volatile compounds in pandan leave were depended on surrounding factors such as quality of soil, sun exposure, plant species and stress [127].

Interestingly, the principal fragrant in pandan leave, 2-AP, was not found in this study, which can be explained as follows. Firstly, the plant material of our study was different from the previous report, which used cloned pandan containing gene expression of maximum 2-AP content. Secondly, the extraction conditions of the previous report contained sample that was ground into fine powder, resulting in increased sample surface area to enhance the volatile compounds. Lastly, the previous study used high concentration of sample per vial, which would provide high intense of volatile compounds [87]. However, 2-acetyl-1H-pyrrole, which is 2-AP derivative, was detected in our study. This compound might possess similar ability as 2-AP.

Some volatile compounds in pandan leave can act as antioxidants, induce brain relaxing and improve respiratory systems [137, 138]. Besides, octanal and nonanal in *Tadarida brasiliensis* hair and *Citrus reticulate* can be used as antimicrobial agents against mammalian skin bacteria and fungal [139, 140]. In addition, benzaldehyde derivative could be used as antivirulence against persistent *S. aureus* infection [141]. Mostly, volatile compounds can act as antioxidants with antimicrobial and antifungal properties, while anti-AD agents were not reported to be related to volatile compounds.

6.3.2 Phenolic acids and flavonoids

Phenolic acids and flavonoids are plant secondary metabolite, composing of aromatic ring and hydroxyl group. The quantity and quality are depended on surrounding stresses including wounding, pH stress, cold stress and infection, which induce phenolics and flavonoids accumulation in higher plants [142]. These phytochemicals possess several structural analogues, which are widely distributed in plants. They act as antioxidants to prevent oxidative stress, leading to the biological application as natural medicine [143]. Therefore, phenolic acids and flavonoids in pandan leaves were investigated regarding anti-AD capacities.

In this study, the different cultivated locations of pandan leaves were extracted with 50% (v/v) aqueous methanol, while HCl was added for hydrolysis of large compound into small compounds. Ascorbic acid and tBHQ were used as stabilizers to prevent oxidation that may disturb chemical structures of phenolics and flavonoids. Lastly, the extracts were purified and analyzed by HPLC method.

As results, three phenolic compounds in pandan leaves including caffeic acid, *p*-coumaric acid and sinapic acid were discovered. These compounds were expressed differently in each cultivated location. Caffeic acid and sinapic acid presented in the highest concentration in the South, while *p*-coumaric acid was the highest in the East. Previous study showed the redox potentials and antioxidant activities (DPPH assay) of these phenolics. The DPPH IC_{50} of caffeic, sinapic and *p*-coumaric acid were 16.6, 32.2 and >100 μM, respectively (Table 6.1) [144]. These antioxidant activities were corresponded with results of TPCs and antioxidant activities of pandan leaves from different cultivated locations. The pandan leaves from the South only possessed two phenolics, caffeic acid and sinapic acid, but expressed the highest TPCs and antioxidant activities due to the caffeic acid and sinapic acid were stronger antioxidants than *p*-coumaric acid.

In addition, it was found that pandan leaves contain flavonoids, quercetin and kaempferol with flavonols group. These two compounds were present in pandan leaves from every cultivated location in exception of the Northeast region, in which quercetin was not detected. The highest concentrations of both flavonoids were presented in the Central region. Prior study had reported that antioxidant activities of quercetin and kaempferol as being detected by DPPH radical scavenging assay were 6.9 and 52.4 μM, respectively (Table 6.1) [145]. These flavonoids might lead to the second rank of high TPCs and antioxidant activities being detected in pandan leaves from the Central.

Table 6.1 The previous study of phenolics and flavonoid abilities to prevent oxidative stress and AD occurrence [144, 145, 146, 147, 148, 149]

Method	**Phenolic acids**			**Flavonoids**	
	Caffeic acid	**Sinapic acid**	***p*-Coumaric acid**	**Quercetin**	**Kaempferol**
Antioxidant activities					
- DPPH assay	16.6 µM	32.2 µM	>100 µM	6.9 µM	52.4 µM
Anticholinesterase					
- AChE	0.7 µM	3.66 µM	29%*	50.9 µM	130.07 µM
- BChE	1 µM	-	36%*	-	-
Anti-β secretase	-	-	>400 µM	-	-

The results showed of antioxidant activity and anticholinesterase were showed IC_{50} (µM) and % inhibitory of enzyme activity.

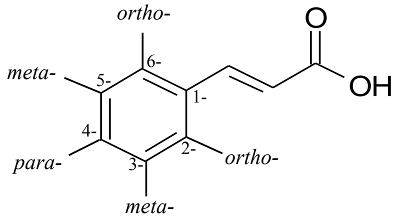
*The concentration of p-coumaric acid that showed percent inhibition was 12 µM

Unlike our study, various phenolics including gallic acid, cinnamic acid and ferulic acid and flavonoids including rutin, epicatechin, catechin and naringin were previously detected in pandan leaves [124]. It was suggested that the quality and quantity of phenolics and flavonoids were varied according to many factors such as sun exposure, quality of soil, weather and cultivated location [124]. Besides, the extraction condition might affect the quality of phenolics, since some might be stabilized in different solvents. It was previously reported that phenolics are likely dissolved in acidic solvent rather than alkaline condition [150]. Moreover, previous study had used different flavonoid standards (catechin, kaempferol, epicatechin, naringin, rutin) from our study (quercetin, kaempferol, isorhamnetin, myricetin, apigenin, luteolin, naringenin and hesperetin) in the HPLC performance, leading to different flavonoids being detected in the HPLC chromatogram [124].

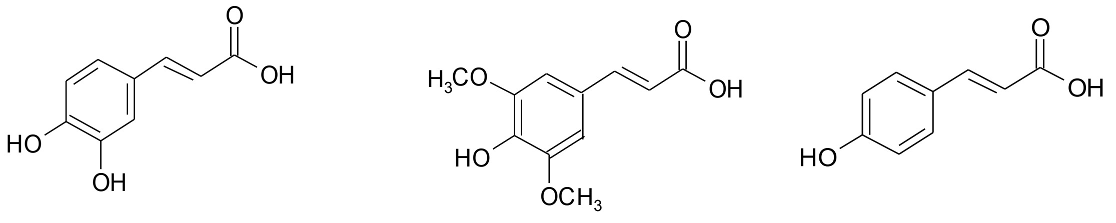
Phenolics and flavonoids play an important role as antioxidants, anti-cholinesterase and anti-β secretase agents. Previous studies suggested that these compounds could prevent oxidative stress and AD occurrence through reduction of ROS levels and improvement of heath function in human [151], increment of CAT (acetylcholine converting enzyme) activity, enhancement of ACh capacities and reduction of AChE function [152]. Besides, these compounds can inhibit $A\beta_{1\text{-}42}$

induced protein, disturb neurofibrillary tangle formation and suppress nitric oxide synthase expression [153, 154, 155].

The structure of phenolics and flavonoids are a key dominant of their radical scavenging called "structure-activity relationships or SAR" [156]. The antioxidant activity of phenolic acids is depended on the number and position of hydroxyl (-OH) group. The hydroxy groups at the *ortho-* or *para-*position on their aromatic ring are not associated with antioxidant activity. On the other hand, *meta-*position of hydroxyl group in phenolic compounds plays a key role in antioxidant activity. However, the antioxidant activity can be reduced by methoxyl (-OCH_3) groups in 3- and 5-position on aromatic ring substitution, thus the highest antioxidant activity in caffeic acid, followed by sinapic acid and *p*-coumaric acid, respectively, were observed [156]. This information is corresponded with antioxidant activities in our results (Figure 6.1).



Hydroxycinnamic structure



Caffeic acid Sinapic acid *p*-Coumaric acid

Figure 6.1 The hydroxycinnamic acid structure and phenolic acids, including caffeic acid, sinapic acid, *p*-coumaric acid. They showed different aromatic substitution of their structures [156].

The SAR of flavonoids associated antioxidant activity was similar to phenolic acids. The antioxidant activity of flavonoid is depended on degree of hydroxylation and the positions of the hydroxyl groups in aromatic B ring (Figure 6.2). The presence of hydroxyl groups at the 3', 4', and 5'-positions of ring B has been reported to enhance more antioxidant activity comparing to a single hydroxyl group [156]. This information is corresponded to our results, which showed high antioxidant activity in quercetin than kaempferol.

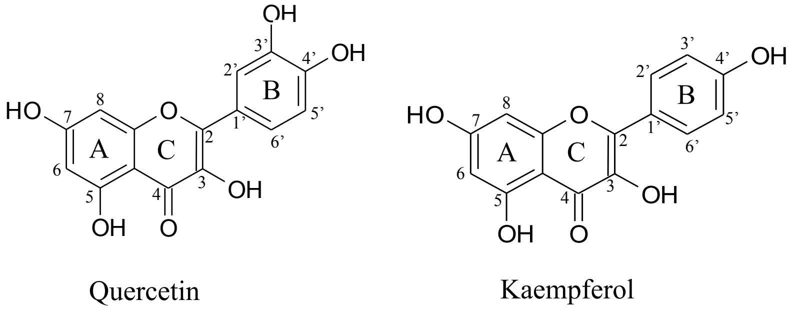


Figure 6.2 Flavonoid structures, including quercetin and kaempferol. They showed different hydroxyl (-OH) group substitution on B ring of their structures [156].

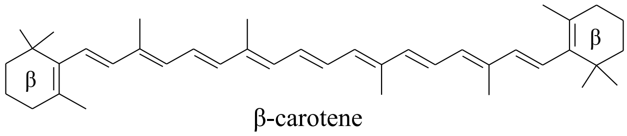
Many studies had proved that the phenolics and flavonoids play a crucial role in AD controlling through oxidative stress, cholinergic termination and Aβ formation. However, the results in phenolics and flavonoids contents were not highly presented in the North region, but showed the highest percentage of ChE inhibition. It was suggested that the phenolics and flavonoids contents might not function as strong anti-ChE agents.

6.3.3 Carotenoids

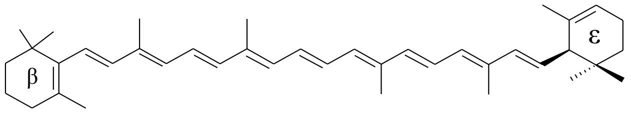
Plant carotenoids are natural fat-soluble pigment in plant cell membrane. Carotenoid is usually lipophilic and present as unsaturated chain in fatty cell. To extract carotenoids from pandan leaves, the freeze-dried samples in different cultivated locations of pandan were hydrolyzed with base (KOH) to provide non-binding structure of carotenoids. The sample was then extracts with non-polar solvent (hexane), which was a suitable solvent for carotenoid extraction [5]. Moreover, the sample was then added ascorbic acid to stabilize antioxidant and against oxidative stress during extraction procedure. Lastly, they were analyzed by HPLC for detected quantity and quality of carotenoid contents.

Three carotenoid compounds including lutein, α-carotene and β-carotene in pandan leaves were detected in our study. The different cultivated locations presented various quality and quantity of carotenoid contents. Lutein was significantly presented in high concentration in every region except the Central region, while α-carotene and β-carotene were significantly the highest in the Northeast and East regions, respectively. Thus, total carotenoid contents were particularly the highest in the Northeast and East regions. Previous study had reported that pandan leaves contained five carotenoids including lutein (470 μg/g), α-carotene (190 μg/g), β-carotene (120 μg/g), neoxanthin (60 μg/g), violaxanthin (50 μg/g) and zeaxanthin (20 μg/g) [5]. These results were different from ours, in which neoxanthin, violaxanthin and zeaxanthin were not found. The first two were not included in our standards, while the last one was reported to be detected in trace amount [5]. Besides, extraction condition might be another factor affecting the detection of carotenoids. In the previous study, high amount of pandan leaves (5-15 g) was employed, while lower quantity of sample (0.2 g) was used in our study.

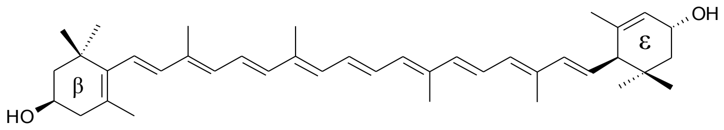
All carotenoids play roles as antioxidants, which prevent some diseases such as cardiovascular disease, cancer and other chronic diseases [158, 159]. Carotenoids that were act as antioxidant are lutein and β-carotene. These two compounds exhibited antioxidant activity *via* inhibition of lipid peroxidation (IC_{50} 2.5 μM for lutein and 3.0 μM for β-carotene), suggesting that the type of terminal group (β-ring or ε-ring), number of C-double bonds (11 bonds) and the number of hydroxyl groups (2 or none) might be associated with antioxidant capacities [160] (Figure 6.3).



β-carotene



α-carotene



Lutein

Figure 6.3 Carotenoid structures, including β-carotene, α-carotene and lutein with different types of aromatic rings and number of hydroxyl groups [160].

Nevertheless, there are only a few researches of carotenoid associated with AD. In a clinical trial study, it was found that the patients under moderated and severe progression of AD possessed significantly lower lutein and β-carotene level in plasma than those of healthy people. Moreover, AD volunteers had lower score in Mini Mental State Examination, a tool for evaluated cognitive function, and impairment of lutein and β-carotene level [161]. In addition, β-carotene might be a key compound for AD therapy and prevention. Interestingly, β-carotene, a hydrophobic symmetrically compound with no oxygen atom, could bind Aβ peptide and inhibit Aβ formation [162, 163, 164].

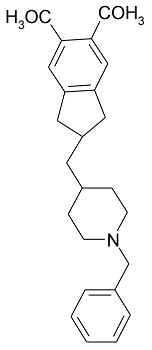
Although the function of carotenoids as cholinesterase inhibitors was not previously reported, these compounds exhibited potential role as Aβ aggregation inhibitors. However, pandan leaves in our study were extracted with polar solvent (DI and ethanol), trace amount of carotenoids was possibly isolated, leading to low effect of pandan extract against AD hypotheses.

6.3.4 Other compounds

Pandan leaves do not only contain phenolic, flavonoid and carotenoid compounds, but also exhibit other compounds such as α-tocopheral, γ-tocopherol and alkaloid [5, 103]. The α-tocopherol and γ-tocopherol are lipid-soluble vitamins that play a significant role as strong antioxidants to prevent oxidative stress [165]. Their abilities are to act as neuroprotection against oxidative stress, which is associated with Aβ production [166, 167, 168, 169]. Comparison of plasma between healthy people and AD patients with MCI indicated that AD patient had lower level of total tocopherols and total tocotrienols than healthy people [170]. However, α-tocopheral (2000 IU/d) could improve and slow down progression of AD with MCI [171, 172]. Conversely, a double-blind randomized controlled study in large group of subjects was proposed that vitamin E was insignificantly affected AD patients [173]. Nowadays, reliable evidence on efficacy of vitamin E in the prevention or treatment of people with AD is unavailable, thus this area of research is needed for further study.

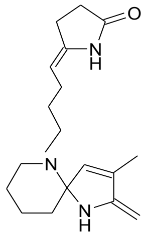
Nowadays, the pharmacological strategy was followed cholinergic hypothesis. Several AChE inhibitors are synthesized to maintain ACh level in brain cell. Alkaloids were proposed to improve cognitive decline in AD patients [104]. The first AChE inhibitor, physostigmine, is an alkaloids isolated from natural sources and used to inhibit cholinesterase. Besides, two alkaloids, galantamine and huperzine A, are also cholinesterase inhibitors isolated from *Galanthus nivalis* and *Huperzia serrata*, respectively. In addition, donepezil is AD drug that synthesized from the alkaloid base structure (Figure 6.4) [174].

The alkaloid activities are particularly associated with AD causes, especially cholinergic hypothesis. In previous studied, alkaloids in pandan leaves were observed including pandamarine, pandamarilactone, pandamarilactam, pandamarilactonine, norpandamarilactonine and pandanamine (Figure 6.5) [92, 103]. The basic structures of these alkaloids are piperidine-, pyrrolidine-, pyrrolidinone- and amine-type moieties [103]. It was found that the piperidine-type alkaloid is involved donepezil, which acts as reversible non-competitive inhibitor of AChE [175]. In previous study, energies interaction between piperidines and AChE and its IC_{50} were investigated. Besides, BACE1 inhibitor from pyrrolidine- and piperidine-based structure was synthesized, and the results suggested high potential inhibition of BACE1 in *in vitro* study [176]. Thus, the pandan alkaloids this study might be developed into new AChE inhibitors to prevent AD occurrence.

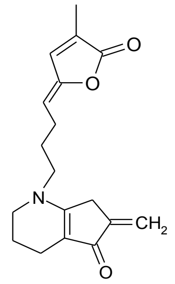


Donepezil

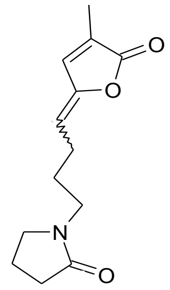
Figure 6.4 Donepezil structure. It composed of piperidine-type alkaloid base, which is similar to alkaloid in pandan leave [177].



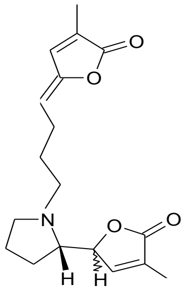
Pandamarine



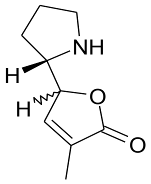
Pandamarilactone



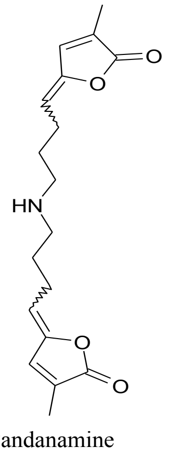
Pandamarilactam



Pandamarilactonine



Norpandamarilactonine



Pandanamine

Figure 6.5 Alkaloid structures of pandan leaves including pandamarine, pandamarilactone, pandamarilactam, pandamarilactonine, norpandamarilactonine and pandanamine [103].