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IN VITRO SCREENING TECHNIQUES FOR THAI HERBAL EXTRACTS
ON ALZHEIMER'S DISEASE PREVENTION

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โรคอัลไซเมอร์ เป็นโรคในกลุ่มอาการสมองเสื่อมที่พบในผู้สูงอายุ ปัจจัยหลักที่เป็นสาเหตุของโรคนี้
คือ การสะสมและการรวมตัวกันของโปรตีนแอมีลอยด์บีตา (Aβ) ภายนอกเซลล์ประสาท อันเนื่องมาจากความไม่
สมดุลของกระบวนการสร้างและสลายของ Aβ ปัจจุบันแนวทางในการรักษาโรคอัลไซเมอร์มุ่งเน้นไปที่การหา
สาร โดยเฉพาะอย่างยิ่งสารจากผลิตภัณฑ์ธรรมชาติที่สามารถออกฤทธิ์ได้หลายรูปแบบในการต้านการสะสมของ
Aβ ซึ่งเป็นสาเหตุหลักของการเกิดโรคอัลไซเมอร์ ในการศึกษาครั้งนี้ได้จัดระบบการคัดกรองสารที่มีฤทธิ์
ดังกล่าวและทดสอบในสมุนไพรไทย 9 ชนิด ที่มีรายงานในตำราพฤกษศาสตร์พื้นบ้านถึงสรรพคุณในการรักษา
อาการที่สัมพันธ์กับการเสื่อมของระบบประสาทและสมอง รวมทั้งมีรายงานผลการวิจัยเบื้องต้นต่อระบบประสาท
และสมอง ได้แก่ ฝาง (CS), รางจืด (TL), ทองพันชั่ง (RN), พุดจีบ (TD), หญ้าแห้วหมู (CR), สมอไทย (TC),
สะเดา (AI), ดีปลี (PR) และรากสามสิบ (AR) นำส่วนลำต้น ใบ ราก หรือผลมาสกัดและคัดกรองฤทธิ์อย่างเป็น
ลำดับขั้นในหลอดทดลอง แบ่งการทดลองออกเป็น 2 ขั้นตอนหลัก คือ การทดสอบทางชีวเคมี และการทดสอบ
ทางชีววิทยาระดับเซลล์ ในการทดสอบทางชีวเคมี ได้คัดกรองสมุนไพรที่มีฤทธิ์ในการยับยั้งการรวมตัวและการ
สลายการรวมกันของ Aβ และฤทธิ์ต้านอนุมูลอิสระ พบว่าสารสกัดสมุนไพรทั้ง 8 ชนิด ยกเว้น AR มีศักยภาพใน
การออกฤทธิ์ดังกล่าว จากนั้นจึงนำสารสกัดทั้ง 8 ชนิด มาทดสอบต่อในการทดสอบทางชีววิทยาระดับเซลล์ โดย
ทดสอบฤทธิ์ในการป้องกันการตายของเซลล์ประสาทจากการเหนี่ยวนำด้วย Aβ ในเซลล์ประสาทของมนุษย์ชนิด
SH-SY5Y พบว่าจากสารสกัดทั้งหมด 8 ชนิด มีเฉพาะ RN, TD, TC และ PR เท่านั้นที่แสดงฤทธิ์ดังกล่าว
จากนั้นจึงนำสารสกัดทั้ง 4 ชนิดนี้มาทดสอบฤทธิ์ในการควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการสร้าง
(*App*, *Bace1* และ *Adam10*) และการสลาย (*Ide* และ *Nep*) Aβ ในเซลล์ประสาท SH-SY5Y พบว่าสารสกัด PR
มีศักยภาพในการลดการสร้าง Aβ ผ่านการลดและเพิ่มการแสดงออกของยีน *Bace1* และ *Adam10* ตามลำดับ
ในขณะที่สารสกัด TC มีศักยภาพในการสลาย Aβ ผ่านการเพิ่มการแสดงออกของยีน *Ide* ผลการศึกษาในครั้งนี้
บ่งชี้ว่า PR และ TC เป็นพืชสมุนไพรที่มีศักยภาพสูงสำหรับนำไปพัฒนาต่อไปเพื่อใช้เป็นยารักษาโรคอัลไซเมอร์
ที่ออกฤทธิ์ได้ในหลากหลายจุดของการเกิดโรค นอกจากนี้ยังเสนอแนะให้นำระบบในการคัดกรองนี้ไป
ประยุกต์ใช้เป็นเครื่องมือมาตรฐานในการคัดกรองสมุนไพรไทยที่มีศักยภาพในการรักษาโรคอัลไซเมอร์ ในเชิง
พาณิชย์และอุตสาหกรรมยา

ภาควิชา	ชีววิทยา	ลายมือชื่อนิสิต ....
สาขาวิชา	สัตววิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

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RUEDEEMARS YUBOLPHAN: *IN VITRO* SCREENING TECHNIQUES FOR THAI HERBAL EXTRACTS ON ALZHEIMER'S DISEASE PREVENTION. ADVISOR: ASST. PROF. SUKANYA JAROENPORN, Ph.D., CO-ADVISOR: PROF. SUCHINDA MALAIVIJITNOND, Ph.D., 71 pp.

Alzheimer's disease (AD) is a neurodegenerative disorder that is commonly found in elderly people. The major causative factor of AD is an extracellular accumulation and aggregation of amyloid β-peptide (Aβ) which results from the imbalance between its production and clearance in the brain. Thus, the potential strategies in the battle against AD focus on the discovery of multifunctional agents, especially from the natural products, which can act against multiple pathogenic steps of Aβ. Therefore, the screening system was set up in this study and nine Thai herbs; *Caesalpinia sappan* Linn., 1753 (CS)*, Thunbergia laurifolia* Lindl., 1867 (TL), *Rhinacanthus nasutus* (Linn.) Kurz., 1870 (RN)*, Tabernaemontana divaricata* (Linn.) R.Br. ex Roem. & Schult., 1819 (TD)*, Cyperus rotundus* Linn., 1753 (CR)*, Terminalia chebula* Retz., 1788 (TC), *Azadirachta indica* A. Juss., 1830 (AI)*, Piper retrofractum* Vahl., 1804 (PR), and *Asparagus racemosus* Willd., 1799 (AR) were selected for the screening based on their properties for remedy the cognitive symptoms in ethnopharmacopoeia and have been researched in the fields of neurological disorders. The stems, leaves, roots or fruits of these herbs were extracted and then performed the 2 consecutive steps of the *in vitro* test; biochemical- and cell-based assay. Anti-Aβ aggregation, Aβ fibril disaggregation and antioxidant potencies of the extracts were investigated by the biochemical-based assay. The results indicated that, except AR, all eight herbal extracts had potential to inhibit Aβ aggregation and stimulate Aβ fibril disaggregation, and also exhibited high antioxidant activity. Hence, the next step of the investigation using cell-based assay was conducted, and the protective effect of these herbal extracts on Aβ-induced neuronal toxicity in SH-SY5Y neuroblastoma cell was determined. Among the eight herbal extracts, only RN, TD, TC and PR effectively protected against Aβ-induced neurotoxicity. Therefore, these four herbal extracts were then employed to investigate the modulating potential on the expression of genes associated with Aβ production (*App*, *Bace1* and *Adam10*) and clearance (*Ide* and *Nep*) in SH-SY5Y cells. The result showed that PR had a potential to suppress Aβ production by decreasing β-secretase *Bace1* and increasing α-secretase *Adam10* mRNA expression levels, respectively, while TC had a potential to enhance the degradation of Aβ by increasing mRNA expression level of *Ide*. Altogether, this study indicates that PR and TC possibly particular candidate herbal medicines for developing the multifunctional anti-AD agents for human use in the future. In addition, this screening system has been suggested to be applied as a standardized tool to screen the high potential Thai herbs on AD therapy at a commercial and pharmaceutical scale.

		Student's Signature	
Department:	Biology	Student's Signature	....
Field of Study:	Zoology	Advisor's Signature	....
Academic Year:	2015	Co-Advisor's Signature	....

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CHAPTER I
GENERAL INTRODUCTION

Alzheimer's disease (AD) is the most common age-related neurodegenerative diseases in elderly people. Clinically, it is characterized by a progressive decline in memory, language, problem-solving and cognitive functions that affect a person's abilities to carry out their daily activities (Association, 2015). Most patients develop AD after the age of 65 years old and the prevalence of AD doubles every five years beyond the age of 65 (Hebert et al., 2013). During the 20th century, the proportion of the older people to the younger ones continued to increase and this trend has kept continuing in the 21st century (Qiu et al., 2009). Therefore, AD which strongly associated with increasing age is becoming much more prevalent and being the one of the leading causes of death in the elderly. In 2010, the number of older people affected by AD is estimated at 35.6 million and is projected to double in every 20 years to 65.7 million in 2030 (Duthey, 2013). It is anticipated that this neurodegenerative disease will place a huge burden on the public health care systems and the finance in all countries across the world. The neuropathological hallmark of AD is a deposition of extracellular senile plaques, composed primarily of a various sizes of small peptide namely 'amyloid beta' (Aβ) (Hardy and Selkoe, 2002; Murphy and LeVine, 2010). Aβ is a 4-kDa peptide which is derived from proteolysis of transmembrane protein, amyloid precursor protein (APP) at the cell surface. Generally, sequential proteolytic processing of APP occurs by two distinct pathways: non-amyloidogenic and amyloidogenic pathways. In non-amyloidogenic pathway, APP is enzymatically cleaved by α-secretase followed by γ-secretase. This pathway produces non-toxic fragment of p3 peptide and precludes Aβ production. Conversely, in amyloidogenic pathway, APP is sequentially cleaved by β- and γ-secretase, and generates toxic fragment into extracellular space known as Aβ, which is regarded as a key player in pathological processes of AD (O'Brien and Wong, 2011). Normally, Aβ is degraded by enzymes such as insulin-degrading enzyme (IDE) and neprilysin (NEP) to maintain its homeostasis in the brain. However, the imbalance between Aβ production and clearance can lead to Aβ accumulation in central nervous system. There are two major forms of Aβ: Aβ40 and Aβ42, which contain 40 and 42 amino acid residues, respectively. Aβ40 is produced in large abundance (80-90% of all

Aβ forms) whereas Aβ42, though produced in a small proportion of 5-10%, is exerted a higher propensity to aggregate into fibrils and plaques and being more neurotoxic than Aβ40 species (Ahmed et al., 2010). In AD patients, the conformation of monomeric Aβ changes from native α-helix to non-native β-sheet structure and becomes prone to aggregation due to hydrogen bonding interaction between Aβ molecules (Knowles et al., 2014). Aggregated Aβ exist in multiple forms: oligomers, protofibrils, fibrils and plaques which trigger a series of neurotoxic events in AD progression (Montoliu-Gaya and Villegas, 2015; Rauk, 2008). Aβ can initiate toxic signal into neurons through direct binding with receptors (e.g., α7 nicotinic receptor, N-methyl-D-aspartic acid receptor, p75 neurotrophin receptor) on cell membrane or forming pores and internalizing into cell to display cellular deficiency (Kam et al., 2014; Sotthibundhu et al., 2008). Aβ-binding receptors generate toxicity through inducing calcium dyshomeostasis, endoplasmic reticulum stress, mitochondrial dysfunction, oxidative damage and synaptic degeneration. In addition, internalized Aβ generates a malfunction of many intracellular organelles (e.g. lysosome, Golgi apparatus), neurofibrillary tangles and chronic microglial neuroinflammation response resulting in neuronal death associated with cognitive impairment (Butterfield et al., 2013; Cai et al., 2014; Cavallucci et al., 2012; Kawahara, 2010; Shankar and Walsh, 2009). Generally, Aβ begins to accumulate in cerebral cortex and hippocampus approximately 15-20 years before cognitive impairment occurs (Jagust et al., 2009). Hence, the neuroprotective strategies are focusing on the discovery of multifunctional agents for preventing or reversing the amyloid-based pathology including (i) inhibiting and reversing Aβ aggregation processes, (ii) reducing neurotoxicity induced by Aβ, and (iii) decreasing Aβ production by suppressing β-secretase activity or by enhancing α-secretase activity and increasing Aβ clearance by stimulating Aβ-degrading enzyme activities such as IDE and NEP. However, up to now, no drugs or treatments that can prevent or cure AD are reported (Salomone et al., 2012; Yiannopoulou and Papageorgiou, 2013). The only currently drugs available for AD are to temporarily alleviate symptoms, or slow down the progression of AD.

At present, many plant species and its biologically-active compounds have been discovered and provided in healthcare system as therapeutic agents for prevention and

treatment of various aging diseases worldwide. This is because the plants have more therapeutic benefits and fewer adverse effects than synthetic agents (Eckert, 2010). Thailand is located in the tropical area abound with diverse compounds with high potency in plants. Herbs are valuable natural resources that have long been traditionally used for remedy many illnesses, especially cognitive disorders (Mitra, 2007). Although those herbs have high potential on the prevention and therapy of AD, no systematically and scientifically screening protocol has been set up. In this study, nine Thai herbs; *Caesalpinia sappan* (CS)*, Thunbergia laurifolia* (TL)*, Rhinacanthus nasutus* (RN)*, Tabernaemontana divaricata* (TD)*, Cyperus rotundus* (CR)*, Terminalia chebula* (TC), *Azadirachta indica* (AI)*, Piper retrofractum* (PR), and *Asparagus racemosus* (AR) were selected based on their traditional uses for remedy cognitive symptoms from Thai ethnopharmacopoeia and has been previously investigated in the fields of neurological disorders and injuries (Brimson et al., 2012; Kubo et al., 2013; Kumar and Khanum, 2013; Moon et al., 2010; Na et al., 2004; Nakdook et al., 2010; Ojha et al., 2010; Raghavendra et al., 2013; Tangpong and Satarug, 2010). Crude extracts of these herbs were systemically examined *in vitro* in 2 steps; biochemical- and cell-based assay. For biochemical assay, anti-Aβ aggregation, Aβ disaggregation, and antioxidant potency of the extracts were determined. Subsequently, only the effective herbal extracts, which possessed anti-Aβ aggregation, disaggregating Aβ fibrils and antioxidant activities, were selected and then proceed to investigate the protective efficiency against Aβ-induced neurotoxicity and the potential modulation of Aβ production and clearance using cell-based assay. If this *in vitro* screening protocol is possible, it should be practically applied for the pharmaceutical companies to use it to screen a large number of plants in a single time which is cost- and time-effectiveness.

OBJECTIVE

To set up the systematic and scientific screening system for Thai herbal extracts on AD prevention and treatment using *in vitro* biochemical- and cell-based assays.

CHAPTER II
LITERATURE REVIEW

1. Alzheimer's disease (AD)

AD is a severe neurodegenerative disease that is characterized clinically by loss of memory and cognitive abilities because of a gradual death of neurons involved in degeneration and a loss of neuronal functions and synaptic connections. In the AD's brain, a neurodegeneration initiates from hippocampus that is associated with formation of new memories and recalling short-term memories. Subsequently, it is proceeded into cerebral cortex that is responsible for storage of long-term memories, thought, consciousness, attention, language, and reasoning (Huang and Mucke, 2012).

1.1 Neuropathological hallmarks of AD

The cause of AD is still mostly unknown. Three proposed hypotheses describe the cause of AD as follows:

1.1.1 Extracellular amyloid-β (Aβ) plaques

Aggregation of Aβ into oligomers, fibrils, and plaques is central in the molecular pathogenesis of AD (Hardy, 2009). Aβ is produced by the sequential cleavage of amyloid precursor protein (APP) by two enzymes, β-site APP-cleaving enzyme 1 (BACE1), also called β-secretase, and the γ-secretase complex. The prevailing hypothesis for AD pathogenesis is called the amyloid cascade hypothesis. It proposes that Aβ aggregation is the initiating mechanistic event in which the different stages of aggregates, from soluble oligomers to insoluble fibrils in plaques, are believed to impair synaptic function and ultimately damage neurons which result in chronic neurodegeneration, lead to cognitive impairment and finally dementia (Hardy, 2009; Hardy and Selkoe, 2002; Murphy and LeVine, 2010).

1.1.2. Intracellular neurofibrillary tangles (NFTs)

In the brain of AD patients, tau is abnormally hyperphosphorylated and aggregated, which marked as NFTs in neuronal soma (Grundke-Iqbal et al., 1986; Iqbal et al., 2016; Tomlinson et al., 1970). NFTs are one of the three main histopathological

hallmarks of AD, and directly correlate with dementia severity (Tomlinson et al., 1970). Generally, the function of tau protein is to promote assembly of the microtubule protein subunit–tubulin into microtubules and stabilize their structure (Drubin and Kirschner, 1986). However, hyperphosphorylation of tau leads to destabilization of the microtubule structure, dysfunction of axonal transport, synaptic transmission, neuronal communication and eventually results in cytoskeleton abnormalities, loss of cell viability and neuronal death (Iqbal et al., 2016).

1.1.3. Loss of basal forebrain cholinergic neurons

During early stages of AD, various neurotransmitters including acetylcholine (ACh) are decreased in the brain involved in encoding of short-term memory, which is caused by the increment of the activity of Ach-degrading enzyme, acetylcholinesterase (AChE). This pathogenic event in combination with depositions of Aβ plaques and NFTs lead to degeneration of cholinergic neurons in the basal forebrain that is associated with cognitive dysfunction in patients with AD (Vendersteen, 2012).

There are many hypotheses have been put forward for AD pathogenesis. Among them, the amyloid beta cascade which is related to the extracellular senile plaques is the widely been accepted hypothesis (Selkoe and Hardy, 2016).

1.2 Origin of Aβ peptide in AD

Aβ, a small soluble peptides composed of 39-43 amino acids, is derived from the proteolytic cleavage of APP by β- and γ-secretase (Eisele and Duyckaerts, 2016; O'Brien and Wong, 2011; Thal et al., 2015). The major forms of Aβ are Aβ40 and Aβ42 monomer, which contain 40 and 42 amino acid residues, respectively. In physiological condition, 80-90% of Aβ is in the form of Aβ40 whereas 5-10% is generated as Aβ42. However, Aβ42 is in a higher propensity to aggregate into insoluble fibrils and more neurotoxic than Aβ40 (O'Brien and Wong, 2011).

In mammals, APP is a type I transmembrane protein belongs to a protein family that includes APP-like protein 1 (APLP1) and 2 (APLP2) (O'Brien and Wong, 2011). These proteins composed of a large N-terminal extracellular domain, a membrane-spanning domain and a C-terminal short cytoplasmic domain (O'Brien and Wong,

2011). Physiological function of APP has been proposed in cell growth, neurite outgrowth, synaptogenesis and others (Zheng and Koo, 2011). APP is produced via the constitutive secretory pathway (Haass et al., 2012). After synthesis in the endoplasmic reticulum, APP is translocated through Golgi apparatus and trans-Golgi-network and then immediately transported into secretory vesicles to cell surface (Haass et al., 2012). Proteolytic cleavage of APP occurs at cell surface resulting in the production of various protein fragments. This cleavage can be divided into two pathways, the amyloidogenic and the non-amyloidogenic. The APP processing is depicted in Figure 2.1.
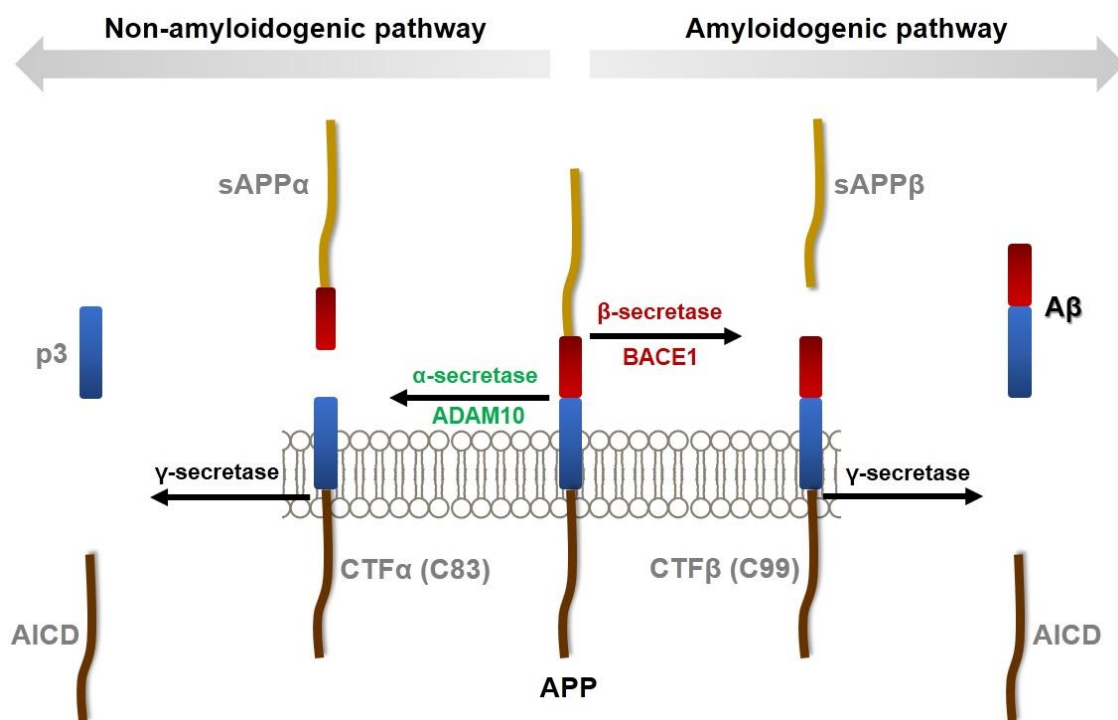
Figure 2.1 APP processing. Sequential proteolytic processing of APP occurs by two distinct routes: non-amyloidogenic and amyloidogenic pathways. In the non-amyloidogenic pathway (left), APP is cleaved by α-secretase followed by γ-secretase to generate sAPPα, AICD and p3 fragments that preclude Aβ production. In the amyloidogenic pathway (right), APP is cleaved by β-secretase followed by γ- secretase to produce sAPPβ, AICD and neurotoxic Aβ fragments implicated in AD (Modified from Thathiah and De Strooper, 2011).

In the non-amyloidogenic pathway, APP is cleaved by α-secretase at amino acid position 83 from C-terminus to produce extracellular liberation of large soluble N-

terminal ectodomain–sAPPα, and the transmembrane carboxyl-terminal fragment of 83 amino acids–CTFα (C83) (Kojro and Fahrenholz, 2005). The CTFα is subsequently cleaved by the γ-secretase to generate a short fragment (3 kDa) of p3, which is secreted into extracellular space, and APP intracellular domain (AICD). In this pathway, α-secretase cleaves APP inside of Aβ domain thus precludes the production of Aβ peptide (Kojro and Fahrenholz, 2005).

In the amyloidogenic pathway, APP is cleaved by β-secretase at N-terminus of the Aβ domain within full-length of APP, giving rise to a secreted sAPPβ fragment and to the transmembrane fragment–CTFβ (C99). Then, subsequent cleavage of CTFβ by γ-secretase releases various length of toxic fragment of Aβ peptide (Barage and Sonawane, 2015).

The α-secretase is a membrane-bound and zinc dependent protease. There are three enzymes of the disintegrin and metalloproteinase (ADAM) family which has been suggested as the constitutive α-secretase: ADAM9, ADAM10 and ADAM17 (Allinson et al., 2003). Among these proteases, ADAM10 is a representative major α-secretase that competes directly with β-secretase for cleavage of APP to promote the non-amyloidogenic pathway (Allinson et al., 2003). Thus, stimulating ADAM10 activity is considered as a key target for reducing Aβ production and preventing AD pathogenesis (Allinson et al., 2003).

The β-secretase (also known as a β-site APP-cleaving enzyme 1, BACE1) is an aspartyl protease belonging to pepsin family. Several studies have been reported that mRNA and protein levels of BACE1 were elevated in the brain of AD patients (Vassar et al., 2014). Hence, inhibiting cleavage of APP by β-secretase via decreasing BACE1 activity may contribute to AD pathology treatment (Vassar et al., 2014).

1.3 Misfolding and aggregation of Aβ peptide

Aβ peptides can form dimers, trimers, higher order oligomers, protofibrils as well as amyloid fibril (O'Brien and Wong, 2011). The pathogenic role of soluble, misfolded Aβ species and of insoluble Aβ fibrils is partially known. Monomeric Aβ peptides, amino acid residue 1-16, are mainly hydrophilic regions and presumably unfolded, but

the central region, residue 12-13, has been categorized as self-recognition site for aggregation of Aβ (Ahmed et al., 2010). The monomeric, normal protein generally includes α-helical and unordered structures. A misfolded, amyloidogenic protein is enriched in β-sheets. The transition from α-helical or unordered structure to β-sheets is associated with the exposure of hydrophobic amino-acid residues, promoting protein aggregation (Eisele and Duyckaerts, 2016). Residue 11-40 of Aβ40 and residue 18-42 of Aβ42 are backbone residues that support hydrogen bonding within the core of Aβ fibrils. In Aβ40 fibrils, the side chain intermolecular contacts are occurred between His13 and Val40, Gln15 and Val36 as well as Phe19 and Ile32/Lue34/Val36. On the other hand, side chains packing are appeared in Aβ42 fibrils between Phe19 and Gly38 and Met35 and Ala42 (Vendersteen, 2012). As a consequence, soluble protein may become insoluble and form aggregates without any change in its amino-acid sequence (Eisele et al., 2015).

The aggregation of monomeric Aβ into fibrils occurs through nucleated polymerization reaction in a pattern of sigmoid growth curve (Wilson et al., 2008). This process is initiated by lag or nucleation phase, that is, soluble prefibrillar oligomers, nuclei and protofibrils are formed (Wilson et al., 2008). After nucleation, these protofibrils rapidly exponential grow to from insoluble mature Aβ fibrils termed fibril elongation phase. Lastly, the plateau phase that is no further fibril growth has been reached. The Aβ fibrils are long, unbranched and twisted structures with 6–12 nm in diameter (Wilson et al., 2008). Although, the misfolded or aggregated proteins can be degraded by specific cellular pathways, some amyloidogenic proteins escape degradation when aggregated and accumulated (Eisele and Duyckaerts, 2016).

1.4 Neurotoxic of Aβ aggregation

Strong evidence suggests that dispersible Aβ oligomers or protofibrils seem to be responsible for Aβ toxicity rather than Aβ plaques (Eisele and Duyckaerts, 2016; O'Brien and Wong, 2011; Thal et al., 2015). The mechanism by which Aβ oligomers can cause neuronal death is currently not fully understood. Recently, several reports have indicated that Aβ oligomer can increase neuronal membrane conductance or leakage, resulting in a progressive degeneration and/or death of neurons (Chimon and

Ishii, 2005; Yu et al., 2009). On the other hand, other studies suggest that accumulated Aβ becomes aggregated on the neuronal membranes and leads to a formation of discrete ion channels or pores (Kayed and Lasagna-Reeves, 2013), and to a disruption of calcium and other-ion homeostasis which promotes the numerous degenerative processes including free radical formation and phosphorylation of tau, thus accelerates neurodegeneration (Takashima et al., 1993; Yatin et al., 1998). The free radicals are also capable of inducing lipid peroxidation resulting in generation of peroxidation products such as lipid hydroperoxides and another lipid radical (Butterfield and Lauderback, 2002). The lipid radical can initiate propagation of chain reaction leads to alteration in biological properties of lipid bilayer in cell membrane and cellular organelles including nucleus, mitochondria, lysosomes and endoplasmic reticulum (Butterfield and Lauderback, 2002). Furthermore, Aβ oligomers can bind to the cell-surface receptors such as the N-methyl-d-aspartate receptor (NMDAR) and other receptors which culminate into synaptic dysfunction and neurodegeneration (Palop and Mucke, 2010; Snyder et al., 2005). Particularly, Aβ oligomers also induce nerve growth factor (NGF) receptor-mediated neuronal death (Yamamoto et al., 2007).

1.5 Clearance of Aβ peptide

In general, Aβ can be degraded by Aβ-degrading enzymes such as IDE and NEP to maintain normal homeostasis of Aβ in the brain. IDE is the most abundant secreted Aβ-degrading enzyme and is capable to degrade the monomeric form of Aβ whereas NEP is found to degrade both monomeric and oligomeric forms of Aβ (Miners et al., 2011). Nevertheless, deficient clearance of Aβ owing to decrease in mRNA and protein levels (as representative markers of enzyme activity) of IDE and NEP could contribute to an excessive accumulation of Aβ in the brain and involve in AD progression (Miners et al., 2011). Therefore, stimulating both IDE and NEP activities could be a possible therapeutic targeting in AD.

1.6 Current trends in pharmacological treatment of AD

Although many approved drugs for treating AD such as cholinesterase inhibitors (donepezil, rivastigmine, and galantamine), are currently sold, but these drugs can only

delay the cognitive and behavioral symptoms, but did not affect the progression of the disease (Mangialasche et al., 2010). Based on the amyloid hypothesis, Aβ is a crucial factor in AD pathogenesis and serves as biological targets for disease-modifying treatments (Christensen, 2007). The therapeutic approach for AD is previously focused on the discovery of drugs which are capable of disrupting each pathological step of Aβ (known as one specific target-one drug approach) such as reduction of Aβ production or prevention of Aβ aggregation. However, none of the development candidate drugs have succeeded in phase 3 clinical trials of drug discovery and development processes (Salomone et al., 2012). The failure of clinical trials with these drugs is due to the heterogeneous in terms of clinical presentation and multiple pathophysiological processes of AD (Salomone et al., 2012). Therefore, within the last three years (2012-2015), the focus in AD drug development is shifted to search for multifunctional drugs which can be interact with more than one step of amyloid cascade and result in reduction of accumulation of Aβ (Guzior et al., 2015).

2. Herbs of Thailand

In recent years, researchers are more interested in searching for biologically active compounds from natural products as a source of new drugs because of high cost of synthetic drug and its unpredictable adverse effects. The Kingdom of Thailand is one of the richest countries in natural resources and biodiversity. It covers around 15,000 plant species, representing 8% of total number of plants worldwide (Mitra, 2007). Thailand has a long history of using plants as key ingredients in traditional medicines for treatment of various symptoms and diseases including cognitive and neurological disorders. Currently, about 1,800 species of plants, in particular herbs, known in Thai as Samunprai, were used in healthcare system in Thailand. However, no systematic and scientific screening methods for Thai herbs on prevention and therapeutics of AD have been reported. Therefore, nine herbs, which have traditionally been used for remedy cognitive symptoms in Thai ethnopharmacopoeia and have been previously studied in the fields of neurological disorders and injuries, were selected for screening their potential against amyloid-based pathology in this thesis.

2.1 *Caesalpinia sappan* **Linn., 1753**

Caesalpinia sappan (CS), a flowering tree, is a member of Leguminosae-Caesalpiniodeae family, which is distributed in Southeast Asia, India and China (Figure 2.2). CS is commonly known as sappan wood in English or Faang in Thai. The heartwood of CS is extensively used as a traditional ingredient of food or beverages and also used in Indian Ayurveda and oriental folk medicine as anti-inflammatory agent for treatment of wounds, arthritis and traumatic disorders and as the promoting agent for blood circulation and cerebral apoplexy therapy (Nirmal and Panichayupakaranant, 2015). In Thailand, it has long been used for the treatment of diarrhea, dysentery, skin rashes and anemia (Sireeratawong et al., 2010). Besides, it has been listed in psychotropic plants that are possessed sedative property (Wongprasert, 2003).

Figure 2.2 Photograph of *Caesalpinia sappan* plant.
(Available from: www.wijayalintassamudra.blogspot.com)

The main chemical constituents of CS's heartwood are phenolic compounds including brazilin, protosappanin, sappanchalcone, sappanol and sappanone (Nirmal et al., 2015). Pharmacological activities of CS extracts and the phenolic compounds are summarized and listed hereafter.

Brazilin-rich CS extract exhibited high DPPH radical scavenging activity (IC_{50} = 6.2 µg/mL) almost the same as standard quercetin (IC_{50} = 3.5 µg/mL). This strong

antioxidant activity was due to its dibenzoxocin structure together with ortho-substitued diphenol group (Nirmal and Panichayupakaranant, 2015).

Activated microglia can produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) during inflammation-mediated neurodegenerative diseases leading to enlarged neuronal injury. However, protosappanin A, obtained from CS extract, showed dose-dependent inhibition of OH, H_2O_2 and also NO production by suppression of NADPH oxidase and inducible nitric oxide synthase (iNOS) activity in LPS-induced neuroinflammation model of BV-2 microglia and primary cortical neurons co-culture system (Zeng et al., 2012). Additionally, deoxysappanone B from CS extract also protected neurons from microglia-mediated inflammatory injuries in the co-culture system through suppression of two major signaling pathways of neuroinflammation including IκB kinase (IKK) mediated NF-κB and p38/ERK mediated mitogen-activated protein kinase (MAPK) cascades (Zeng et al., 2015b).

Brazilin, found in CS extract, inhibited Aβ42 aggregation by binding to Aβ42 molecules via hydrophobic interactions and hydrogen bonding and remodeled Aβ42 fibrils disaggregation by disrupting the intermolecular salt bridge Asp23-Lys28 via hydrogen bonding (Du et al., 2015).

Brazilin, isolated from methanol extract of CS, revealed dose-dependent inhibition of cell proliferation and apoptosis in glioma U87 cells which related to most malignant brain cancer termed glioblastoma multiform through suppression of caspase-3 and caspase-7 (Lee et al., 2013).

3'-deoxy-4-O-methylepisappanol, isolated from methanol extract of CS, inhibited the glutamate-induced toxicity in primary culture of mixed rat cortical cells (composed of both neuronal and glial cells) in dose-dependent manner by maintaining cell viabilities of 50-70% as compared to glutamate-treated cell (0%) (Moon et al., 2010). Moreover, sappanchalcone, isolated from methanol extract of CS, exhibited protection against glutamate-induced oxidative stress in HT22 rat hippocampal cells via triggering of an important enzyme mediated-antioxidant defense system called heme oxygenase (HO)-1 (Jeong et al., 2009).

Protosappanin B from CS extract manifested protection against neuronal anoxia and ischemic injury on oxygen–glucose deprivation (OGD)-induced rat pheochromocytoma (PC12) cells model by up-regulation of mitochondrial membrane potential (MMP), inhibition of cytochrome c release from mitochondria and inactivation of mitochondrial caspase-9/3 apoptosis pathway (Zeng et al., 2015a).

2.2 *Thunbergia laurifolia* Lindl., 1867

Thunbergia laurifolia (TL) belongs to the Acanthaceae family. It is a vigorous climber widely cultivated in Southeast Asian countries and India (Figure 2.3). TL is commonly known as laurel clock vine or blue trumpet vine in English or Rang Jeud in Thai. Based on its detoxifying effects, TL leaves have been used as antidote for various types of poison such as pesticides, arsenic and strychnine and used in the treatment of drug addiction in Thailand (Tejasen and Thongthapp, 1980). Furthermore, it was reported as nerve tonic treatment for migraines and chronic headaches in monograph of Abhaibhubejhr Thai traditional medicine (Thatsanamit, 2012).

Figure 2.3 Photograph of *Thunbergia laurifolia* plant.
(Available from: www.en.wikipedia.org/wiki/Thunbergia_laurifolia.com and www.gpoproduct.com)

The main chemical constituents of TL's leaves are apigenin, apigenin glucosides and phenolic acids, including caffeic acid, gallic acid, protocatechuic acid and

rosmarinic acid (Tangpong and Satarug, 2010). Pharmacological activities of TL extracts and the chemical constituents are summarized and listed hereafter.

Rosmarinic acid, isolated from ethanol extract of TL, and standard trolox performed equally well in DPPH-scavenging capacity with an EC_{50} value of 2.71 µg/ml and 3.51 µg/ml, respectively. The two orthodihydroxyphenyl groups and multiple conjugated double bonds in the structure of rosmarinic acid possessed its ability to eliminate free radicals (Suwanchaikasem et al., 2014).

There is no report in term of neuroprotective property of TL in cell culture. Nonetheless, the extract of TL leaves had neuroprotective effects in animal models. It reduced acetylcholinesterase dysfunction, neuronal cell death and cognitive impairment caused by lead uptake in mice and ameliorated non-spatial short-term memory deficits and depression-like behavior in olfactory bulbectomized mice (Phyu and Tangpong, 2013; Rojsanga et al., 2015).

2.3 *Rhinacanthus nasutus* (Linn.) Kurz., 1870

Rhinacanthus nasutus (RN) is a plant belonging to the family of Acanthaceae. It is a perennial shrub widely distributed in the region of Southeast Asia, India and South China (Figure 2.4). RN is commonly known as snake jasmine in English or Thong Phan Chang in Thai. The whole plant of RN, especially leaves and roots, is used in Thai traditional medicine for treating of diabetes, hypertension, hepatitis, abscess and various skin diseases including herpes, eczema, and ringworm (Farnsworth and Bunyapraphatsara, 1992). Additionally, the leaves of RN is used as CNS stimulant or antidepressant for anxiety and depression in traditional herbal remedies for primary healthcare (Subjaroen, 2005).

Figure 2.4 Photograph of *Rhinacanthus nasutus* plant.
(Available from: www.toptropicals.com and www.greenclinic.in.th)

The main chemical constituents of RN's leaves are naphthoquinones such as rhinacanthins and rhinacanthone, anthraquinones, and glycosides (Bukke, 2011). Pharmacological activities of RN extracts and the chemical constituents are summarized and listed hereafter.

The methanolic extract of RN showed profound antioxidant activity in DPPH free radical scavenging assay. The IC_{50} value of the RN extract was similar to ascorbic acid (Vitamin C) at 34.4 μg/mL and 40.8 μg/mL, respectively (Rao, 2010).

Rhinacanthin-C, a naphthoquinone derivative isolated from ethyl acetate extract of RN, demonstrated anti-inflammatory activity against LPS-induced $^{\bullet}O$ and prostaglandin E_2 (PGE_2) releases from RAW264.7 cells via down-regulation of iNOS and cyclooxygenase (COX-2) gene expression (Tewtrakul et al., 2009).

The ethanol extracts of RN, contained the highest concentrations of lupeol and β-sitosterol, attenuated the HT-22 hippocampal cell death caused by both glutamate and Aβ25-35 toxicity (Brimson et al., 2012).

2.4 *Tabernaemontana divaricata* (Linn.) R.Br. ex Roem. & Schult., 1819

Tabernaemontana divaricata (TD), an evergreen shrub in Apocynaceae family which is distributed in tropical parts of the world including Brazil, Egypt, India, Sri Lanka, and Southeast Asia (Figure 2.5). TD is commonly known as crepe jasmine or pinwheel flower in English or Pud Jeep in Thai. TD is used in Chinese, Ayurvedic and Thai traditional medicine for fever, pain and dysentery. Moreover, the root of TD is used as rejuvenating and neurotonic agents for forgetfulness and improving memory in Thailand (Ingkaninan et al., 2003).

Figure 2.5 Photograph of *Tabernaemontana divaricata* plant.
(Available from: www.mydokhome.com and www.nanagarden.com)

The main chemical constituents of TD's root are bis-indole alkaloids such as 19,20-dihydrotabernamine and 19,20-dihydroervahanine A, steroids, flavonoids, phenyl propanoids, phenolic acids and enzymes (Pratchayasakul et al., 2008). Pharmacological activities of TD extracts are summarized and listed hereafter.

The methanol extract of TD, contained the highest concentrations of 19,20-dihydrotabernamine and 19,20-dihydroervahanine A, maintained the acetylcholine level in the brain by inhibiting more than 90% of AChE activity (Ingkaninan et al., 2006). Moreover, the ethanol extract of TD also elevated acetylcholine level at the cholinergic synapses via repression of synaptic responses in glutamatergic projections,

which act as intervener of cholinergic transmission in hippocampal slices of normal rats (Pratchayasakul et al., 2010).

In vivo study reported that subchronic administration of ethanol extract of TD can prevent the Aβ25–35-induced memory deficits in mice by decreasing the AChE activity (Nakdook et al., 2010).

2.5 *Cyperus rotundus* **Linn., 1753**

Cyperus rotundus (CR) belongs to the Cyperaceae family. It is a widespread weed distributed in tropical and subtropical regions of the world, principally Africa and Eurasia (Figure 2.6). CR is commonly known as nut grass or purple nut sedge in English or Yaa Haeo Muu in Thai. The rhizomes and tubers of CR are used as traditional medicine for the treatment of stomach and bowel disorders, diabetes, dysentery, pyretic symptoms and malaria, inflammatory diseases and menstrual irregularities in China, Japan, India and Arab countries (Pirzada et al., 2015). In Thailand, it has been credited with the ability to relieve meningitis in medicine texts of King Rama V (Department, 1999).

Figure 2.6 Photograph of *Cyperus rotundus* plant.
(Available from: www.plants.uaex.edu and www.balajitraders.info)

The main chemical constituents of CR's rhizomes and tubers are cyperol, cyperene, cyperone, alkaloids flavonoids, terpenoids, sesquiterpenes, valencene, furochromones, nootkatone, starch, saponins, glycerol, sitosterol, stearic acid, linolenic acid, ascorbic acid, myristic acid, fatty oils and essential oils (Pirzada et al., 2015). Pharmacological activities of CR extracts are summarized and listed hereafter.

The ethanol extract of CR showed profound antioxidant activity in DPPH free radical scavenging assay. The percentage of DPPH scavenging of the 250 µg/mL CR extract (81.27%) was similar to 18 µg/mL ascorbic acid (95.12 %) (Yazdanparast and Ardestani, 2007).

The ethanol extract of CR exhibited protection against H_2O_2-induced neuroblastoma SH-SY5Y cell damage through its antioxidant and anti-apoptotic activities and also restored nuclear and mitochondrial morphologies as well as increased the expression of brain derived nerve growth factor (BDNF) (Kumar and Khanum, 2013).

2.6 *Terminalia chebula* Retz., 1788

Terminalia chebula (TC), a deciduous tree, is a member of Combretaceae family, which is distributed in Middle East and tropical regions such as India, China, and Thailand (Figure 2.7). TC is commonly known as ink tree, or chebulic myrobalan in English or Samo Thai in Thai. The fruit of TC is used in oriental folk medicine for treating digestive, urinary, skin and heart diseases, diabetes, irregular fevers, constipation, ulcers, vomiting, colic pain and hemorrhoid. Additionally, TC has been documented in Materia Medica of Ayurveda as King of medicines and also used to treat geriatric diseases and improve memory and brain function (Bag et al., 2013).

Figure 2.7 Photograph of *Terminalia chebula* plant.
(Available from: www.i.ytimg.com and www.jogindernursery.com)

The main chemical constituents of TC's fruit are hydrolysable tannins (i.e., gallotannins, ellagitannin, chebulagic acid and chebulinic acid), phenolic compounds (i.e., ellagic acid, gallic acid, tannic acid, chebulic acid and anthraquinone glycosides), and miscellaneous compounds (i.e., palmitic, stearic, oleic, linoleic and arachidic acids) (Afshari et al., 2016). Pharmacological activities of TC extracts are summarized and listed hereafter.

The methanol extract of TC exhibited high DPPH radical scavenging activity (IC_{50} = 8.5 µg/mL) almost the same as standard ascorbic acid (IC_{50} = 2.5 µg/mL). This antioxidant activity is due to phytochemical compounds such as ascorbic acid and polyphenols like gallic acid and ellagic acid which found in the fruits of TC (Mathew and Subramanian, 2014).

The 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, isolated from methanol extract of TC, showed inhibitory effects against AChE with IC_{50} values of 29.9 ± 0.3 µM in Ellman's colorimetric method (Sancheti et al., 2010).

Chebulagic acid showed a protective effect against the 1-methyl-4-phenylpyridinium (MPP^+)-induced cytotoxicity of SH-SY5Y cells which mimics the

pathological symptom of Parkinson's disease by enhancing autophagy which increased the degradation of aggregated or misfolded proteins from cells (Kim et al., 2014).

2.7 *Azadirachta indica* A. Juss., 1830

Azadirachta indica (AI) belongs to the Meliaceae family. It is a large evergreen tree native to tropical and semitropical areas such as India, Bangladesh, Pakistan and Nepal (Figure 2.8). AI is commonly known as neem in English or Sa Dao in Thai. The leaves of AI are used as Ayurvedic herbal medicine for the treatment of oncologic diseases, microbial infections, ulcer and inflammation, skin diseases, dental disorders and hypoglycemia. Furthermore, it was reported as sedative-hypnotics for mental symptoms such as apathy, dysphoria, anxiety and depression in Thai traditional medicine (Muangman, 2002).

Figure 2.8 Photograph of *Azadirachta indica* plant.
(Available from: www.prota4u.org and www.botany.hawaii.edu)

The main chemical constituents of AI's leaves are azadirachtin, nimbin, nimbanene, nimbandiol, nimbolide, nimbiol, 6-desacetylnimbinene, 7-desacetyl-7-benzoylazadiradione, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione, quercetin and ß-sitosterol, ascorbic acid, n-hexacosanol and amino acid (Alzohairy, 2016). Pharmacological activities of AI extracts are summarized and listed hereafter.

The leaf aqueous extract of AI exhibited high DPPH radical scavenging activity (EC_{50} = 26.48 µg/mL) almost the same as standard butylated hydroxytoluene (IC_{50} = 14.87 µg/mL) (Sithisarn et al., 2005).

There is no information available regarding the neuroprotective property of AI in cell-based assay. However, in *in vivo* study reported that subchronic administration of aqueous extract of AI attenuates ibotenic acid and colchicine-induced dysfunction and loss of basal forebrain cholinergic neurons that is related to severe impairment of learning and memory in rat (Raghavendra et al., 2013).

2.8 *Piper retrofractum* **Vahl., 1804**

Piper retrofractum (PR), a flowering vine, is a member of Piperaceae family, which is distributed in tropical and subtropical regions of the world, particularly in Southeast Asia (Figure 2.9). PR is commonly known as Javanese long pepper in English or Di Pli in Thai. The fruits of PR are widely used in ethnomedicine to treat asthma, bronchitis, hemorrhoids, fever and abdominal pain in Southeast Asian countries (Kubo et al., 2013). Besides, it has been used as one of the ingredients in medicine texts of King Rama V to alleviate inflammation of the meninges (Department, 1999).

Figure 2.9 Photograph of *Piper retrofractum* plant.
(Available from: www.yingthai-mag.com and www.frynn.com)

The main chemical constituents of PR's fruits are piperidine alkaloids (i.e., piperine, pipernonaline, and dehydropipernonaline) and amide alkaloids (i.e., piperodione, (2E,12E)-pipertridecadienamide and N-isobutyl-(2E,4E,10Z)-hexadeca-2,4,10- trienamide) (Kim et al., 2011; Kubo et al., 2013). Pharmacological activities of PR extracts are summarized and listed hereafter.

Piperodione, isolated from methanol extract of PR, possessed neurotropic effect by promoting nerve growth factor-mediated signal transduction and neurite outgrowth in PC12 cells (Kubo et al., 2013).

2.9 *Asparagus racemosus* Willd., 1799

Asparagus racemosus (AR), a woody climber, belongs to the family Asparagaceae which is found in abundance in tropical climates throughout Asia, Australia and Africa (Figure 2.10). AR is commonly known as shatavari in English or Rag Samsib in Thai. The roots of AR have long been traditionally used in Ayurveda as rejuvenation drug for enhancing fertility and relieving menopausal symptoms. It is also used for dry coughs, gastric ulcers and especially nervous system disorders as nerve tonics (Goyal et al., 2003). In Thailand, it was reported for the treatment of lactation failure, dysentery, peptic ulcer, pruritus, rheumatic diseases and geriatric syndromes (Potduang et al., 2008). Moreover, AR was reported to possess phytoestrogenic activity and used as a hormone modulator for treatment of post-menopausal symptoms (Singh and Kulkarni, 2002).

Figure 2.10 Photograph of *Asparagus racemosus* plant.
(Available from www.technoayurveda.com)

The main chemical constituents of AR's roots are steroidal saponins (i.e., shatavarins I–IV), sapogenins (i.e., sarsasapogenin), polycyclic alkaloid (i.e., asparagamine), racemofuran, racemosol and kaempferol (Bopana and Saxena, 2007). Pharmacological activities of AR extracts are summarized and listed hereafter.

There is report in term of neuroprotective property of AR in cell culture. Nevertheless, the methanolic extract of AR roots has been reported neuroprotective effects in *in vivo* rat models. It reversed scopolamine and sodium nitrite-induced acetylcholinesterase deficiency and amnesia in rat. It also diminished pentylenetetrazol-induced kindling-associated depression and memory deficit in rat through elevating acetylcholinesterase activity (Ojha et al., 2010; Pahwa and Goel, 2016).

CHAPTER III

ANTI-Aβ AGGREGATION, Aβ DISAGGREGATION AND ANTIOXIDANT POTENCIES OF THAI HERBAL EXTRACTS: BIOCHEMICAL ASSAY

1. Introduction

The progressive extracellular aggregation of Aβ which subsequently forms amyloid fibrils and plaques in the brain is identified as crucial events of the pathogenesis of AD. An Aβ monomeric form is derived from the proteolysis of APP by sequential cleavage of β- and γ-secretases (O'Brien and Wong, 2011). There are two major forms of Aβ, namely Aβ40 and Aβ42, of which Aβ42 is more neurotoxic than Aβ40 and shows a higher propensity to aggregate into fibrils and plaques between neurons (Ahmed et al., 2010). In general, Aβ monomer favors the α-helix conformation, whereas an excess Aβ monomer can cause its structural change to β-strand conformation which has high tendency to interact with hydrophobic part of other Aβ molecule and aggregate into large molecular weight species of Aβ (Vendersteen, 2012). The aggregated forms of monomeric Aβ, including soluble oligomers, protofibrils and insoluble fibrils, can generate neurotoxicity through multiple pathways, especially stimulating the excessive production of neurotoxin molecules such as ROS which result in oxidative stress, cellular damage and consequently neuronal death (Cervellati et al., 2016). Therefore, inhibiting and reversing the Aβ aggregation processes are attractive strategy in the development of agents for the AD treatment. Besides, the current drugs available in the pharmaceutical markets for AD such as acetylcholinesterase inhibitor do not prevent or reverse the Aβ fibril formation (Salomone et al., 2012; Sgarbossa, 2012; Yiannopoulou and Papageorgiou, 2013).

Due to the great interest in plants as potential sources for drug development against AD, the ethnopharmacological screening of the plants is one of the useful tools for the discovery of the new drugs (Dastmalchi, 2007). Numerous species of herbs have long been traditionally used to cure the cognitive symptoms in Thailand. However, no scientific research supports the neurophramacological effects of these Thai herbs on Aβ-based pathology. In this study, nine Thai herbs; *Caesalpinia sappan* (CS)*, Thunbergia laurifolia* (TL)*, Rhinacanthus nasutus* (RN)*, Tabernaemontana divaricata*

(TD)*, Cyperus rotundus* (CR)*, Terminalia chebula* (TC)*, Azadirachta indica* (AI)*, Piper retrofractum* (PR), and *Asparagus racemosus* (AR) were selected based on their ethnobotanical uses to remedy the cognitive symptoms recorded in Thai scriptures or gazettes and the previous studies on the fields of nervous system disorders (Brimson et al., 2012; Kubo et al., 2013; Kumar and Khanum, 2013; Moon et al., 2010; Na et al., 2004; Nakdook et al., 2010; Ojha et al., 2010; Raghavendra et al., 2013; Tangpong and Satarug, 2010). These herbs were extracted with 95% ethanol using Soxhlet extractor and screened *in vitro* for anti-Aβ aggregation and Aβ fibril disaggregation potency using the biochemical-based testing (Thioflavin T (ThT) fluorescence assay).

Additionally, many researches demonstrated that oxidative stress, which caused by Aβ, also plays a key role in AD pathogenesis that underlies the neurotoxicity and consequent neuronal loss (Greenough et al., 2013; Hajieva and Behl, 2006). Apart from the direct neurotoxic effect, oxidative stress may enhance the β-secretase activity, and leads to the increased Aβ production and accumulation (Contestabile, 2001). Therefore, the selected nine Thai herbs were also screened for their antioxidant activity using 2,2-diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging assay.

2. Materials and Methods

2.1 Collection and identification of Thai herbs

Nine Thai herbs were collected in October 2013 - July 2014. Details in each herbs used in this study are given in Table 3.1. Herbs were authenticated by a specialist in plant taxonomy at the Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. Voucher specimens were deposited at the Professor Kasin Suvatabhandhu Herbarium, Chulalongkorn University (Figure 3.1).

2.2 Extraction of Thai herbs

Plant materials were dried at 40 °C and pulverized into a fine powder. The 500 g of each plant powder was extracted with 3 L of 95% ethanol twice in Soxhlet apparatus following Ong (2004). The apparatus was heated at 60 °C and the extraction process was performed successively for 6 to 8 h (until the solvent in siphon tube becomes colorless). The ethanol extracts were pooled and filtrated through Whatman No.4 filter paper with vacuum suction pump and evaporated in a rotary evaporator

under vacuum in a 60 °C heated water bath. The extraction yield of crude herbal extract was calculated in the following equation:

Extraction yield (%) = (Extracted weight/Dried weight of plant powder) $\times$ 100.

To eliminate dregs and lipophilic substances from the extracts, an additional extraction was performed with 95% ethanol and hexane (1:1 v/v) by using separating funnel. In brief, 25 mg of each crude herbal extract was dissolved with 250 mL of 95% ethanol, mixed with 250 mL of hexane, placed in the funnel for 30 to 60 minutes to allow the mixture separated into two layers. Ethanol-soluble layer in a lower phase was collected, evaporated and stored in amber glass bottle at -20 °C for subsequent uses.

Table 3.1 Scientific name, code, family, used part, location and date of collection, voucher number and extraction yield of nine Thai herbs selected for this study**.**

Scientific name	Code	Family	Used part	location and date of collection	Voucher number	Yield (%)
Caesalpinia sappan	CS	Leguminosae-Caesalpiniodeae	Stem	Khonkaen, Sept. 2013	A 015242/BCU	14.53
Thunbergia laurifolia	TL	Acanthaceae	Leaves	Kalasin, Oct. 2013	A 015240/BCU	17.52
Rhinacanthus nasutus	RN	Acanthaceae	Leaves	Chachoengsao, Oct. 2013	A 015280/BCU	16.37
Tabernaemontana divaricata	TD	Apocynaceae	Root	Chonburi, July 2014	A 015241/BCU	20.16
Cyperus rotundus	CR	Cyperaceae	Root	Nakhon Pathom, Jan. 2014	A 015318/BCU	10.72
Terminalia chebula	TC	Combretaceae	Fruit	Kalasin, Dec. 2013	A 015243/BCU	48.22
Azadirachta indica	AI	Meliacea	Leaves	Kalasin, Oct. 2013	A 015281/BCU	12.57
Piper retrofractum	PR	Piperaceae	Fruit	Kanchanaburi, Oct. 2013	A 015242/BCU	14.15
Asparagus racemosus	AR	Asparagaceae	Root	Maha Sarakham, Jan. 2014	A 015239/BCU	24.54

Caesalpinia sappan (CS)
Voucher number: A 015242/BCU

Thunbergia laurifolia (TL)
Voucher number: A 015240/BCU
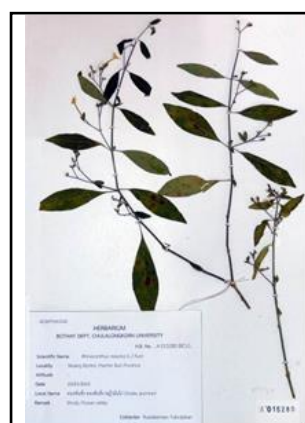
Rhinacanthus nasutus (RN)
Voucher number: A 015280/BCU

Tabernaemontana divaricate (TD)
Voucher number: A 015241/BCU

Cyperus rotundus (CR)
Voucher number: A 015318/BCU

Terminalia chebula (TC)
Voucher number: A 015243/BCU

Azadirachta indica (AI)
Voucher number: A 015281/BCU

Piper retrofractum (PR)
Voucher number: A 015242/BCU

Asparagus racemosus (AR)
Voucher number: A 015239/BCU

Figure 3.1 Photograph of nine Thai herbs selected for this study. All herbal specimens were collected and photographed by Miss Ruedeemars Yubolphan. The specimens were deposited at the Professor Kasin Suvatabhandhu Herbarium, Chulalongkorn University (BCU).

2.3 Preparation of crude herbal extracts

One-hundred mg of each crude herbal extract was dissolved in 100% dimethylsulfoxide (DMSO) at a concentration of 100 mg/mL and stored at -20 °C for subsequent use.

2.4 Preparation of chemicals and reagents

2.4.1 Human amyloid-β peptide

Human amyloid-β peptide which contains 42 amino acids (referred to as Aβ42) was purchased from Abcam, MA, USA (catalog no. ab120301). The peptide was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP; Merck, Darmstadt, Germany) at a concentration of 200 μM. Then, it was sonicated in a water bath for 10 min, dried under gentle stream of nitrogen, resuspended with 100% DMSO and stored at -20 °C for subsequent use.

2.4.2 Resveratrol

Resveratrol was purchased from Merck, Darmstadt, Germany. This phenolic compound was used as positive control because of its ability to inhibit Aβ aggregation and stimulate Aβ fibril disaggregation (Feng, 2009). Resveratrol was dissolved in 100% DMSO at a concentration of 20,000 μM and stored at -20 °C for subsequent use.

2.4.3 Thioflavin T fluorescence dye

ThioflavinT (ThT), a cationic benzothiazole dye, has been most widely used to quantify the structure of Aβ fibrils. ThT can insert within the grooves throughout the length of Aβ fibrils which come from the β-strand interaction between each Aβ molecule and aggregate to form the fibrils (Biancalana and Koide, 2010). The binding of ThT and Aβ fibrils induces the fluorescence excitation (Ex) and emission (Em) wavelengths of ThT dramatically shift to longer values (from 385 nm to 450 nm in Ex and from 445 nm to 482 nm in Em, respectively). This change leads to the increase in fluorescence intensity which can be detected by fluorescence spectrophotometer.

In order to prepare ThT stock solution, ThT dye (Sigma-Aldrich, MO, USA) was dissolved in absolute ethanol (Merck, Darmstadt, Germany) at a concentration of

100 µM, aliquot into 1.5 mL amber microcentrifuge tube and stored at -20 °C for subsequent use. When being assay, ThT stock solution was diluted to 10 µM with 500 mM glycine-NaOH buffer, pH 8.5.

2.5 Thioflavin T (ThT) fluorescence assay

To determine the inhibitory effects of the nine candidate herbal extracts on Aβ42 aggregation, ThT fluorescence assay was performed. ThT exhibits enhanced fluorescence upon binding to β-strand structures of Aβ fibrils and is commonly used as a standard to diagnose Aβ species which aggregate into Aβ fibrils form (Biancalana and Koide, 2010).

To assess the effect of crude herbal extracts on Aβ aggregation, crude herbal extracts were diluted to 0.01, 0.1, 1, 10 and 100 µg/mL with phosphate-buffered saline (PBS buffer; 137 mM NaCl, 2.7 mM KCl, 6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Subsequently, all concentrations of the extracts, 0.1% DMSO (a negative control) and 10 µM resveratrol were mixed with 10 µM Aβ42 in microcentrifuge tubes and incubated at 37 °C without shaking for 28 h (Feng, 2009). At the end of the incubation, 80 µL of Aβ42 from the incubated samples were pipetted into 96-well white flat bottom plate and mixed with 20 µL of 10 µM ThT (Sigma-Aldrich, MO, USA) in 50 mM glycine-NaOH buffer, pH 8.5. The ThT fluorescence intensity (in arbitrary units, a.u.) was measured using Cary Eclipse fluorescence spectrophotometer (Varian, CA, USA) with 450/485 nm excitation/emission filter set (LeVine, 1993). The experiment was run in three replicates. The value of the fluorescence intensity of the negative control group, Aβ42 peptide in the absence of the extracts, was set as 100% aggregation of Aβ42. For the treatment groups, Aβ42 peptide in the presence of the extracts, % aggregation of Aβ42 was calculated based on the following equation:

$$\% \text{ Aggregation of } A\beta42 = (F_{Extract}/F_{A\beta}) \times 100.$$

Where $F_{Extract}$ is the average value of fluorescence intensity of the Aβ42 peptide in the presence of the extracts, and $F_{A\beta}$ is the average value of fluorescence intensity of Aβ42 peptide in the absence of the extracts.

Furthermore, data were analyzed and calculated for the half-maximal effective concentration (EC_{50}). Briefly, the dose-response curve which demonstrates the relationship between the log of concentrations of the extract and the %change in Aβ42 aggregation was constructed. Thereafter, the curve was fitted with non-linear regression model using built-in equation of dose-response stimulation: log(agonist) *vs*. response (Y=Bottom + (Top-Bottom)/(1+10$^{(LogEC50-X)}$)). The EC_{50} value was calculated by a non-linear, least squares curve-fitting algorithm using GraphPad Prism 5.0 software (GraphPad Software, Inc., CA, USA).

To assess the effect of crude herbal extracts on Aβ fibril disaggregation, the Aβ42 was first incubated in microcentrifuge tubes at 37 °C without shaking for 28 h. Then the crude herbal extracts, 0.1% DMSO and 10 μM resveratrol were added into the Aβ42 fibrils solution and incubated at 37 °C for another 28 h (Feng, 2009). Afterwards, the aggregation of the fibrils was measured by ThT fluorescence assay as described above. The experiment was run in three replicates and % aggregation of Aβ42 and EC_{50} were calculated as done in the Aβ aggregation experiment mentioned above.

2.6 Antioxidant activity determination

For quantitating antioxidant activity, free stable radicals which generally used in this kind of evaluation is 2,2-diphenyl-1-Picrylhydrazyl (DPPH; Sigma-Aldrich, MO, USA). An ability of herbal extracts to donate hydrogen atom to unpaired electron of DPPH· free radical molecule (deep violet color) and turn it into reduced form (pale yellow color) is the basis of this assay.

The protocol of determination of antioxidant activity of each crude herbal extract was slightly modified from the method used by Aluko and Monu (2003). In a 96-well plate, 40 μl of each extract (0.01, 0.1, 1, 10 and 100 μg/mL) was combined with 160 μl of 100 mM DPPH dissolved in 95% methanol and kept to react in the dark for 15 min at room temperature. The absorbance was measured at the wavelength of 517 nm using microplate reader (BioTek, VT, USA). Vitamin C (L-ascorbic acid) was used as the positive control. Higher DPPH radical scavenging activity conveys a lower absorbance. The percentage of inhibition of radical scavenging activity was calculated from the following equation:

$$\% \text{ Inhibition} = [(Ac - Acb) - (As - Asb)]/(Ac - Acb) \times 100$$

Where Ac is the absorbance of deionized water plus DPPH (in methanol), Acb is the absorbance of the blank (deionized water plus methanol without DPPH), As is the absorbance of the sample plus DPPH (in methanol), and Asb is the absorbance of the sample plus methanol without DPPH. Different sample concentrations were used to obtain a calibration curve and calculated the half-maximal inhibitory concentration (IC_{50}) using GraphPad Prism 5.0 software with built-in equation of dose-response inhibition: log(agonist) *vs*. response (Y=Bottom + (Top-Bottom)/(1+10$^{(LogIC50-X)}$)).

2.7 Statistical analyses

The results were expressed as mean ± standard error (SE) from three replicates in each treatment. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Turkey's HSD post-hoc comparison tests using the Statistical Package for the Social Sciences (SPSS) program. The level of significance for all statistical tests was accepted at $p<0.05$. The EC_{50} and IC_{50} values were calculated using GraphPad Prism program with built-in equations for non-linear regression model of dose-response stimulation and dose-response inhibition, respectively.

3. Results

3.1 Inhibition effect of crude herbal extracts on Aβ42 aggregation

From Table 3.2, the aggregation of Aβ42 into fibril form was significantly reduced by all nine herbal extracts in a concentration-dependent manner. It is worthily noted that the stem extract of CS, and the leaves extract of RN and TL inhibited Aβ42 aggregation in the greatest degree among the nine herbal extracts with EC_{50} values of 10.21±0.55, 45.80±6.19 and 52.29±6.59 μg/mL, respectively (Figure 3.2). Moreover, at the concentration of 1 μg/mL, CS showed the higher anti-Aβ aggregation ability than that of the 10 μM or 2.28 μg/mL resveratrol, a natural-based chemical which elicits a potent anti-amyloidogenic activity (Sgarbossa, 2012).

Table 3.2 Effect of the nine crude herbal extracts, *Caesalpinia sappan* (CS), *Thunbergia laurifolia* (TL), *Rhinacanthus nasutus* (RN), *Tabernaemontana divaricate* (TD), *Cyperus rotundus* (CR), *Terminalia chebula* (TC), *Azadirachta indica* (AI), *Piper retrofractum* (PR) and *Asparagus racemosus* (AR), on Aβ aggregation of The extracts at concentration 0.01-100 μg/mL were incubated with 10 μM Aβ42 for 28 h. Resveratrol (10 μM) was used as a positive control. % Aggregation of Aβ42 in resveratrol treated-group = 81.20±0.92%.

Plant extract	% Aggregation of Aβ42 †					
	0 μg/mL (Control)	0.01 μg/mL	0.1 μg/mL	1 μg/mL	10 μg/mL	100 μg/mL
CS	100±0.41	97.10±0.69	93.41±1.13***	80.74±1.48***	50.31±1.16***	20.52±1.20***
TL	100±0.50	97.71±0.54	94.87±0.76**	90.14±0.33***	71.66±1.82***	42.19±1.86***
RN	100±0.50	101.61±1.30	93.31±1.18	96.25±0.26*	74.24±1.91***	36.50±2.44***
TD	100±0.71	91.61±5.22	94.38±1.22	90.96±1.64**	86.66±2.32***	72.41±1.42***
CR	100±0.50	104.02±2.19	103.00±1.37	100.64±0.78	79.48±0.69***	49.15±0.96***
TC	100±0.41	104.96±1.72	102.38±1.14	100.14±1.65	86.53±2.86***	59.85±3.43***
AI	100±0.41	100.92±1.47	100.01±0.63	100.88±1.64	93.64±1.17***	75.64±1.16***
PR	100±0.41	103.60±1.70	103.20±1.36	103.96±1.75	99.15±1.03	87.99±1.81***
AR	100±0.50	104.94±1.93	105.41±0.98	105.84±1.15	100.99±1.00	86.08±2.78***

† Aβ42 aggregation was expressed as percentage of the control (no-herbal extract) group. The data are expressed as mean±SEM in three replicates, where *, ** and *** represent $p < 0.05$, 0.01 and 0.001, respectively, compared to the Aβ-treated control groups.

Figure 3.2 Dose-response curve of the nine herbal extracts; Caesalpinia sappan (CS), Thunbergia laurifolia (TL), Rhinacanthus nasutus (RN), Tabernaemontana divaricate (TD), Cyperus rotundus (CR), Terminalia chebula (TC), Azadirachta indica (AI), Piper retrofractum (PR) and Asparagus racemosus (AR), on Aβ aggregation. Resveratrol (10 μM) was used as a positive control. EC_{50} values of herbal extracts were calculated by a non-linear, least squares curve-fitting algorithm using GraphPad Prism software. The EC_{50} of CS, TL and RN were 10.21±0.55, 52.29±6.59 and 45.80±6.19 μg/mL, respectively.

3.2 Effect of crude herbal extracts on Aβ42 fibril disaggregation

With regard to the inhibitory effect on Aβ42 aggregation, only the crude herbal extracts at concentrations that exhibited anti-Aβ aggregation potency were selected for the investigation on Aβ42 fibril disaggregation. From the nine extracts that were examined the Aβ fibril disaggregation potency, only eight extracts, except the root extract of AR, were significantly reduced Aβ42 fibrils. As shown in Table 3.3, the percentage of Aβ42 aggregations after incubated for 28 h with the extracts were reduced, ranging from 20.59±1.45 to 93.64±0.79% as compared with the control (Aβ42 fibrils alone) group which denoted as 100% aggregation. Similar to the anti-Aβ aggregation potency presented above, the extracts of CS (EC_{50} = 11.95±2.20 μg/mL), RN (EC_{50} = 56.46±5.81 μg/mL) and TL (EC_{50} = 72.49±10.74 μg/mL) also elicited the highest disaggregation ability on Aβ fibril formation (Figure 3.3).

Table 3.3 Effect of the nine crude herbal extracts; *Caesalpinia sappan* (CS), *Thunbergia laurifolia* (TL), *Rhinacanthus nasutus* (RN), *Tabernaemontana divaricate* (TD), *Cyperus rotundus* (CR), *Terminalia chebula* (TC), *Azadirachta indica* (AI), *Piper retrofractum* (PR) and *Asparagus racemosus* (AR), on Aβ fibril disaggregation. Only the crude herbal extracts at the anti-aggregation concentrations were selected for this study. Aβ42 was incubated for 28 h to allow Aβ42 monomers aggregated into mature fibrils, and then the extracts were added into the Aβ42 fibrils and incubated for another 28 h. Resveratrol (10 μM) was used as a positive control. % Aggregation of Aβ42 in resveratrol treated-group = 89.43±1.90%.

Plant extract	% Aggregation of Aβ42 [†]				
	0 μg/mL (Control)	0.1 μg/mL	1 μg/mL	10 μg/mL	100 μg/mL
CS	100±1.51	94.64±1.95	83.40±0.95 ***	52.49±4.46 ***	20.59±1.45 ***
TL	100±1.51	94.48±1.59	89.60±0.33 ***	76.07±1.76 ***	47.35±2.82 ***
RN	100±1.51	-	93.64±0.79 **	77.88±2.45 ***	40.21±1.97 ***
TD	100±1.51	-	93.28±4.80	91.44±1.79 *	77.82±3.31 ***
CR	100±1.51	-	-	77.73±2.12 ***	45.50±2.28 ***
TC	100±1.51	-	-	92.80±1.38 **	67.24±0.93 ***
AI	100±1.51	-	-	96.46±1.80	74.42±2.41 ***
PR	100±1.51	-	-	-	88.11±2.50 ***
AR	100±1.51	-	-	-	92.64±2.51

[†] Aβ42 aggregation was expressed as percentage of the control (no-herbal extract) group. The data are expressed as mean±SEM in three replicates, where *, ** and *** represent p<0.05, 0.01 and 0.001, respectively, compared to the control groups.
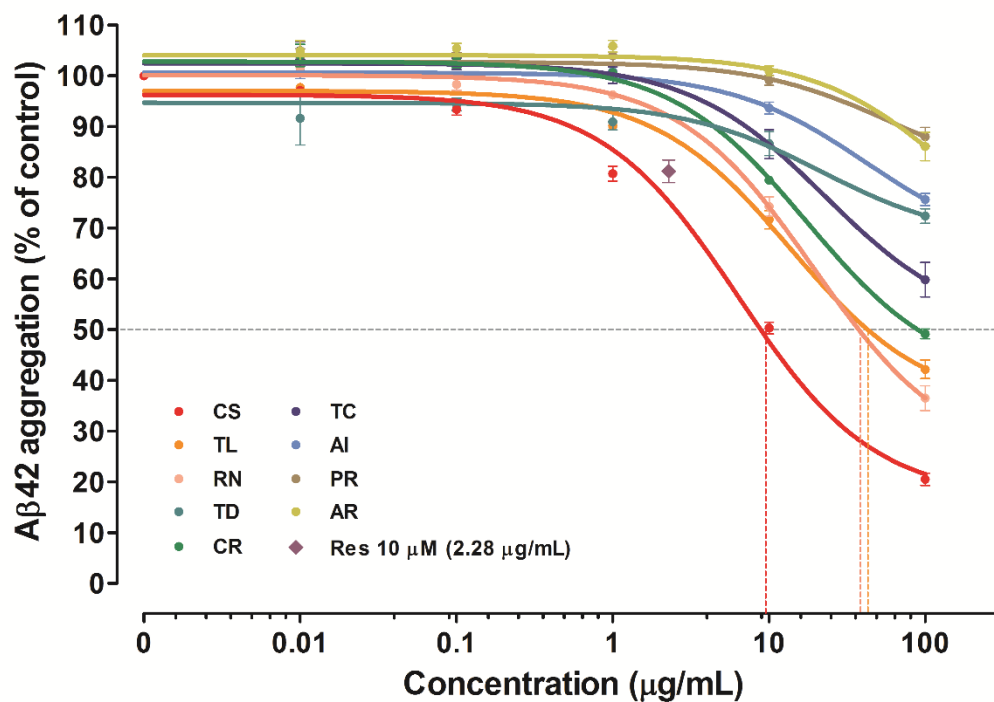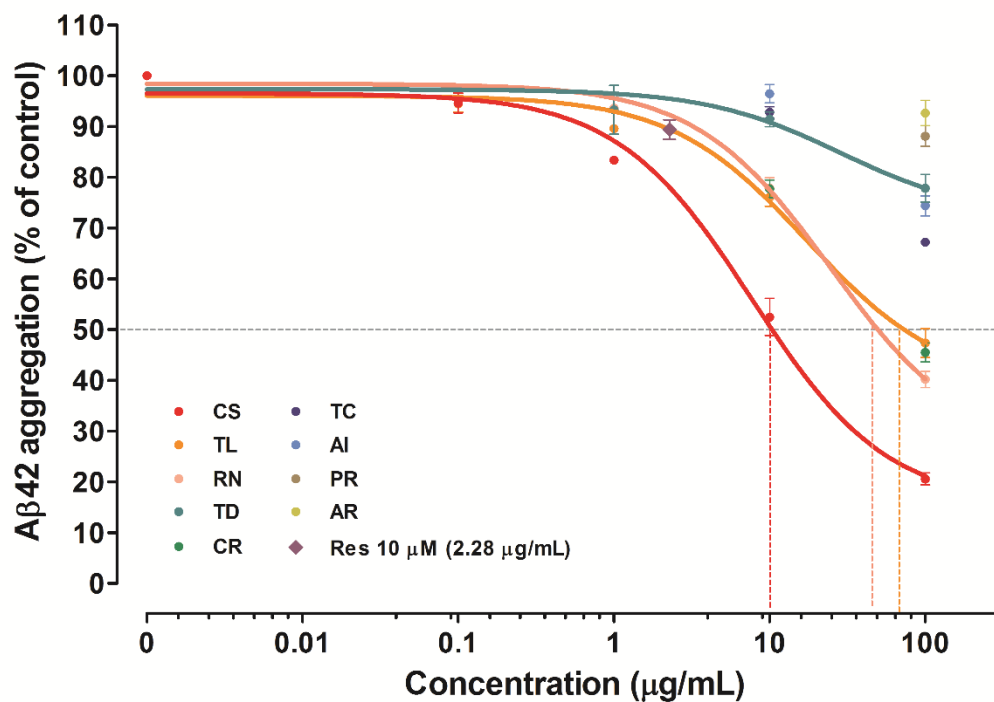
Figure 3.3 Dose-response curve of the nine herbal extracts; Caesalpinia sappan (CS), Thunbergia laurifolia (TL), Rhinacanthus nasutus (RN), Tabernaemontana divaricate (TD), Cyperus rotundus (CR), Terminalia chebula (TC), Azadirachta indica (AI), Piper retrofractum (PR) and Asparagus racemosus (AR), on Aβ fibril disaggregation. Resveratrol (10 μM) was used as a positive control. EC_{50} values of herbal extracts were calculated by a non-linear, least squares curve-fitting algorithm using GraphPad Prism software. The EC_{50} of CS, TL and RN were 11.95±2.20, 72.49±10.74 and 56.46±5.81 μg/mL, respectively.

3.3 Antioxidant activity of crude herbal extracts

From Table 3.4, all nine herbal extracts could decrease DPPH free radicals in the following order; TL > CR > AI > TD > L-ascorbic acid (positive control) > PR > CS > TC > RN = AR. It is worthily noted that TL, CR, AI and TD extracts (with IC_{50} values of 0.17 ± 0.01, 0.19 ± 0.001, 0.49 ± 0.01 and 2.57 ± 0.07 µg/mL, respectively) exhibited antioxidant activity higher than that of L-ascorbic acid ($IC_{50} = 3.52\pm0.35$ µg/mL), which has been reported to possess strong antioxidant activity. In regard to the classification of Kuete and Efferth (2010) that they categorized the plant extracts which possess antioxidant activity against free radicals in different degree into three groups; high ($IC_{50} < 50$ µg/mL), moderate ($50 < IC_{50} < 100$ µg/mL) and low antioxidant activity ($IC_{50} > 100$ µg/mL), all nine herbal extracts in this study belong to the group of high antioxidant activity.

Table 3.4 Antioxidant activity determination by DPPH assay of the nine crude herbal extracts; *Thunbergia laurifolia* (TL), *Cyperus rotundus* (CR), *Azadirachta indica* (AI), *Tabernaemontana divaricate* (TD), *Piper retrofractum* (PR), *Caesalpinia sappan* (CS), *Terminalia chebula* (TC), *Rhinacanthus nasutus* (RN), *Asparagus racemosus* (AR), and L-ascobic acid (Positive control).

Plant extract	Antioxidant activity IC_{50} (µg/mL)
TL	0.17±0.010
CR	0.19±0.011
AI	0.49±0.010
TD	2.57±0.070
L-ascobic acid (Positive control)	**3.52±0.350**
PR	3.75±0.040
CS	3.92±0.100
TC	8.93±0.270
AR	>10
RN	>10

4. Discussion

Most of therapeutic strategies for AD were focused on inhibiting Aβ aggregation, stimulating Aβ fibril disaggregation and also reducing the accumulation of ROS, main types of free radical that is caused by Aβ aggregates. In these recent years, several studies have been reported that many compounds elicited their beneficial effects against aggregation processes of Aβ. However, the efficacy of those compounds was not high enough to meet the requirements for AD treatment (Stefani and Rigacci, 2014). Consistent with their low toxicity and absence of significant side effects, natural-based products such as herbs represent an interesting alternative in the search for new anti-AD agents.

Using the biochemical-based testing, it revealed that eight herbal extracts: CS, TL, RN, TD, CR, TC, AI and PR exert anti-Aβ aggregation and Aβ fibril disaggregation properties. Among the eight herbal extracts, the CS, RN and TL extracts exhibited the highest potential against Aβ fibril formation, which might be due to the active components containing in the extracts.

CS stem has been listed in Thai herbal monograph as psychotropic plant possessing sedative property (Wongprasert, 2003). Bibliographic information about the biological activities and the chemical constituents isolated from CS extract revealed that brazilin is the main component which has various biological activities such as anti-inflammatory and anti-platelet aggregation activity (Nirmal et al., 2015). It was reported that brazilin possesses anti-amyloidogenic properties by binding with phenyl ring in Phe20 of Aβ42 monomer and convert the Aβ to unstructured form which rarely aggregates with other Aβ molecules. Furthermore, brazilin can interfere with the intermolecular bonding of Asp23-Lys28 between Aβ molecules within Aβ fibrils and ultimately lead to destabilizing fibrils to non-toxic forms (Du et al., 2015).

RN leaves have long been used as CNS stimulant or antidepressant for anxiety and depression (Subjaroen, 2005). It was revealed that the main phytochemical constituents of RN leaves are naphthoquinones (Bukke, 2011). Naphthoquinone and its derivatives could exhibit anti-Aβ aggregation property and stimulate Aβ fibril

disaggregation through the disruption of hydrophobic interactions between Aβ molecules (Bermejo-Bescos et al., 2010).

TL leaves were reported in monograph of Thai traditional medicine as nerve tonic treatment for migraines and chronic headaches (Thatsanamit, 2012). The main chemical components of TL are apigenin and phenolic acids. Previously, it was reported that apigenin exhibited potency on aggregation processes of Aβ. It can interact with protofibrillar form of Aβ and then inhibit fibrillogenesis together with transform Aβ fibrils into non-toxic aggregated Aβ (Thapa et al., 2011). Recent study was also revealed that rosmarinic acid, one of phenolic acid found in TL, also possessed anti-Aβ aggregation and Aβ fibril disaggregation properties (Ono et al., 2012). It can inhibit aggregation by stabilizing Aβ monomer into native form (α-helix) and reverse Aβ fibrils aggregation by transforming it into large aggregated Aβ (Ono et al., 2012).

Taken together, brazilin from CS stem, naphthoquinones from RN leaves, and apigenin and rosmarinic acid from TL leaves might be the candidate compounds acting against Aβ aggregation and Aβ fibril disaggregation. However, if these compounds are responsible to these properties, it needs investigation.

Recently, an interest in the role of oxidative stress in neurological disorders has been highlighted. There is evidence that free radicals produced by several redox reactions in the human body are implicated in contributing to protein oxidation, DNA damage, inflammation, and neurodegenerative disorders including AD (Huang et al., 2016; Markesbery, 1997). Moreover, accumulated Aβ also promotes free radical formation in the brain (Takashima et al., 1993; Yatin et al., 1998). The natural compounds such as vitamins, polyphenolics and flavonoids which play a role in the defense against free radicals have been used as supplementary antioxidants for the AD treatment (Ansari and Khodagholi, 2013). The results of this study showed that all nine herbs elicited antioxidant activity tested by DPPH radical scavenging assay. Remarkably, TL, CR, AI and TD extracts displayed higher antioxidant activity than the L-ascorbic acid, a strong antioxidant agent. The high antioxidant activities shown by TL, CR and AI might be due to the presence of abundant phenolic compounds which have been reported in the previous studies (Jing et al., 2012; Sithisarn et al., 2006;

Tangpong and Satarug, 2010; Yazdanparast and Ardestani, 2007). However, no reports regarding antioxidant activity of TD have been found so far. Only a sole *in vivo* study reported that alkaloid-rich TD extract increased the levels of anti-oxidant agents such as glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) (Gupta, 2004). It is interesting to note that the ethanol extract of TD exhibited antioxidant activity with DPPH in this study. It is most likely that this work offers the first report of scavenging activity against free radicals of the TD.

According to the combination of results of all biochemical-based assays mentioned above, it can propose that TL and CR had the highest potential to counteract the AD pathogenesis. This is because TL and CR possessed a high potency on anti-Aβ aggregation and Aβ fibril disaggregation, together with elicited a strong antioxidant potency.

In conclusion, from the nine herbal extracts that were screened in this study, only eight extracts had a potential to inhibit Aβ aggregation, stimulate Aβ fibril disaggregation and display antioxidant activity. Nonetheless, since only the *in vitro* biochemical-based assay was performed here, it might not be certain to tell whether or not these eight herbal extracts should be developed further for AD therapeutics. The next step of test on neuronal cells will be conducted and reported in Chapter IV.

CHAPTER IV
PROTECTIVE EFFICACY AGAINST Aβ-INDUCED NEUROTOXICITY AND POTENTIAL MODULATION ON Aβ PRODUCTION AND CLEARANCE OF THAI HERBAL EXTRACTS: CELL-BASED ASSAY

1. Introduction

AD is clinically characterized by a decline in memory and cognitive function which affects geriatric people at the age over 65 years (Association, 2015). The neuropathological feature of AD is an extracellular deposition of senile plaques, which primarily composes of various sizes of toxic peptide namely Aβ, in hippocampus and cortex (Ferrer, 2012). Naturally, Aβ occurs in the brain and cerebrospinal fluid throughout human lifetime; however, the imbalance between their production and clearance can lead to the increment of Aβ in multiple forms such as monomers, oligomers, insoluble fibrils, and plaques (Hardy and Selkoe, 2002). An excessive accumulation of Aβ can subsequently trigger a series of neurotoxic events in AD such as synaptic dysfunction, acetylcholine diminution, free radical generation, oxidative damage, neurofibrillary tangle formation and chronic inflammatory. These events contribute to a malfunction of many intracellular organelles, e.g., mitochondria, lysosome, Golgi apparatus, and are responsible for the induction of apoptotic cell death (Cavallucci et al., 2012).

Aβ is derived from proteolysis of transmembrane protein–APP. Actually, APP can be processed by two metabolic pathways: non-amyloidogenic and amyloidogenic. In non-amyloidogenic pathway, APP is enzymatically cleaved by α-secretase such as ADAM10 and followed by γ-secretase. This pathway produces non-toxic fragment and precludes Aβ production. Conversely, in amyloidogenic pathway, APP is sequentially cleaved by β-secretase or BACE1 and then γ-secretase which generates Aβ toxic fragment into extracellular space (O'Brien and Wong, 2011). Within the extracellular space, Aβ can be removed by Aβ-degrading enzymes such as IDE and NEP. IDE is found to degrade only the monomeric form of Aβ whereas NEP is able to hydrolyze both monomeric and oligomeric forms of Aβ (Miners et al., 2011). Hence, the potential strategies in the battle against AD focus on the discovery of agents which can prevent

Aβ-induced neurotoxicity and regulating the balance between production and clearance of Aβ.

Regarding the results of Chapter III, the eight crude herbal extracts which possessed the potency of anti-Aβ aggregation, disaggregating Aβ fibrils and antioxidation were selected and tested their effects on (i) cytotoxicity (ii) Aβ-induced neurotoxicity and (iii) expression of genes associated with Aβ production (*App*, *Bace1* and *Adam10*) and Aβ clearance (*Ide* and *Nep*) in SH-SY5Y human neuroblastoma cell.

2. Materials and Methods

2.1 Crude herbal extracts

The crude extracts of eight herbs; CS, TL, RN, AI, TD, CR, TC and PR were selected for this study. The process of the ethanol extraction and the elimination of a dregs and lipophilic substances from the extracts were described in Chapter III.

2.2 Resveratrol

Resveratrol was used as positive control in Aβ-induced neurotoxicity assay because it can effectively attenuate the cytotoxicity of Aβ42 to SH-SY5Y cells (Feng, 2009). The resveratrol stock solution was prepared as described in Chapter III.

2.3 SH-SY5Y human neuroblastoma cell culture

SH-SY5Y human neuroblastoma cells (Figure 4.1) have been widely used as an *in vitro* model for research related to neurodegenerative diseases including AD (Agholme et al., 2010). The cells were derived from the parental line of SK-N-SH cells which were originally established from a bone marrow biopsy of a neuroblastoma patient (Biedler et al., 1978).

SH-SY5Y cells were obtained from the American Type Culture Collection, VA, USA (ATCC; catalog no. CRL-2266, and batch no. 3475264). Cells were plated on 100×20 mm dishes and cultured in a 1:1 mixture of minimal essential medium (MEM) and Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 1% MEM non-essential amino acids and 1% penicillin (100 Units/mL)/streptomycin (100 µg/mL)

in a humidified incubator with 5% CO_2 at 37 °C for 6-7 days. When the cells have covered approximately 80-90% of the surface area of the dish, they were detached with 0.25% trypsin/EDTA and then cell count was performed using hemocytometer and 0.4% trypan blue solution. Subsequently, the cells were maintained in MEM/Ham's F-12 media (1:1) with 1% FBS and plated in 96-well plate or 6-well plate for 24 h prior to treatment. All chemicals used in this study were purchased from Invitrogen, CA, USA.
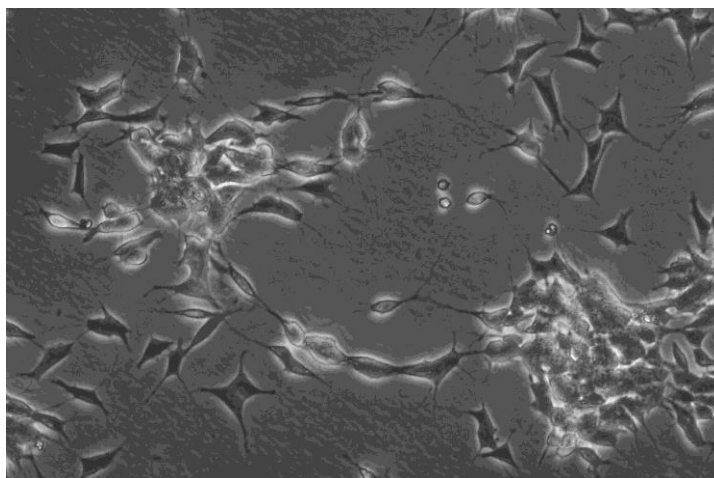
Figure 4.1 Photograph of SH-SY5Y human neuroblastoma cells under an inverted phase- contrast microscope at 200× magnification. The cells grow in clusters of neuroblastic cells with multiple and extended short neurites.

2.4 Cytotoxicity assay

SH-SY5Y cells were seeded in 96-well plate at a density of 1×10^4 cells per well. After 24 h of seeding, cells were treated with 0.01, 0.1, 1, 10 and 100 µg/mL of each crude herbal extract and 10 µM of resveratrol for 48 h. Cell viability was determined by MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Datki et al., 2003).

2.5 MTT assay for cell viability

The MTT colorimetric assay is extensively used for measurement of cell viability. A yellow tetrazolium salt namely MTT is transported into the cell by endocytosis and converted into insoluble purple formazan products by mitochondrial

reductase enzyme which is found only in viable cells. At the end of incubation period, the cells were replaced with 90 µL of serum-free MEM/Ham's F-12 media, then 10 µL of 5 mg/mL MTT solution (Amresco, OH, USA) was added to each well and incubated at 37 °C for 4 h. Afterwards, the supernatant was carefully removed and the remaining formazan was dissolved with 150 µL of DMSO. The colorimetric changes (OD) were measured using microplate reader (Thermo Fisher Scientific, MA, USA) at wavelength of 540 nm. The experiment was performed in triplicate upon each of six independent samples. Cell viability of each treatment group was expressed as a percentage of the untreated control group which was calculated by the following equation:

$$\text{Cell viability (\%)} = (A_{\text{Extract}}/A_{\text{control}}) \times 100$$

Where A_{Extract} is the absorbance of treatment group, and A_{control} is the absorbance of untreated control group.

2.6 Aβ-induced neurotoxicity assay

SH-SY5Y cells were seeded in 96-well plate at a density of 1×10^4 cells per well for 24 h. Following the cytotoxicity assay, non-toxic concentrations of each crude herbal extract and 10 µM of resveratrol were selected and co-incubated with 1 µM Aβ42 in the SH-SY5Y cells. After 48 h of treatment, cell viability was determined by using MTT assay as described above. The assay was performed in four replications upon each of six independent samples.

2.7 Expression of genes associated with Aβ production and clearance

In order to quantify whether the herbal extracts affect Aβ production by modulating mRNA expression of enzymes involved in amyloidogenic and non-amyloidogenic pathways of APP processing, changes in mRNA levels of *App*, β-secretase *Bace1* and α-secretase *Adam10* in SH-SY5Y cells were determined by Real-time quantitative reverse transcription PCR (qRT-PCR). Likewise, mRNA expression levels of *Ide* and *Nep* genes were also determined.

SH-SY5Y cells at a density of 4.5×10^5 cells per well were seeded in 6-well plate and incubated for 24 h. Regarding the cytotoxicity and Aβ-induced neurotoxicity assay, the highest and non-toxic concentrations of each crude herbal extract, which possessed protective potency against Aβ-induced neurotoxicity, were selected and treated to the SH-SY5Y cells. After 24 h of incubation, cells were collected and immediately processed for RNA extraction.

2.8 RNA extraction and cDNA synthesis

The total RNA was extracted using Trizol reagent (Invitrogen, VA, USA) according to manufacturer's protocol. Briefly, cell samples were washed twice with PBS, and then lysed by 500 µL of Trizol reagent). The solution was transferred into 1.5 mL microcentrifuge tube and 100 µL of chloroform (Merck, Darmstadt, Germany) was added into each tube. The tube was shaken vigorously for 15 sec, incubated at room temperature for 3 min and centrifuged at 12,000 ×g, 4 °C for 15 min. The mixture was separated into three layers, top (RNA), middle (DNA) and bottom (protein). The clear aqueous solution at the top layer was carefully taken and placed into a new 1.5 mL microcentrifuge tube. The 300 µL of isopropanol (Merck, Darmstadt, Germany) were added into each tube, mixed the tube gently for 15 sec, incubated at 4 °C for 10 min and centrifuged at 12,000 ×g, 4 °C for 20 min for RNA pellet precipitation. Then, the supernatant was completely removed. The remaining RNA pellet was washed by 500 µL of 80% ethanol (Merck, Darmstadt, Germany) and centrifuged at 12,000 ×g, 4 °C for 10 min, and repeated this step for another round. All leftover ethanol was removed as much as possible, the RNA pellet was air-dried for 10-20 min and dissolved with 10 µL of DEPC-treated water (Invitrogen, CA, USA).

The quantity and purity of the RNA samples were confirmed by measuring the absorbance at a wavelength of 260 and 280 nm using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, MA, USA), where a A260/A280 ratio of 1.8–2.0 was accepted. Subsequently, 2 µg of the total RNA was reversed transcribed to cDNA in a 20 µL reaction mixture using Tetro cDNA synthesis kit (Bioline, CA, USA) which contained 1 µL oligo-dT primers, 1 µL dNTP mix (at 10 mM), 1 µL RiboSafe RNase Inhibitor, 1 µL Tetro Reverse Transcriptase and 4 µL 5x RT Buffer. The samples were

incubated at 25 °C for 10 min followed by 45 °C for 30 min, and finally at 85 °C for 5 min.

2.9 qRT-PCR

The expression mRNA levels of *App*, *Bace1*, *Adam10*, *Ide* and *Nep* were examined by qRT-PCR with the previously reported primers as listed in Table 4.1. β-actin was used as a housekeeping gene. The cDNA (1 µL) was mixed with SensiFAST™ SYBR Hi-ROX Master Mix (Bioline, CA, USA) and 0.4 µM each of the forward and reverse primers for a final volume of 20 µL. The mixer was then performed with system of Step One™ Plus Real-time PCR (Applied Biosystem, CA, USA) with following cycling conditions: 95 °C for 2 min, and 40 cycles at 95 °C for 5 sec, 55 °C (for *App*, *Bace1*, and *Adam10*) or 61 °C (for *Ide* and *Nep*) for 30 sec, followed by the melting curve determination between 60 °C and 95 °C to differentiate between the desired amplicons and any primer dimers or DNA contaminant. Fold changes were calculated relative to untreated control group using the $2^{-\Delta\Delta CT}$ equation. Each qRT-PCR was performed with six independent samples.

2.10 Statistical analyses

The results were presented as mean ± standard error (SE) from three or four replications in each treatment. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Turkey's HSD post-hoc tests using the Statistical Package for the Social Sciences (SPSS) program. The level of significance for all statistical tests was accepted at $p<0.05$.

Table 4.1 Oligonucleotide sequences of the human primers used in qRT-PCR analyses.

Target genes	Accession no.		Primer sequences (5'-3')	Product size (bp)	References
Aβ production					
App	NM_000484	Forward	ACGAAGAAGCCACAGAGAGA	152	Yasojima et al.,
		Reverse	TTCATTCTCATCCCCAGGTG		2001
Bace1	NM_012104.4	Forward	CCGGCGGGAGTGGTATTATG	130	Li et al., 2010
		Reverse	GCAAACGAAGGTTGGTGGT		
Adam10	NM_001110.3	Forward	TTTGATGATGGCGTACTTGG	313	Marshall, 2006
		Reverse	AGTTTGTCCCCAGATGTTGC		
Aβ clearance					
Ide	NM_004969.3	Forward	TGCCCTAGACAGGTTTGCAC	119	Grimm et al., 2015
		Reverse	CTCCAGGCATCATTCATCACAT		
Nep	NM_007289.2	Forward	GATCAGCCTCTCGGTCCTTG	133	Grimm et al., 2015
		Reverse	TGTTTTGGATCAGTCGAGCAG		
Housekeeping gene					
β-actin	NM_001101.3	Forward	CTTCCTGGGCATGGAGTC	101	Grimm et al., 2015
		Reverse	AGCACTGTGTTGGCGTACAG		

3. Results

3.1 Cytotoxic effect of herbal extracts

Prior to investigating the effect of the eight herbal extracts on Aβ-induced neurotoxicity and expression of genes associated with Aβ production and clearance, cytotoxicity of the extracts on SH-SY5Y cells was first evaluated. This is to assure that the further results were not caused by the innate toxicity of the extracts. Based on the %cell viability after 48 h exposure with the extracts, CS, RN and TC at concentrations of 10 and 100 µg/mL and TD, TL and AI at concentrations of 100 µg/mL displayed cytotoxicity as indicated by a significant decrease in %cell viability compared to the untreated control group ($p<0.001$; Figure 4.2 A-F). Up to 100 µg/mL of CR and PR and 10 µM of resveratrol (positive control) treatments, no reduction of cell viability was detected (Figure 4.2 G-I). Interestingly, exposure of SH-SY5Y cells to 1 µg/mL of TC for 48 h showed the increase in %cell viability ($p<0.01$; Figure 4.2 C). Thus, the non-toxic concentrations of each herbal extract were selected for subsequent Aβ-induced neurotoxicity study.
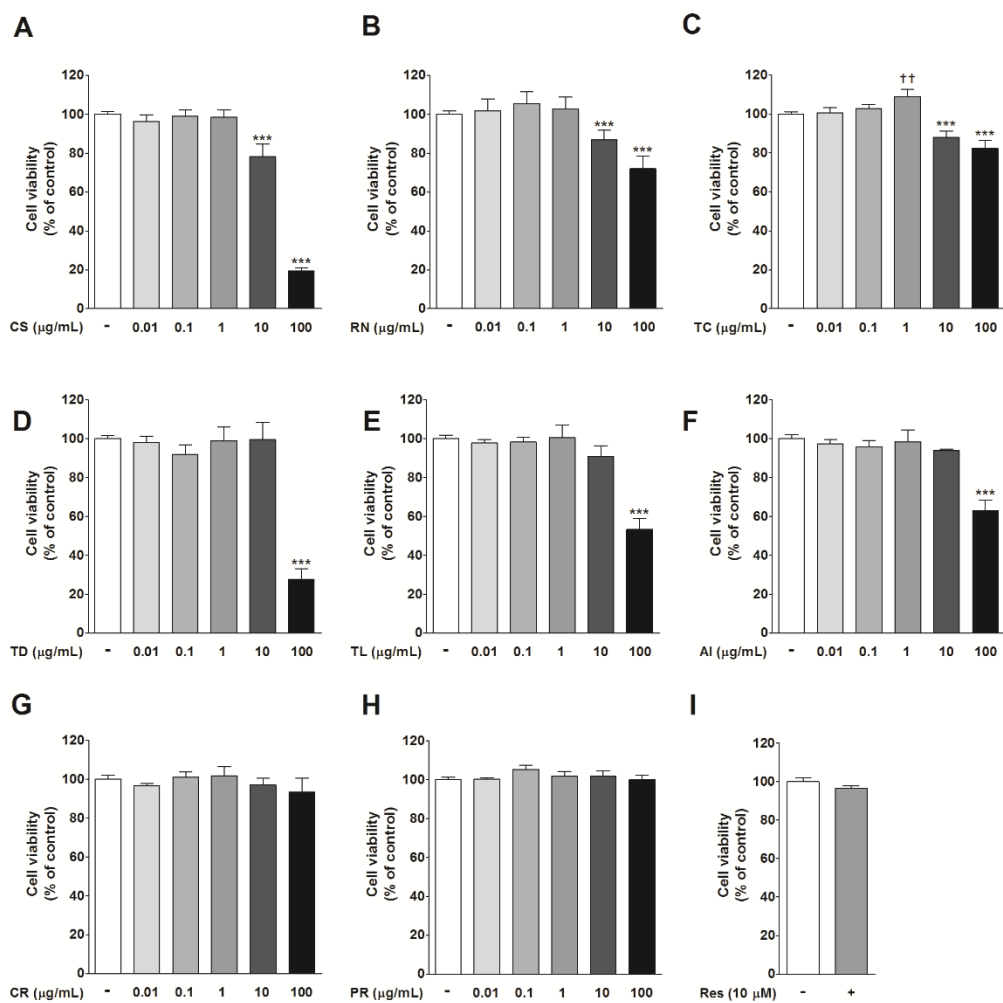
Figure 4.2 Cytotoxic effect of the eight crude herbal extracts; *Caesalpinia sappan* (CS, A), *Rhinacanthus nasutus* (RN, B), *Terminalia chebula* (TC, C), *Tabernaemontana divaricata* (TD, D), *Thunbergia laurifolia* (TL, E), *Azadirachta indica* (AI, F), *Cyperus rotundus* (CR, G) and *Piper retrofractum* (PR, H). SH-SY5Y cells were incubated with the extracts at concentrations of 0.01-100 µg/mL for 48 h. Resveratrol at concentration of 10 µM was used as a positive control (Res, I). Cell viability was determined by MTT assay. The data are expressed as mean±SEM in four replications. ** and *** indicate the %cell viability lower than the untreated control groups for p<0.01 and 0.001, respectively. †† indicates the %cell viability higher than the untreated control groups for p<0.01.

3.2 Protective efficacy against Aβ42-induced neurotoxicity of the herbal extracts

Exposure to 1 µM of Aβ42 for 48 h was apparently cytotoxic to SH-SY5Y cells as observed from cell morphology and viability. Neurons with typical appearances of irregular shape such as rounding, blebbing, shrinkage and fragmented neurites were clearly seen after exposure to Aβ42 (Figure 4.3). Moreover, cell viability was also reduced to 76.19 - 85.8% as compared to the untreated control group (Figure 4.4).

To investigate the protective effects of the herbal extracts, cultured SH-SY5Y cells were exposed to 1 µM of Aβ42 for 48 h in combination with non-toxic concentrations of the eight herbal extracts prior to determination of the cell viability by MTT assay. As shown in Figure 4.4, co-exposure with CS, TL, AI and CR at all concentrations tested did not induce significant changes in cell viability compared with the Aβ42-treated group. Nevertheless, co-exposure with RN, TC, TD and PR increased the percentage of cell viability compared with the Aβ-treated group (Figure 4.4 B-D, H). The degrees of cell viability ranged from $91.37\pm2.08\%$ to $96.87\pm3.27\%$ which were comparable to that of the resveratrol-treated group ($96.40\pm2.20\%$; Figure 4.4 I). It is worthily noted that RN (0.1 and 1 µg/mL), TC (1 µg/mL), TD (10 µg/mL) and PR (100 µg/mL) manifested the greater protective efficacy against Aβ-induced neurotoxicity than the others because those concentrations enhanced cell viability equal to the untreated control group. Thus, the highest concentration showing the greatest degree of the protective efficacy against Aβ-induced neurotoxicity of four herbal extracts; 1 µg/mL of RN, 10 µg/mL of TD, 1 µg/mL of TC and 100 µg/mL of PR, were selected for the expression of genes associated with Aβ production (*App*, *Bace1*, and *Adam10*) and clearance (*Ide* and *Nep*).
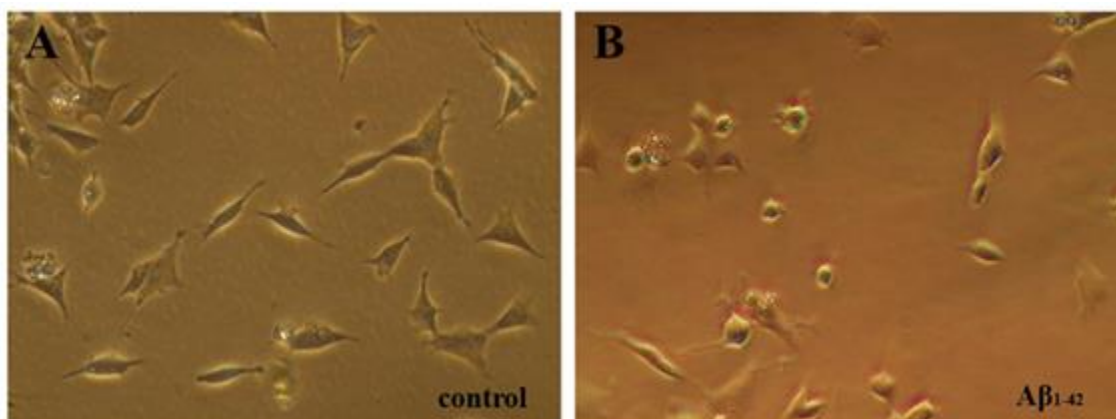
Figure 4.3 Photograph of SH-SY5Y human neuroblastoma cells exposed to 0.1% DMSO (A) and 1 μM of Aβ42 (B) for 48 h. Cell morphology was observed with an inverted phase-contrast microscope at 200× magnification.
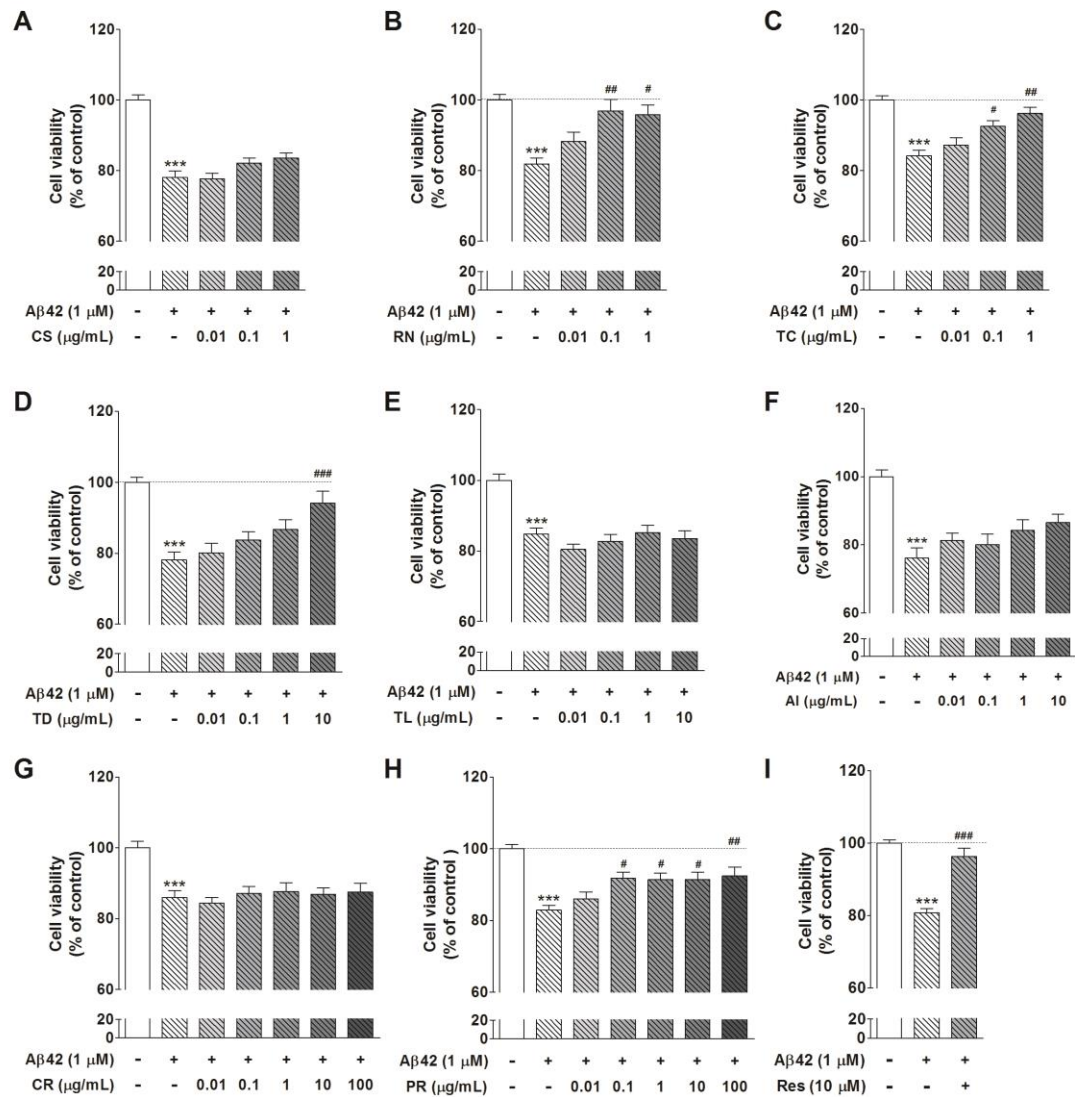
Figure 4.4 Protective efficacy of the eight crude herbal extracts; *Caesalpinia sappan* (CS, A), *Thunbergia laurifolia* (TL, B), *Rhinacanthus nasutus* (RN, C), *Tabernaemontana divaricata* (TD, D), *Azadirachta indica* (AI, E), *Cyperus rotundus* (CR, F), *Terminalia chebula* (TC, G) and *Piper retrofractum* (PR, H) on Aβ42-induced neurotoxicity. SH-SY5Y cells were treated with 1 μM of monomeric Aβ42 in the absence or presence of the extracts at various concentrations for 48 h. Resveratrol at concentration of 10 μM was used as a positive control (Res, I). Cell viability was determined by MTT assay. The data are expressed as mean±SEM in four replications, where *** represent $p<0.001$ compared to untreated control group, and #, ## and ### represent $p<0.05$, 0.01 and 0.001, respectively, compared to Aβ-treated group.

3.3 Effect of the herbal extracts on expressions of genes associated with Aβ production

Among the four herbal extracts, only the PR exhibited the suppressive effect against Aβ production. SH-SY5Y cells exposed with PR at a concentration of 100 µg/mL for 24 h significantly decreased *Bace1* (0.25 ± 0.08 folds; $p<0.05$; Figure 4.5 B) and increased *Adam10* (2.39 ± 0.46 folds; $p<0.01$; Figure 4.5 C) mRNA levels. No statistically significant changes in the expression of *App* mRNA levels in all three treatment groups (Figure 4.5 A).
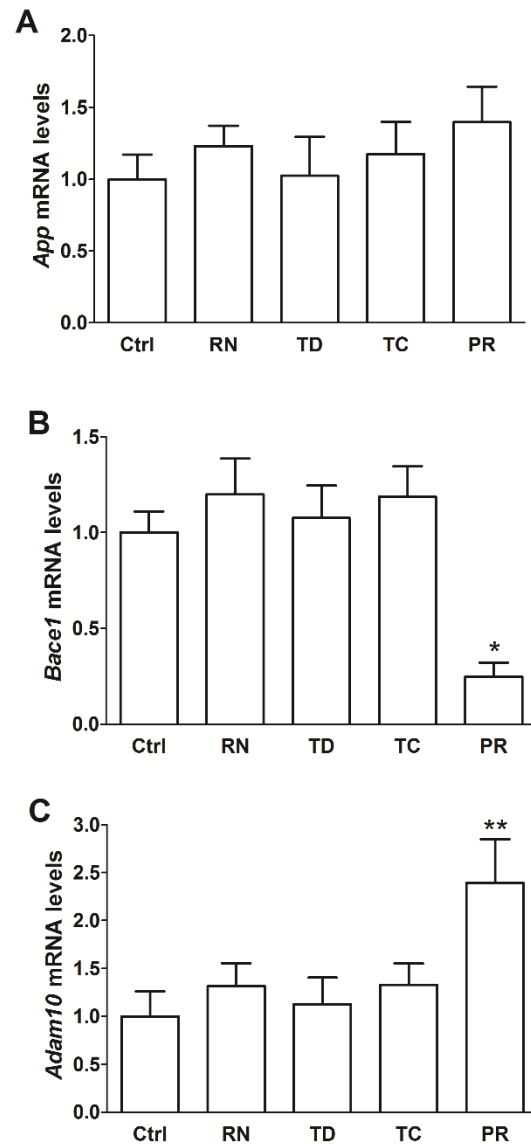
Figure 4.5 Expression of mRNA levels of genes associated with Aβ production; *App* (A), *Bace1* (B) and *Adam10* (C) in SH-SY5Y cells after incubation with *Rhinacanthus nasutus* (RN; 1 μg/mL), *Tabernaemontana divaricata* (TD; 10 μg/mL), *Terminalia chebula* (TC; 1 μg/mL) and *Piper retrofractum* (PR; 100 μg/mL) for 24 h and expression of genes were quantified by qRT-PCR. The data are expressed as mean±SEM (n=6), where * and ** represent $p < 0.05$ and 0.01, respectively, compared to the untreated control group.

3.4 Effect of the herbal extracts on expressions of genes associated with Aβ clearance

No significant changes in the expression mRNA levels of *Nep* and *Ide* genes in all four herbal extract treatments (Figure 4.6 A and B). However, the *Ide* mRNA level was marginally increased in the TC group (1.43 ± 0.09 folds; $p=0.077$; Figure 4.6 A).

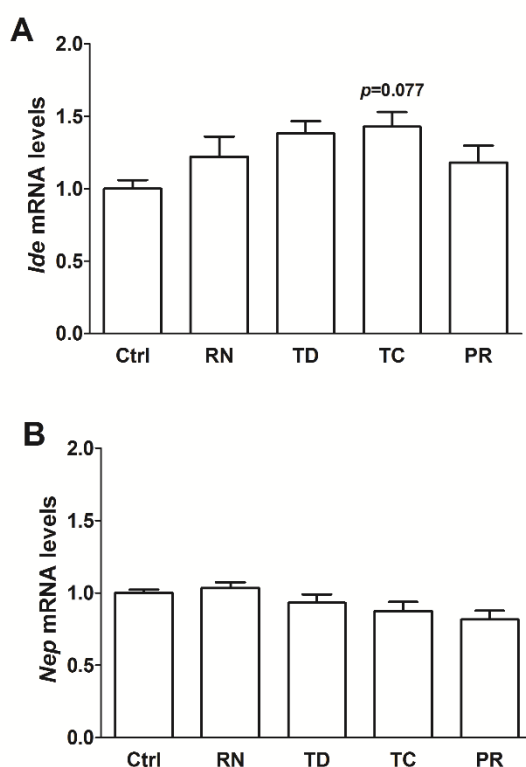**Figure 4.6** Expression of mRNA levels of genes associated with Aβ clearance; *Ide* (A) and *Nep* (B) in SH-SY5Y cells after incubation with *Rhinacanthus nasutus* (RN; 1 µg/mL), *Tabernaemontana divaricata* (TD; 10 µg/mL), *Terminalia chebula* (TC; 1 µg/mL) and *Piper retrofractum* (PR; 100 µg/mL) for 24 h and expression of genes were quantified by qRT-PCR. The data are expressed as mean±SEM (n=6).

4. Discussion

   In the present study, the ethanol extracts derived from the eight Thai herbs which possessed anti-Aβ aggregation, Aβ fibril disaggregation and antioxidant potency were selected and determined the protective efficacy against Aβ-induced neurotoxicity and the potential modulation on Aβ production and clearance using cell-based assay. The SH-SY5Y human neuroblastoma cell line exposed to Aβ42 was counted as an *in vitro* neurodegeneration paradigm because of its high sensitivity and susceptibility to oxidative damages and the relevance to the pathology of AD (Cavallucci et al., 2012; Yatin et al., 1998). The results demonstrated that incubation of 1 μM Aβ42 for 48 h could induce neurotoxicity toward SH-SY5Y and only four herbal extracts; RN (0.1 and 1 μg/mL), TD (1 μg/mL), TC (10 μg/mL) and PR (100 μg/mL), elicited protective effect against Aβ-insulted neurotoxicity. Based on the results from biochemical-based assay in Chapter III, these four herbs exhibited high antioxidant activity against free radicals which can cause oxidative damage to many intracellular organelles and subsequent neuronal cell death. Thus, one possible underlying mechanism is that these herbal extracts protect against Aβ-induced neurotoxicity via the scavenging of free radicals and reducing oxidative damage. Furthermore, the literature reviews on pharmacological properties of these four herbs indicated that RN and TC extracts exhibited strong antioxidant activity in the cell culture (Mathew and Subramanian, 2014; Rao, 2010). Likewise, the ethanol extract of RN leaves, which contained highly condensed lupeol and β-sitosterol, could attenuate the oxidative damage in both glutamate and Aβ25-35-insulted HT-22 hippocampal cells (Brimson et al., 2012). The ethanol extract of TC fruit has also possessed antioxidant effects on H_2O_2-induced oxidative stress in PC12 neuroblastoma cell (Chang and Lin, 2012). It was also reported that piperine, a major alkaloid found in the PR fruit, could reduce the ROS and prevent the apoptosis of SH-SY5Y cells from Aβ42-induced oxidative stress via stimulating the scavenging enzyme action (Chonpathompikunlert et al., 2011). Although TD exhibited high antioxidant activity tested by biochemical-based assay in the Chapter III, it has been no previous cellular studies reporting that TD exerted neuroprotection through scavenging the oxidants. Alternatively, since these four herbs showed the anti-Aβ aggregation and Aβ fibril disaggregation potency tested in the Chapter III, biochemical-based assay, it is possibly that these extracts could prevent Aβ induced neurotoxicity

directly via the induction of the unstructured and non-toxic forms of Aβ. Previous study denoted that punicalagin, a minor component isolated from TC, exerted anti-amyloidogenic properties by binding with exterior surface of Aβ42 oligomer and modifying Aβ to non-toxic conformation which afforded neuroprotection to PC12 cells (Das et al., 2016). As such, the neuroprotective mechanism of TC against Aβ toxicity might pass through both anti-aggregative and antioxidant actions. It is worthily noted that 1 μg/mL of TC treatment also increased the %viability of SH-SY5Y cells which was significantly higher than untreated control group. This is consistent with the previous reported result of 100 μM of punicalagin treatment which increased the %viability of PC12 cells (Das et al., 2016). Thus, the increase in cell viability in the TC-treated group may cause by a proliferative effect of punicalagin in TC. However, the testing of cell proliferation such as BrdU assay should be investigated further.

Interestingly, CS and TL, although possessed highest anti-Aβ aggregation and Aβ fibril disaggregation potency in the Chapter III, did not show the protective efficacy on neurotoxicity in the cell-based assay determined here. These might be due to the selected non-toxic doses on cellular viability of CS and TL contained low amounts of bioactive compounds which could not reach the threshold of therapeutics. Taken together, the protective effects of RN, TD, TC and PR on Aβ induced neurotoxicity observed in Chapter IV might occur directly by inhibiting and/or reversing Aβ aggregates or indirectly by activating antioxidative defense mechanisms. Consequently, the detailed underlying mechanism of these four herbal extracts on neuroprotection needs to be carried on further.

To further investigate the ability of these herbal extracts on Aβ production and clearance, the highest and non-toxic concentrations of RN, TD, TC and PR which had the greatest protective effect against Aβ-induced neurotoxicity were selected and determined the expression of genes associated with Aβ production and clearance. For the first time, this study found that the ethanol extract of PR had a potential to diminish Aβ production through modulating metabolic pathways of APP. PR at a non-toxic concentration (100 μg/mL) decreased the β-secretase *Bace1* mRNA level together with increased the α-secretase *Adam10* mRNA level, suggesting that PR might shift APP processing from the amyloidogenic pathway toward the non-amyloidogenic pathway

and then reduced the Aβ production. A literature search on a phytochemical study revealed that the main chemical constituents of PR fruit are piperidine alkaloids (i.e., piperine, pipernonaline, and dehydropipernonaline) and amide alkaloids (i.e., piperodione) (Kim et al., 2011). Piperine was later identified as β-secretase inhibitor owing to its inhibitory effect against β-secretase activity (Murata et al., 2015). Consequently, piperine might be one of the active compounds of PR which is responsible for the reduction in mRNA expression level of β-secretase *Bace1* in SH-SY5Y cells. So far, there have been no reports of any chemical constituents isolated from PR fruit which can act as α-secretase stimulator. In addition, piperine has also been previously reported to reduce AChE activity in the AF64A-induced memory deficits in rats which can mitigate the AD progression (Chonpathompikunlert et al., 2010). Thus, PR might only inhibit Aβ-based pathogenic cascade but it should also modulate another causative factor of AD.

Furthermore, TC at concentration of 1 μg/mL tended to increase the mRNA expression level of *Ide*, a key Aβ-degrading enzyme. Thus, it is interesting to determine if the higher doses of TC, but non-toxic to the SH-SY5Y cells, which are in between 1 and 10 μg/mL, could significantly upregulate the *Ide* expression. This result highlights for the first time in the modulation on Aβ degrading enzyme of TC as another potential neuroprotective mechanism. Besides, an individual active compound of TC should also be isolated and tested for its mechanisms. Previously, it was reported that chebulagic acid, a major constituent of TC fruit, exhibited potency in degradation of neurotoxin via enhancing autophagy, a lysosomal degradation pathway (Kim et al., 2014). It therefore should be noted that TC might facilitate Aβ clearance through other pathways.

In conclusion, the cell-based screening of the four herbal extracts in this study illustrates the multifunctional activities of PR and TC as a potential treatment of Aβ-based pathology in AD. However, since this is a preliminary study tested solely in *in vitro* SH-SY5Y cells, the next step of research in laboratory animals should be carried out. I also suggest that the bioactive compounds of PR and TC should also be isolated and determined their effects on Aβ-based pathology.

CHAPTER V
GENERAL DISCUSSION AND CONCLUSION

AD is the result of multiple pathophysiological processes that lead to the neurodegenerative cascade. Hence, multifunctional drugs which are able to interact with several targets are a focal point of interest for the treatment of this disease. Therefore, the scientifically virtual screening system to generate multifunctional hits is a promise for the treatment of AD. Moreover, the screening system should also be simple, inexpensive, less time consuming and able to perform a large scale of test because many candidate species of plants in tropical country, as in Thailand, are awaited for being tested. Particularly, this screening was focused on the action of the plant extracts against multiple steps of Aβ-based pathology including (i) inhibiting and reversing Aβ aggregation processes, (ii) reducing neurotoxicity induced by Aβ, and (iii) decreasing Aβ production and increasing Aβ clearance. The crude extracts of nine Thai herbs were systematically tested *in vitro* in 2 steps; biochemical- and cell-based assay (Figure 5.1).

In the first step, biochemical-based assay, the herbal extracts were screened for anti-Aβ aggregation, Aβ disaggregation, and antioxidant potencies using ThT fluorescence assay and DPPH free radical scavenging assay, respectively. These methods offer the advantage of focusing on a single target with simple technique, inexpensive, no time-consuming processes and high adaptability to high throughput. Among the nine herbal extracts which were selected in this studies, CS, TL, RN, TD, CR, TC, AI, PR and AR; except the AR, all eight herbs had a potential to inhibit Aβ aggregation and stimulate Aβ fibril disaggregation and also possessed antioxidant potency. Subsequently, only these eight herbal extracts were selected and processed to the next step.

In the second step, cell-based assay, cytotoxicity of the herbal extracts on SH-SY5Y human neuroblastoma cells was first evaluated, and then non-toxic concentrations were selected and investigated further for the protective effect against Aβ-induced neurotoxicity. Cell viability of SH-SY5Y cells measured by MTT assay was used as a judging parameter for these two experiments. In this step, SH-SY5Y cells

were used as a neuronal cellular model to provide more multiplexed biologically relevant surrogates of the precisely specific response in organism and also provide a better understanding of the complex effects of the extracts in the living cells. From the eight extracts that were screened, only four extracts; RN, TD, TC and PR, elicited protective effects against Aβ induced neurotoxicity. The proposed underlying mechanisms of action of these protections are through directly inhibiting and/or reversing aggregated Aβ into unstructured and non-toxic forms, or indirectly reducing oxidative damage caused by Aβ. Lastly, the non-toxic concentrations which had the greatest protective effect against Aβ-induced neurotoxicity were selected and investigated the potential modulation on expression of genes associated with Aβ production (*App, Bace1 and Adam10*) and clearance (*Ide and N*ep) using qRT-PCR analysis. The malfunction of those selected genes were previously reported as a key player in the pathogenesis of AD. Among the four herbal extracts, PR displayed the suppressive effect against Aβ production by decreasing β-secretase *Bace1* and increasing α-secretase *Adam10* mRNA expression levels, while TC had potential to degrade the monomeric form of Aβ by increasing mRNA expression level of *Ide*, a key Aβ-degrading enzyme.

Consequently, PR and TC seem to be promising herbal candidates to be developed further as multifunctional agents targeting multiple steps of Aβ-based pathology for disease-modifying therapies in AD. While other herbal extracts, except AR, may serve as a valuable inspiration in the search for new effective therapies for AD in view of one target-one drug therapeutic approach. Also, it is interesting to test if those remaining herbs are effective in other pathways such as cholinergic pathway to battle with neurodegenerative diseases. However, it should be noted that only the non-cytotoxic concentrations of the extracts were selected and tested in the SH-SY5Y cell-based assay. It is possible that these non-toxic doses contain very small amount of the bioactive compounds and could not reach the threshold of the effective window on protection of neurotoxicity or modulation of Aβ production and clearance. Thus, it is recommended to isolate the bioactive compounds from those extracts and determine their effects on Aβ pathogenic cascade directly.

In conclusion, the screening system from this study can apply as a standardized and high-throughput tool to screen the high potential compounds on AD at a commercial and pharmaceutical scale for the development of new anti-AD agents for human use in the future.

Recommendations for further research

1. As can be seen that the PR and TC changed the mRNA expression of Aβ production and clearance, it is better to confirm whether the alteration also occurs at the protein level.

2. As mentioned earlier that only the non-cytotoxic concentrations of the extracts were selected and tested in the SH-SY5Y cell-based assay, it is better to isolate the active compounds from the PR and TC extracts and determine the effect on each step of Aβ-based pathology directly.

3. Since the PR and TC extracts have high possibility to be developed as drugs for AD treatment, thus, these two extracts should be tested further in the laboratory animals
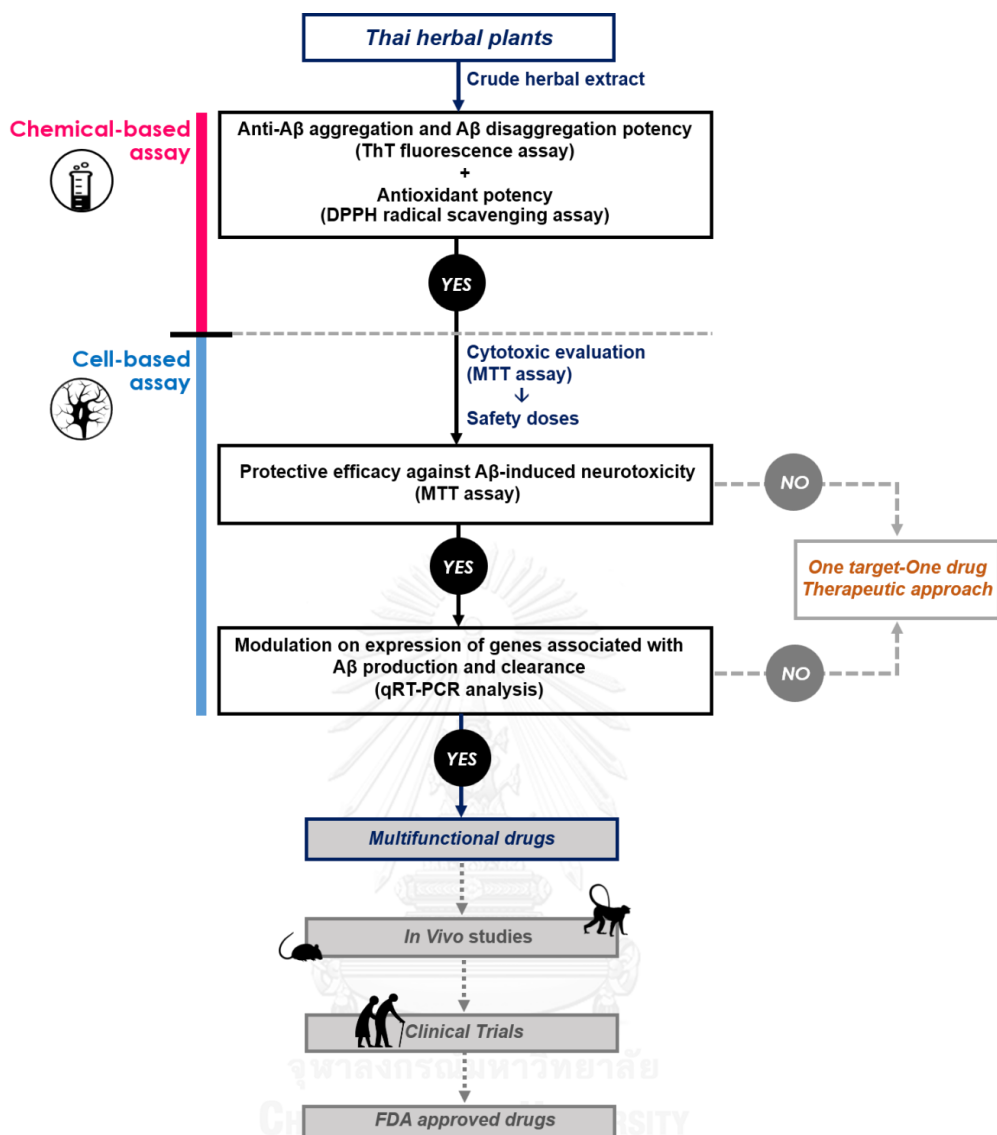
Figure 5.1 Schematic of *in vitro* screening system of Thai herbs for exploring multifunctional agents which target on multiple steps of Aβ-based pathology.

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APPENDIX

VITA

Miss Ruedeemars Yubolphan was born on the 2nd of January, 1990 in Kalasin, Thailand. She graduated with a Bachelor of Science degree in Biology from the Department of Biology, Faculty of Science, Chulalongkorn University in 2012. She was then continued her study in Master's degree Program in Zoology at the same department. As a graduate student in the department, she carried out her research at the Primate Research Unit and was a teaching assistant in General Biology Laboratory and Ethology courses. During her study, she worked on Mini-project in Special Problem coursework, entitled "The neuropathological sequence in estrogen deficient rats" and received the grant from The Ministry of Education, Culture, Sports, Science and Technology, Japan for performing this research at the Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology in 2012. She also had a chance to participate in a research project, entitled "The effect of Kisspeptin (Kiss2) on Arginin vasotocin (AVT) mRNA expression in sexual motivation of male zebra fish" in the 2nd Advanced School of Neuroscience of the International Brain Research Organization (IBRO) at Monash University, Malaysia in 2014. Besides, she had presented a part of her research work as a poster presentation in the 8th Federation of the Asian and Oceanian Physiological Societies Congress (FAOPS) at Bangkok, Thailand in 2015 and she also published a part of her work as a research article in the proceedings of the 11th Science and Technology Conference for Youths: 2016.