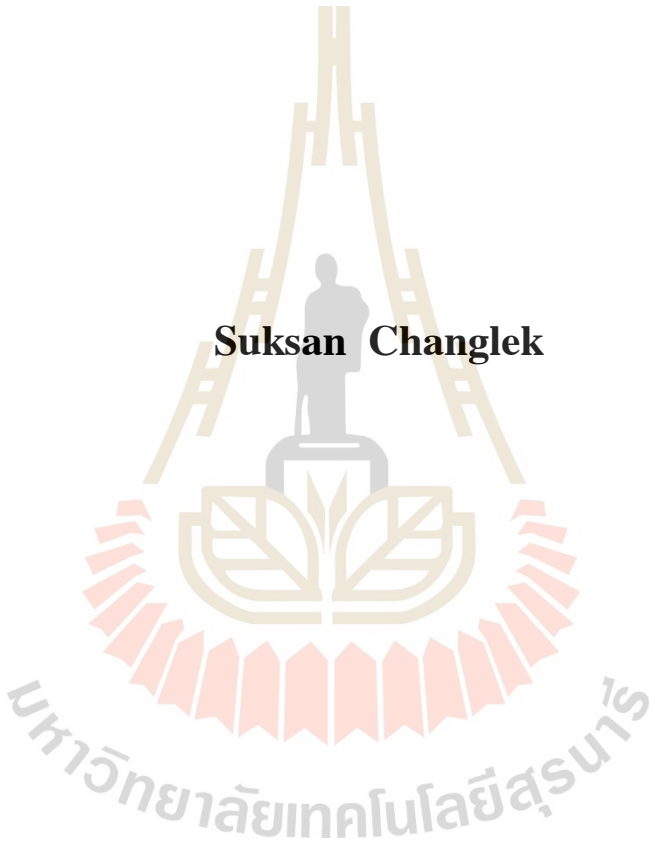
EFFECTS OF α-MANGOSTIN ON LEARNING AND MEMORY IMPAIRMENT IN RATS

Suksan Changlek



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ผลของสารอัลฟา-แมงโกสตินต่อความบกพร่อง ของการเรียนรู้และจดจำในหนู

นายสุขสันต์ ช่างเหล็ก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECTS OF *α*-MANGOSTIN ON LEARNING AND MEMORY IMPAIRMENT IN RATS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Doctor's Degree.

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สุขสันต์ ช่างเหล็ก : ผลของสารอัลฟา-แมงโกสตินต่อความบกพร่องของการเรียนรู้และจดจำในหนู (EFFECTS OF α-MANGOSTIN ON LEARNING AND MEMORY IMPAIRMENT IN RATS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รุ่งฤดี ศรีสวัสดิ์, 198 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารอัลฟา-แมงโกสตินและโดเนเปซิลต่อความบกพร่องของการเรียนรู้และจดจำในหนู ซึ่งประกอบด้วย 3 การทดลองดังนี้

การศึกษาผลของสารอัลฟา-แมงโกสตินและโดเนเปซิลต่อภาวะความจำเสื่อมโดยมีคุณสมบัติในการต้านอนุมูลอิสระและหน้าที่ในการยับยั้งการทำงานของเอนไซม์อะซิติลโคลินเอสเตอเรสในหลอดทดลอง พบว่าสาร อัลฟา-แมงโกสตินและโดเนเปซิลที่ความข้มข้น 100 - 600 ไมโครกรัมต่อมิลลิลิตร มี FRAP value อยู่ระหว่าง 20 - 410 ไมโครโมล (Fe^{2+}) ต่อลิตรและมีค่าความเข้มข้นที่สามารถยับยั้ง ABTS ร้อยละ 50 (IC_{50}) เท่ากับ 21.52 ± 3.45 และ 14.53 ± 1.86 ไมโครกรัมต่อมิลลิลิตร ตามลำดับและ DPPH ร้อยละ 50 (IC_{50}) เท่ากับ 38.12 ± 8.36 และ 29.44 ± 5.13 ไมโครกรัมต่อมิลลิลิตร ตามลำดับและสารอัลฟา-แมงโกสตินและโดเนเปซิลสามารถยับยั้งการทำงานของเอนไซม์อะซิติลโคลินเอสเตอเรสร้อยละ 50 เท่ากับ 64.23 ± 0.22 และ 32.46 ± 0.14 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สารโดเนเปซิลมีความสามารถยับยั้งการทำงานของเอนไซม์อะซิติลโคลินเอสเตอเรสได้ดีกว่าสารอัลฟา-แมงโกสตินเมื่อศึกษาด้วยวิธีของ Ellman แต่ไม่พบว่ามีความแตกต่างกับสารอัลฟา-แมงโกสตินเมื่อศึกษาด้วยวิธี NA-FB เมื่อเทียบกับสารมาตรฐานที่ความเข้มข้นเดียวกัน (50 ไมโครกรัมต่อมิลลิลิตร)

การศึกษาผลของสารอัลฟา-แมงโกสตินและโดเนเปซิลในการกระตุ้นการเรียนรู้และจดจำถูกทดสอบโดยวิธี Morris water maze การศึกษาหนูขาวเพศผู้ที่ถูกเหนี่ยวนำให้ความจำเสื่อมด้วยยาซาโคโปลามีน ร่วมกับสารอัลฟา-แมงโกสติน (50 หรือ 100 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) หรือโดเนเปซิล (2 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) เป็นเวลา 7 วัน ใช้เวลาในการหาแท่นที่ซ่อนอยู่ลดต่ำลงอย่างมีนัยสำคัญทางสถิติและมีผลทำให้เวลาที่ใช้ในพื้นที่ที่เคยมีแท่นและจำนวนครั้งที่เข้าออกบริเวณเป้าหมายเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มหนูที่ถูกเหนี่ยวนำให้เกิดความจำเสื่อมด้วยยาซาโคโปลามีน (P<0.05) ร่วมกับสารอัลฟา-แมงโกสติน (50 หรือ 100 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) หรือโดเนเปซิล (2 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) ไม่มีผลทำให้เกิดการเปลี่ยนแปลงของระดับค่าบ่งชี้ทางชีวเคมีต่าง ๆ ในพลาสมาและค่าการเปลี่ยนแปลงน้ำหนัก พบการเพิ่มการทำงานของเอนไซม์โคลินอะซิติลทรานส์เฟอเรส และการยับยั้งการทำงานของเอนไซม์อะซิติลโคลินเอสเตอเรสในสมองส่วนซีรีบรัลคอร์เท็กซ์และสมองส่วนฮิปโปแคมปัสแต่ไม่พบในสมองส่วนเบซอลฟอร์เบรนอย่างมีนัยสำคัญทางสถิติ (P<0.05) และยับยั้งการทำงานของเอนไซม์

แคสเปส-3 และลดระดับมาลอนไดอัลดีไฮด์ในสมองส่วนซีรีบรัลคอร์เท็กซ์และสมองส่วนฮิปโปแคมปัสแต่ไม่พบในสมองส่วนเบซอลฟอร์เบรนอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุม ($P<0.05$)

การศึกษาผลของการได้รับสารอัลฟา-แมงโกสตินและโดเนเปซิลต่อเนื่องกันเป็นเวลา 7 วันที่มีต่อการแสดงออกของโปรตีนฟอสที่บ่งชี้การตื่นตัวของเซลล์ประสาทโคลิเนอร์จิกของสมองส่วนที่ทำหน้าที่ควบคุมการเรียนรู้และจดจำ สารอัลฟา-แมงโกสติน (50 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) และสารโดเนเปซิล (2 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) สามารถกระตุ้นให้เกิดการแสดงออกของโปรตีนฟอสในเซลล์ประสาทโคลิเนอร์จิกของสมองบริเวณ mPFC แต่ไม่พบในสมองบริเวณ dHip ในกลุ่มที่ได้รับน้ำเกลือเมื่อเปรียบเทียบกับกลุ่มควบคุม ($P<0.05$)

นอกจากนี้โดเนเปซิลสามารถกระตุ้นให้เกิดการแสดงออกของโปรตีนฟอสในเซลล์ประสาทโคลิเนอร์จิกของสมองบริเวณ mPFC และ dHip ในกลุ่มที่ถูกเหนี่ยวนำให้ความจำเสื่อมด้วยยาซาโคโปลามีนแต่ไม่พบในกลุ่มที่ได้รับสารอัลฟา-แมงโกสตินเมื่อเปรียบเทียบกับกลุ่มควบคุม ($P<0.05$)

การศึกษาในครั้งนี้โดยสรุปแสดงให้เห็นว่าสารอัลฟา-แมงโกสตินและโดเนเปซิลมีฤทธิ์ในการต้านอนุมูลอิสระ กระตุ้นความทรงจำ ยับยั้งการทำงานของเอนไซม์อะซิติลโคลินเอสเตอเรส ยับยั้งการตายของเซลล์และกระตุ้นการทำงานของเซลล์ประสาทโคลิเนอร์จิกในสมองส่วนที่ทำหน้าที่ควบคุมการเรียนรู้และจดจำ ดังนั้นการได้รับสารอัลฟา-แมงโกสตินเสริมอาจมีประโยชน์สำหรับการป้องกันการพัฒนาการหรือการดำเนินไปของความจำเสื่อมและโรคที่เกิดจากความเสื่อมทางระบบประสาท

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SUKSAN CHANGLEK : EFFECTS OF α-MANGOSTIN ON LEARNING AND MEMORY IMPAIRMENT IN RATS. THESIS ADVISOR : ASST. PROF. RUNGRUDEE SRISAWAT, Ph.D. 198 PP.

α-MANGOSTIN/ MORRIS WATER MAZE TEST/ LEARNING/ MEMORY/ AMNESIA/ CENTRAL CHOLINERGIC SYSTEM

The present study aimed to investigate the effects of α-mangostin and donepezil on learning and memory impairment in rats. This study consisted of 3 experiments.

The effects of the α-mangostin and donepezil on the antioxidant properties and acetylcholinesterase inhibitory activities *in vitro* were performed. The results showed that 100 - 600 $\mu g \cdot mL^{-1}$ α-mangostin and donepezil gave an FRAP value within the 20 - 410 $\mu mol\ Fe^{2+} \cdot L^{-1}$. The concentrations of the α-mangostin and donepezil required to inhibit 50% (IC_{50}) of ABTS were 21.52 ± 3.45 and 14.53 ± 1.86 $\mu g \cdot mL^{-1}$, and DPPH were 38.12 ± 8.36 and 29.44 ± 5.13 $\mu g \cdot mL^{-1}$, respectively. The concentrations of the α-mangostin and donepezil required to inhibit 50% of acetylcholinesterase activity were 64.23 ± 0.22 and 32.46 ± 0.14 $mg \cdot mL^{-1}$, respectively. Donepezil exhibited acetylcholinesterase inhibitory activity stronger than α-mangostin with the same pattern of inhibitory action by Ellman's method, but not significantly different with α-mangostin by NA-FB method when compared to their reference standard at the same concentration (50 $\mu g \cdot mL^{-1}$).

The effects of α-mangostin and donepezil on enhancing learning and memory performance were investigated using Morris water maze task. The study in adult male Wistar rats found that intraperitoneal administration of α-mangostin (50 or 100 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) or donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) of scopolamine-induced amnesic rats (2

mg·mL^{-1}·kg^{-1}, i.p.) for 7 constitutive days significantly decreased time to find platform and increased number of entry and time spent in target quadrant when compared to the vehicle control (P<0.05). In scopolamine-induced amnesic rats, the α-mangostin (50 and 100 mg·mL^{-1}) and donepezil did not significantly altered plasma levels of biochemical parameters and body weight changes, significantly elevated the choline acetyltransferase (ChAT) activity and inhibited acetyl cholinesterase (AChE) activity in cerebral cortex, hippocampus, but not in basal forebrain (P<0.05), and significantly inhibited caspase-3 activities and reduced the malondialdehyde level in cerebral cortex, hippocampus, but not in basal forebrain when compared to the vehicle control (P<0.05).

The effects of α-mangostin and donepezil treatment for 7 constitutive days on Fos expression that represent the activation of cholinergic neurons in the brain areas involved in the regulation of learning and memory were investigated. In NSS-treated rats, donepezil and α-mangostin (50 mg·mL^{-1}) could significantly induced Fos expression of cholinergic neurons in dorsal hippocampus (dHip), but not in medial prefrontal cortex (mPFC) when compared to the vehicle control (P<0.05). In scopolamine-induced amnesic rats, donepezil, but not α-mangostin could significantly induced Fos expression of cholinergic neurons in mPFC and dHip when compared to the vehicle control (P<0.05).

In conclusion, α-mangostin and donepezil exhibited antioxidant, memory enhancing, and acetylcholinesterase inhibitory, anti-apoptotic activities and induced cholinergic activation in the brain areas involving in regulation of learning and memory. Supplementation of α-mangostin may be beneficial for the prevention of the development or progression of cognitive impairment and neurodegenerative diseases.

School of Preclinic — Student's Signature Suksan Changlek

Academic Year 2017 — Advisor's Signature Rungsudee Siasawat

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CHAPTER I

INTRODUCTION

1.1 Background/Problem

Dementia is the loss of cognitive functioning, which means the loss of the ability to think, remember, or reason, as well as behavioral abilities, to such an extent that it interferes with a person's daily life and activities. Signs and symptoms of dementia result when once-healthy neurons (nerve cells) in the brain stop working, lose connections with other brain cells, and die. While everyone loses some neurons as they age, people with dementia experience far greater loss. Researchers are still trying to understand the underlying disease processes involved in the disorders. Scientists have some theories about mechanisms that may lead to different forms of dementias, but more research is needed to better understand if and how these mechanisms contribute to the development of dementia. While dementia is more common with advanced age (as many as half of all people age 85 or older may have some form of dementia), it is not a normal part of aging. Many people live into their 90s and beyond without any signs of dementia. Memory loss, though common, is not the only sign of dementia. For a person to be considered to have dementia, he or she must meet the following criteria: two or more core mental functions must be impaired. These functions include memory, language skills, visual perception, and the ability to focus and pay attention. These also include cognitive skills, such as the ability to reason and solve problems. The loss of brain function is severe enough that a person cannot do normal, everyday tasks (Boenink *et al*., 2011).

Alzheimer's disease (AD) is the most common form of dementia in those over the age of 65. As many as 5 million Americans age 65 and older may have AD, and that number is expected to double for every 5-year interval beyond age 65. However, AD is only one of many dementia disorders; another 1.8 million people in the United State have some other form of dementia. Among all people with dementia, many are believed to have a mixed type of dementia that can involve more than one of the disorders. Age is the primary risk factor for developing dementia. For that reason, the number of people living with dementia could double in the next 40 years with an increase in the number of Americans who are age 65 or older, from 40 million today to more than 88 million in 2050. Regardless of the form of dementia, the personal, economic, and societal demands can be devastating. Research over the past 30 years has helped us learn more about dementia, possible causes, who is at risk, and how it develops and affects the brain. (Burns and Iliffe, 2009).

AD and vascular dementia are the two most common types of dementia with the former being the most predominant. It is evident that oxidative stress, an environment where pro-oxidant species overwhelm antioxidant species, is involved in the pathogenesis of both forms of dementia. An increased level of reactive oxygen species in the vasculature, reduced nitric oxide bioavailability, and endothelial dysfunction leading to vascular disease is associated with vascular dementia. In AD, an increased amount of amyloid-β precursor protein (APP) or amyloid-β (Aβ) peptide induces elevated reactive oxygen species (ROS) production thereby causing neuronal cell death and damage (Bennett, 2009).

An oxidative stress state is characterized by the generation of reactive oxygen species in a biological system above its capacity to counterbalance them (Finkel and

Holbrook, 2000). ROS are important intermediates in oxidative processes. Unlike ground state oxygen [$^3O_2$ or $O_2(^3\Sigma^-)$], ROS include non-exhaustively, hydroxyl radical ($^{\bullet}OH$), superoxide anion ($^{\bullet}O_2^-$), peroxide anion (O_2^{2-}), electronically-excited dioxygen [$^1O_2$ or $O_2(^1\Sigma^+)$], *i.e*. singlet oxygen [$^1O_2$ or $O_2(^1\Delta)$], peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2), known to be almost as reactive as atomic oxygen and to react directly with proteins and polyunsaturated fatty acids (PUFAs) located in the cytosol and cell membranes to give the corresponding peroxides. The cells are then damaged by oxidization of their subcellular components, which can lead to cell apoptosis or necrosis. Cell apoptosis, bacterial and viral inactivation are consequently identified effects of singlet oxygen action with higher yields than hydroxide or superoxide radicals (Kochevar *et al*., 2000). Exposure to oxidative stress induces the accumulation of intracellular ROS, which in turn causes cell damage in the form of lipid peroxidation (Vileno *et al*., 2010).

The principal mediators of apoptosis are cysteine-aspartic proteases (caspases), which cleave hundreds of proteins. Phosphorylation also plays an important role in apoptosis, although the extent to which proteolytic and phosphorylation pathways crosstalk during programmed cell death (PCD) remains poorly understood. Over 500 apoptosis-specific phosphorylation events are enriched on cleaved proteins and clustered around sites of caspase proteolysis. Caspase cleavage can expose new sites for phosphorylation, and, conversely, that phosphorylation at the +3 position of cleavage sites can directly promote substrate proteolysis by caspase-8. A global portrait of the apoptotic phosphoproteome, revealing heretofore unrecognized forms of functional crosstalk between phosphorylation and caspase proteolytic pathways that lead to enhanced rates of protein cleavage and the unveiling of new sites for

phosphorylation (Dix *et al*., 2012). Specific protein phosphorylation events have been previously demonstrated to occur during apoptosis and play an important role in the regulation of the programmed cell death mechanism (Gjertsen and Døskeland, 1995).

Apoptotic cell death has been the focus of intense research in the last several decades as a means of controlling cell populations in normal development and inflammation through PCD. Failure to control cell numbers through apoptosis is common in cancer, while excessive apoptosis is viewed to play a role in a number of neurological disorders in addition to AD, including stroke and Parkinson disease (Cotman and Anderson, 1995). Apoptosis is an attractive mechanism for neuronal death in neurodegenerative diseases for several reasons. In AD, neuronal loss is prominent in the cerebral cortex and the limbic lobe, while different neuronal populations are vulnerable in other neurodegenerative diseases. Evidence for frank cellular apoptosis in AD is controversial, but there is growing recognition that apoptotic mechanisms may play a role in disease pathogenesis in the absence of overt apoptosis (Stanojevic *et al*., 2009).

Dysfunction of cholinergic neurotransmission in the brain that cause cognitive decline has been reported in AD. The gradual death of cholinergic cells in AD is accompanied by loss of the acetylcholine (Vinutha *et al*., 2007). A cholinergic deficit has been shown to be associated with memory loss and the severity of AD. Loss of the cholinergic markers, acetylcholine (ACh), choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) are neurological changes consistently found in the brains of AD patients (Court and Perry, 1991). Hence, it has been suggested that elevation of ACh level might be helpful in attempt to improve the symptoms of cognitive deficit in AD. One of the most promising approaches for treatment of this disease is to enhance

the acetylcholine level in the brain using acetylcholinesterase inhibitors (Chattipakorn *et al*., 2007; Ingkaninan *et al*., 2003; Vinutha *et al*., 2007).

Compounds isolated from the fruit rind of mangosteen (*Garcinia mangostana*) contain abundant xanthones, especially α-mangostin ($C_{24}H_{26}O_6$), a polyphenolic xanthone derivative from mangosteen (Yodhnu *et al*., 2009). Mangosteen has antioxidative and neuroprotective activities (Weecharangsan *et al*., 2006). γ-Mangostin ($C_{23}H_{24}O_6$) and garcinone B ($C_{23}H_{22}O_6$), are the anti-inflammatory xanthone ($C_{13}H_8O_2$) phytomolecules present in the pericarp of the mangosteen fruit that inhibit cyclooxygenase (COX) and prostaglandin E_2 (PGE2) synthesis in rat glioma cells (Yamakuni *et al*., 2006; Nakatani *et al*., 2004).

The crude extract from the fruit rind of mangosteen demonstrated memory enhancing and acetylcholinesterase inhibitory activities in normal adult rats and normal aging rats (Nontamart *et al*., 2013). α-Mangostin showed ROS scavenging capacity and neuroprotective effect against 3-nitropropionic acid (3-NPA) in cerebellar granule neuron (CGN) (Pedraza-Chaverrí *et al*., 2009). Furthermore, Wang *et al*. (2012) found that α-mangostin attenuates Aβ oligomers-induced neurotoxicity by inhibiting amyloid aggregation. These findings suggest the potential role of polyphenols found in the fruit hull of mangosteen in the treatment of neurodegenerative disease.

A preliminary study has shown that *G. mangostana* has 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Ngawhirunpat *et al*., 2010). Nontamart *et al*. (2013) found that the aqueous ethanol extract from the fruit rind of *G. mangostana* demonstrated memory enhancing and acetylcholinesterase inhibiting activities in normal adult and normal aging rats. Although *G. mangostana* showed an

anti-AD capability which has important medicinal values for the treatment of AD, no studies have yet examined its phenolic compound. α-Mangostin might be responsible for cognitive enhancing activity of *G. mangostana*. Therefore, the effects of α-mangostin on learning and memory and the central cholinergic system in order to treat AD were investigated.

1.2 Research objectives

The experiments were designed to clarify the followings:

1. to study the effects of α-mangostin on the antioxidant and acetylcholine esterase inhibitory activities *in vitro*,

2. to study the effects of α-mangostin on learning and memory performance, plasma biochemical parameters and body weight changes, cholinergic and apoptotic caspase-3 enzyme activities, and lipid peroxidation in cerebral cortex, hippocampus, and basal forebrain in scopolamine- induced amnesic rats, and

3. to study the effects of α-mangostin on Fos expression in the cholinergic neurons of the brain areas regulating learning and memory in scopolamine-induced amnesic rats.

1.3 Research hypotheses

α-Mangostin acts as an antioxidant and a centrally acting reversible acetylcholinesterase inhibitor affecting against learning and memory impairment in amnesic rats by improving memory performance after place learning test by regulating cholinergic enzyme activity, inhibiting apoptotic enzyme activity and lipid peroxidation,

and exhibiting neuronal death prevention and neuronal activation in the brain areas involved in the regulation of learning and memory.

1.4 Expected results

The findings would provide the new evidence of the beneficial effects of α-mangostin against learning and memory impairment in scopolamine-treated rats by enhancing memory after learning performance in a Morris water swimming maze which may have the result of choline acetyltransferase stimulation and the inhibition of acetylcholine esterase, caspase-3, and lipid peroxidation in the cerebral cortex, hippocampus, and basal forebrain. Moreover, α-mangostin may also prevent neuronal cell death and induced Fos expression in the medial prefrontal cortex and dorsal hippocampus. The α-mangostin may have acted as one of the best dietary supplements for the protection of learning and memory impairment.

CHAPTER II

LITERATURE REVIEW

2.1 Central cholinergic system

Acetylcholine (ACh) is the transmitter at the neuromuscular junction, in autonomic ganglia, and in postganglionic parasympathetic nerve-target organ junctions and some post-ganglionic sympathetic nerve-target junctions. It is also found within the brain, including the cerebral cortex, hippocampus, and basal forebrain. ACh is the acetyl ester of choline, is largely enclosed in small, clear synaptic vesicles in high concentration in the terminal boutons of neurons that release ACh (cholinergic neurons). Synthesis of ACh involves the reaction of choline with acetate. ACh is synthesized in the pre-synaptic terminal by choline acetyltransferase (ChAT), and is broken down after release into the synaptic cleft by AChE which is located on post synaptic membrane. ChAT is synthesized in the neuronal cell body and is carried by axonal transport to the presynaptic terminal. Choline and the acetyl group are grouped together by the enzyme ChAT to form ACh. Neurons that synthesize and release ACh are termed cholinergic neuron. When nerve impulse arrive nerve terminal, a voltage-gated calcium channel will open. The influx of calcium ions (Ca^{2+}) will stimulate the exocytosis of presynaptic vesicles containing ACh, which is thereby released into the synaptic cleft. Once released, ACh must be removed rapidly by AChE in order to

allow repolarization to take place, this step, hydrolysis is carried out by the enzyme. AChE metabolizes ACh into acetate and choline (Adamson *et al*., 1997, Figure 2.1).

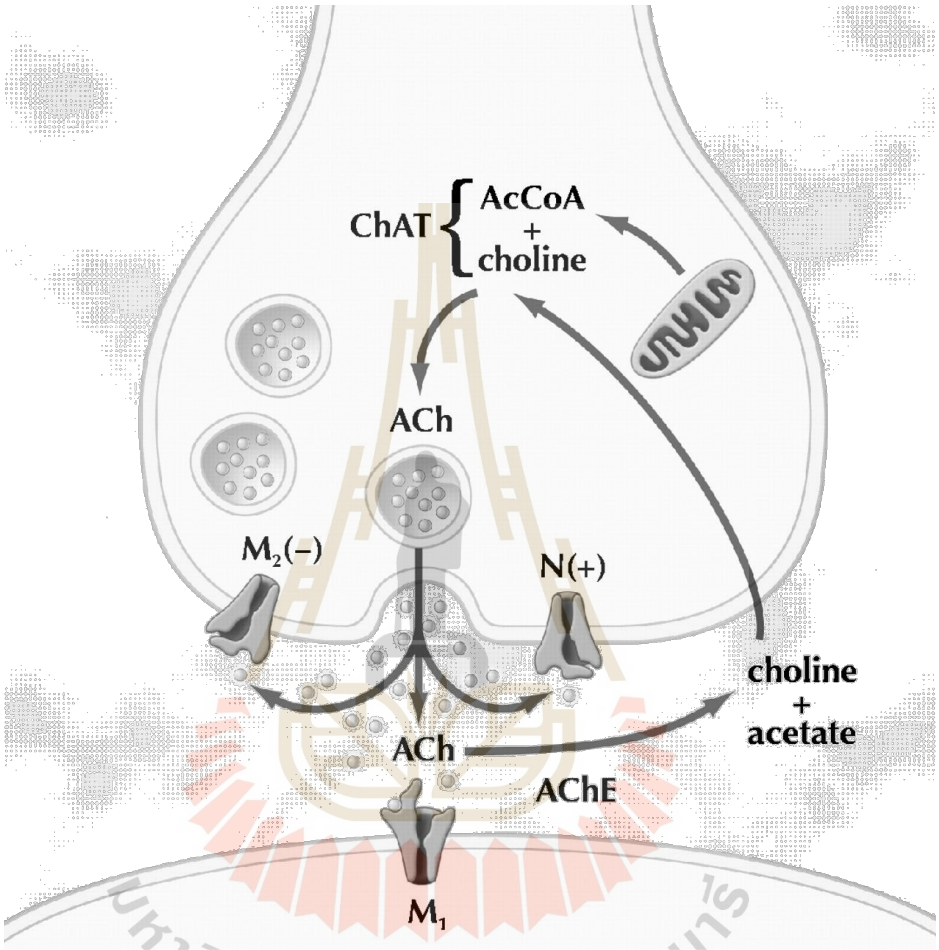

Figure 2.1 Synthesis of the neurotransmitter acetylcholine (ACh) from acetyl coenzyme A (AcCoA) and choline through the action of the enzyme choline acetyltransferase (ChAT). Acetylcholine is released into the synaptic cleft and acts on multiple sites of postsynaptic muscarinic type 1 (M_1) receptors. Acetylcholinesterase (AChE) breaks acetylcholine down into choline and acetate (Gauthier, 2002).

Central cholinergic pathways are ideally suited to regulate global functions of global cerebral cortex; such functions include attention, arousal, motivation, memory and consciousness (Woolf, 1991; Woolf, 1996). There are two groups of forebrain base cholinergic neurons: (1) a group in the middle wall (medial septal nucleus and vertical diagonal band: MS and vDB) that project cholinergic axons to hippocampus and parahippocampal gyrus and (2) the nucleus basalis group (nucleus basalis, horizontal diagonal band, and substantia innominata: nBas, hDb, Si) that project cholinergic axons to all parts of the neocortex, some parts of the cortical limbic and amygdala (Figure 2.2). Moreover, there are pontomesencephalon cholinergic neurons (laterodorsal tegmental and pedunculopontine tegmental nuclei: LDT and PPT) that project cholinergic axons to hindbrain, thalamus, hypothalamus and basal forebrain.

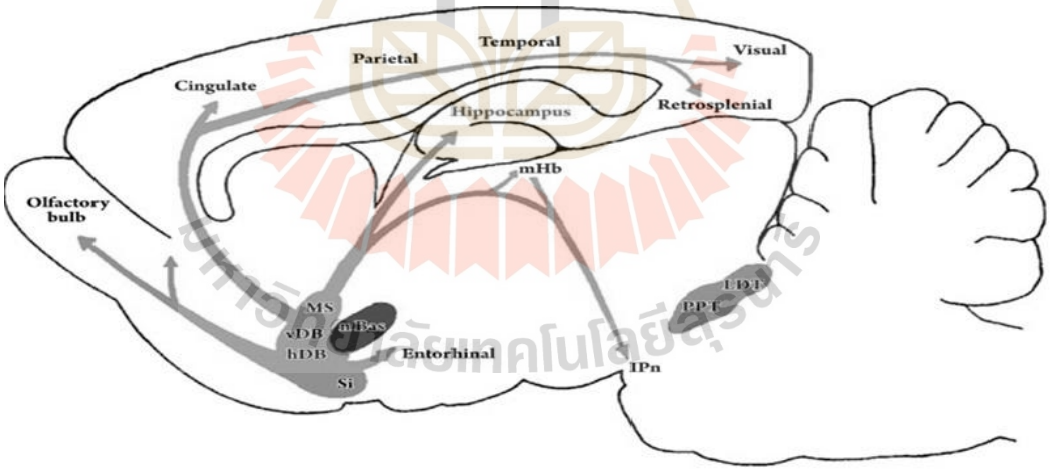

Figure 2.2 Schematic diagram showing the central cholinergic pathway. (MS: medial septum, nBas: nucleus basalis, vDB: vertical diagonal band, hDB: horizontal diagonal band, Si: sublenticular substantia innominata, mHb: medial habenula, LDT: laterodorsal tegmentum, PPT: pedunculopontine tegmentum, IPn: interpeduncular nucleus) (Placzek *et al*., 2009).

The major cholinergic afferent projections to the hippocampus are shown in Figure 2.2. The hippocampus receives cholinergic innervations mainly from the medial septum-diagonal band complex *via* the fimbria-fornix. A fine network of cholinergic fibers projects to the hippocampus and dentate gyrus, and synaptic contacts are made onto pyramidal cells, granule cells, interneurons, and neurons of the hilus. In addition to direct synaptic connections, ACh may spill out of synaptic contacts and also produce volume transmission within the hippocampus *via* non-synaptic signaling (Placzek *et al*., 2009). In humans and other mammals, the hippocampus is located inside the medial temporal lobe, beneath the cortical surface. It belongs to the limbic system and plays important roles in long-term memory and spatial navigation.

The basal forebrain is a collection of structures located ventrally to the striatum**:** the nucleus basalis, diagonal band of Broca, and medial septal nuclei. It is considered to be the major cholinergic output of the CNS. ACh produced from these areas affects the ability of brain cells to transmit information to one another, and also encourages plasticity, or learning. Thus, damage to the basal forebrain can reduce the amount of acetylcholine in the brain and impair learning and memory (Laursen *et al*., 2014).

The cerebral cortex is a sheet of neural tissue that is outer most to the cerebrum of the mammalian brain. It plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness (Kandel *et al*., 2000). It is divided into 5 main lobe: the frontal lobe is involved in conscious thought and plays an important part in processing short-term memories and longer-term memories; the parietal lobe is involved in integrating sensory information from the various senses, and in the manipulations of objects in determining spatial sense and navigation; the temporal lobe is involved with the sense of smell and sound, the processing of

semantics in both speech and vision and plays a key role in the formation of long-term memory; the occipital lobe is mainly involved with the sense of sight and the insula lobe is involved in consciousness and play a role in diverse functions usually linked to emotion or the regulation of the body's homeostasis (Craig, 2009; Craig, 2011).

2.2 Memory

Space and time appear to play key roles in the way that information is organized in short-term memory. Recent behavioral, neurophysiological and imaging studies have sought to investigate the nature of spatial, sequential and duration representations in short-term memory (STM), and how these might break down in disease. Hippocampus and other medial frontal lobe structures play an important role of the in aspects of STM, challenging conventional accounts of involvement of these regions in only long-term memory. Research on STM (storage of information over a few seconds) and working memory (manipulation of information held in STM) has gained new impetus over the last few years. New behavioral techniques have had a strong impact on the field, challenging some influential views of STM and working memory, how attention interacts with STM, even the role of the hippocampus in STM, and how new methods have provided new tools to probe brain mechanisms underlying STM for spatial location, sequences, and temporal durations. Theoretical considerations of the role of space and time in STM/working memory have led to two distinct views. Several different mechanisms have been proposed to support spatial and temporal contexts. Behaving effectively in dynamic settings, where we or other agents are on the move, often requires the use of STM for spatial locations (Manohar *et al*., 2017).

Memory encoding, consolidation and retrieval engage interactions between dispersed neuronal assemblies. The hippocampus is crucial to encode and store declarative-like memories. Several studies supporting a role for hippocampal-medial prefrontal cortex (mPFC) interplay in systems level consolidation show a selective role of the mPFC in retrieval of remote, but not recent memories (Frankland and Bontempi 2005; Winocur *et al*., 2010; Nadel and Hardt, 2011). The first of them (Bontempi *et al*., 1999) labeled glucose metabolism in mice recalling a 3-arm configuration in a radial maze and found more activity in the mPFC at remote (25 days) than recent (5 days) time-point. The implication of the mPFC in remote memory retrieval was confirmed for spatial and fear memory, taste aversion, social transmission of food preference, object recognition and trace eye-blink conditioning (*e.g*. Frankland *et al*., 2004; Maviel *et al*., 2004; Takehara-Nishiuchi *et al*., 2006; Teixeira *et al*., 2006; Ding *et al*., 2008; Restivo *et al*., 2009; Lesburguères *et al*., 2011; Vetere *et al*., 2011; Lopez *et al*., 2012; Weible *et al*., 2012). Other studies support a role for the mPFC in recent memory, as shown with trace fear conditioning (Blum *et al*., 2006; Corcoran and Quirk, 2007), inhibitory avoidance task (Gonzalez *et al*., 2013), active avoidance task (Bravo-Rivera *et al*., 2014) and after spatial paired-associate learning (Lee and Solivan, 2008). Intra-mPFC injections of the MEK inhibitor U0126 impaired fear (Runyan *et al*., 2004) and spatial memory (Leon *et al*., 2010). 5-HT_2A receptor blockade in the mPFC prevented object recognition shortlyafter training (Bekinschtein *et al*., 2013). Notably, most of these studies targeted the prelimbic/infralimbic (PrL/IL) areas of the mPFC. During retrieval, the mPFC might particularly control rule-based contextual memories, behavioral flexibility and strategy selection, but also segregate relevant from non-relevant

associations and more generally guide adaptive responses in close interrelation with the hippocampus (*e.g.*, Rich and Shapiro, 2009; Euston *et al.*, 2012; Preston and Eichenbaum, 2013). These studies suggested an involvement of mPFC regions in memory processes not depending on the age of a memory, in accordance with mPFC implications in functions like working memory, attention, evaluative mechanisms and top-down control of hippocampal processes (*e.g.*, Tomita *et al.*, 1999; Euston *et al.*, 2012). This view also has been supported by the studies showing an implication of the human ventromedial PFC (homolog to the mPFC in rodents) in retrieving relational and inferential memories (Zeithamova *et al.*, 2012) or associative information congruent with established knowledge (van Kesteren *et al.*, 2010, 2012). Churchwell *et al.* (2010) demonstrated the necessity of the hippocampus-mPFC (PrL cortex) circuit for encoding and retrieving allocentric goal locations. This observation was in line with data showing that activity of mPFC and hippocampal cells is highly correlated (Benchenane *et al.*, 2011; Gordon, 2011), and that neurons from the PrL and IL cortices display goal-, location- and time-specific firing patterns (Hok *et al.*, 2005, 2013; Hyman *et al.*, 2012). Finally, some studies showed a role of particularly the PL cortex in both recent and remote memory retrieval using trace fear conditioning (Blum *et al.*, 2006) or inhibitory avoidance task (Gonzalez *et al.*, 2013). The latter series of findings suggested a role for the mPFC in recent memory retrieval. If so, this should keep true for spatial, highly hippocampus-dependent memories. This is why we assessed, in rats, spatial memory retrieval 24 h after the acquisition of a Morris water maze task in a classical protocol. The extent of the induced inactivation was verified by c-Fos immunohistochemistry for the two highest amounts as compared to control.

2.3 Dementia

Dementia is a symptom complex of continuous global weak of intellectual function. It becomes a major medical, economic, and social problem that is deterioration as the number of elderly people in the general population increases (McPhee and Papadakis, 2009). Worldwide, 35.6 million people have dementia, with just over half (58%) living in low- and middle-income countries. Every year, there are 7.7 million new cases. The estimated proportion of the general population aged 60 and over with dementia at a given time is between 2 to 8 per 100 people. The total number of people with dementia is projected to almost double every 20 years, to 65.7 million in 2030 and 115.4 million in 2050. Much of this increase is attributable to the rising numbers of people with dementia living in low- and middle-income countries (Llibre *et al*., 2008).

Dementia is caused by various diseases and conditions that result in damaged brain cells or connections between brain cells. People with dementia may not be able to think well enough to do normal activities, such as getting dressed or eating. They may lose their ability to solve problems or control their emotions. Their personalities may change. They may become agitated or see things that are not there. Dementia is caused by a variety of diseases and injuries that primarily or secondarily affect the brain, such as AD. Dementia is one of the major causes of disability and dependency among older people worldwide. It is overwhelming not only for the people who have it, but also for their caregivers and families. There is often a lack of awareness and understanding of dementia, resulting in stigmatization and barriers to diagnosis and care. The impact of dementia on caregivers, family and societies can be physical and psychological (Brodaty and Donkin, 2009).

Alzheimer's disease (AD), a debilitating progressive neurodegenerative disorder associated with the ageing process, is the most common type of dementia. It is also characterized by loss of cognitive function, behavior and activities of daily living. It is also characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions that primarily affects the elderly population (Howes and Houghton, 2003). Dysfunction of cholinergic neurotransmission in the brain that cause cognitive decline has been reported in AD. The gradual death of cholinergic cells in AD is accompanied by loss of the acetylcholine (Vinutha *et al*., 2007). A cholinergic deficit has been shown to be associated with memory loss and the severity of AD. Loss of the cholinergic markers, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) are neurological changes consistently found in the brains of AD patients (Court and Perry, 1991). Hence, it has been suggested that elevation of acetylcholine level might be helpful in attempt to improve the symptoms of cognitive deficit in AD. One of the most promising approaches for treatment of this disease is to enhance the acetylcholine level in the brain using acetylcholinesterase inhibitors (Chattipakorn *et al*., 2007; Ingkaninan *et al*., 2003; Vinutha *et al*., 2007). AD is a successive age-related disorder that is characterized by the regression of neurological function showed in the loss of cognitive ability, loss of reasoning ability and other disturbances of affect. Progressive lost of acetylcholine occurs during the progression of AD. According to the cholinergic hypothesis, the replacement of acetylcholine can delay the loss of cognitive ability. AChE inhibitors have been shown to function by increasing acetylcholine within the synaptic region, by which restoring deficient cholinergic neurotransmission. In AD, the hippocampus is one of the first regions of the brain to suffer damage; memory problems and disorientation appear among the first symptoms. Damage to the

hippocampus can also result from oxygen starvation (hypoxia), encephalitis, or medial temporal lobe epilepsy. People with extensive hippocampal damage may experience amnesia, the inability to form or retain new memories (Felder *et al*., 2000). Dementia is characterized by loss of memory and cognitive abilities. Dementia is not a disease, but rather a group of symptom caused by disorders that affect the brain manner (Alzheimer's Association, 2011; 2012; 2013).

Since AD had been discovered over a century ago, acetylcholinesterase inhibitor like donepezil has been the mainstay for the symptomatic treatment of the disease. The memory enhancing effect of donepezil (DPZ) in patients with AD is generally ascribed to the elevation of central cholinergic neurotransmission. However, there are indications that DPZ may protect neurons against injury through the prevention of free radical-mediated neuroinflammation that has been implicated in the pathology of AD. Thus, this study was carried out to examine the effect of DPZ on memory impairment and on biomarkers of oxidative stress induced by scopolamine and lipopolysaccharide in mice brains (Umukoro *et al*., 2014).

Oxidative stress plays a key role in the pathogenesis of degeneration of several neuronal populations, especially central cholinergic pathways in AD brains. However, acetylcholine dysfunction is not the primary pathological cause for AD but rather represents one of the consequences of the disease. Diminished level of central cholinergic neurotransmitter has also been reported to be associated with accumulation of beta-amyloids (Aβ), a cardinal mediator of neuroinflammation. Scopolamine-induced memory impairment in rats has also been linked to oxidative stress and neuroinflammation *via* ROS generation (Rahimzadegan and Soodi, 2018).

Momcilović *et al*. (1999) revealed pathognomonic changes of biomolecules such as phosphorylation of lipid in oxidative stress and phosphorylation of protein in apoptotic process of the cortical grey and subcortical white matter from the frontal and temporal brain lobes of patients who had suffered from AD. Carmona *et al*. (2008) analyzed the effects of certain drugs on oxidative stress and protein structure of the brain sections by Fourier transform infrared (FTIR) microspectroscopy in order to find spectroscopic biomarkers for the detection of carbonyl ester group and asymmetric phosphate stretching, and their relationships with other molecular parameters that may be indicative of pathological processes (Vileno *et al*., 2010).

2.4 Plant-derived natural compounds against neurodegeneration

From *in vitro* studies, polyphenols from aqueous tea infusions of strawberry and walnut showed 45% and 42.5% inhibition of AChE activity, respectively (Kulišić-Bilušić *et al*., 2008). The aqueous tea infusions from walnut (*Juglans regia* L.), strawberry (*Fragaria ananassa* L.), peppermint (*Mentha piperita* L.), lemon balm (*Melissa officinalis* L.), immortelle (*Helichrysum arenarium* L.) and sage (*Salvia officinalis* L.) showed high rate of antioxidant activity determined by low-density lipoprotein (LDL) oxidation method, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, β-carotene bleaching method and Rancimat method. The highest inhibition of LDL oxidation was found in aqueous infusions of strawberry and lemon balm. Screening of the AChE inhibitory activity by Ellman's method showed high inhibitory activity in walnut and strawberry aqueous tea infusions, suggesting their possible role on the treatment of Alzheimer´s disease (Kulišić-Bilušić *et al*., 2008). Polyphenols from petroleum ether extract of *Salvia albimaculata* and chloroform

extract of *Salvia cyanescens* potently inhibited AChE activity at 0.2 $mg \cdot mL^{-1}$ (Orhan *et al*., 2007). Phenolic acid found in rosemary (rosmarinic acid) showed 85.8% inhibition against AChE activity at 1.0 $mg \cdot mL^{-1}$ (Orhan *et al*., 2008). Xanthones and benzophenones isolated from the fruit extract of *Allanblackia monticola* and the leaf extract of *Symphonia globulifera* showed potent anti-cholinesterase activity (Lenta *et al*., 2007). The aqueous extract of *Pistacia atlantica* and *Pistacia lentiscus* containing flavonoid showed high AChE inhibitory activity (% inhibition of 81.90%) (Benamar *et al*., 2010). Mukhergee *et al*. (2006) and Yang *et al*. (2008) reported that *N. nucifera* rhizome extract inhibited AChE activity and improved learning and memory by enhancing neurogenesis in the dentate gyrus of hippocampus.

From *in vivo* studies, isoflavones from the extract of soybean improved learning and memory performance, and reduced age-related neuronal loss and cognition decline in elderly male rats that was tested by water maze test. In addition, soybean extract also increased the ChAT activity in basal forebrain but decreased the AChE activity in cerebral cortex and hippocampus of aged rats (Lee *et al*., 2004). Procyanidins extracted from the lotus seed pod significantly reversed scopolamine induced learning and memory impairment in mice. In addition, the extract was found to inhibit AChE activity (Xu *et al*., 2009). Green tea extract could inhibit AChE activity in scopolamine-induced amnesia mice (Kim *et al*., 2004). Polyphenol-rich wild blueberry extract attenuated brain oxidative stress, increased brain ascorbate and glutathione (GSH) levels, and decreased AChE activity in mice whole brain homogenates. This extract also exhibited a significant improvement in learning and memory tested by the passive avoidance behavioral test (Papandreou *et al*., 2009). *Centella asiatica* extract were found to contain triterpenes namely asiaticoside, asiatic

acid and madecassic acid. The extract also contained high phenolic contents, which exhibited strong association with its anti-oxidative activities. The extract could alter the levels of markers of brain oxidative stress: a significant decrease in the rat brain levels of malondialdehyde (MDA) with simultaneous significant increase in levels of GSH. In addition, aqueous extract of whole plants of *Centella asiatica* showed an improvement in learning and memory of the rats in both shuttle box and step through paradigms (Veerendra *et al*., 2002). *Mangifera indica* fruit extract which contained polyphenol, flavonoids, tannins, saponins, and flavonols could prevent cognitive deficits and amnesic effect in aging mice and scopolamine-treated young mice in passive avoidance task and elevated plus maze task (Kumar *et al*., 2009). Grape seed extract containing polyphenol such as proanthocyanidin (condensed tannin) could promote memory in aging as decreased latency time, decreased path length but increased swimming speed as measured by Morris water maze test in male aged rat were demonstrated (Sarkaki *et al*., 2007). Nine weeks administration of grape seed proanthocyanidin extract could increase ChAT activity and decrease AChE activity in adult rat's hippocampus (Devi *et al*., 2006). The co-effect of procyanidins extracted from the lotus seed pod and bilobalide on ameliorating scopolamine-induced learning and memory impairment in young mice showed significantly shorter escape latency and swimming distance in the Morris water maze test (Zhang *et al*., 2009).

Flavonoids are a subclass of the polyphenols, which are consisted of an aromatic ring. There are six groups of flavonoids: anthocyanins, flavones, flavanones, catechins, isoflavonoids, and flavonols. Flavonoids found in fruits and vegetables contribute to the blue, orange or purple colors. Flavonoids found in certain beverages have diverse beneficial biochemical and antioxidant effects (Beecher, 2003; Hooper *et*

al., 2008). The importance of flavonoids in enhancing cell resistance to oxidative stress goes beyond simple scavenging activity and be of most interest in pathologies in which oxidative stress plays an important role (Weinreb *et al*., 2004). Flavonoids were shown to activate key enzymes in mitochondrial respiration and to protect neuronal cells by acting as antioxidants, thus breaking the vicious cycle of oxidative stress and tissue damage. Furthermore, recent data showed favorable effect of flavonoids on neuro-inflammatory events. Whereas, most of these effects have been shown *in vitro*, limited data *in vivo* are available, suggesting a rather low penetration of flavonoids into the brain (Schmitt-Schillig *et al*., 2005). The neuroprotective actions of dietary flavonoids involve a number of effects within the brain, including a potential to protect neurons against injury induced by neurotoxins, an ability to contain neuro-inflammation, and the potential to promote memory, learning and cognitive function (Spencer, 2009). The *in vivo* study of neuro-protective showed that green tea extract and (-)-epigallocatechin-3-gallate (EGCG) possessed highly potent activities in preventing striatal dopamine depletion as well as substantia nigra dopaminergic neuron loss in mice induced by the parkinsonism-inducing neurotoxin, N-methyl-4-pheny l-1,2,3,6-tetrahydropyridine (MPTP). One possible mechanism underlying the effectiveness of green tea and EGCG against MPTP neurotoxicity may involve its catechol-like structure, since it is known that catechol-containing compounds arepotent radical anti-oxidants and chelators of ferric ion (Weinreb *et al*., 2004). Krikorian *et al*. (2010) showed that wild blueberry juice supplementation for 12 weeks improved memory function in older adults with early memory decline. This is the first human trial evaluate the potential benefit of blueberry supplementation on neuro-cognitive function in older adults with increased risk for dementia. The FLDK-P70, a

standardized flavonoid extract from *Diospyros kaki* leaves, significantly increased the survival of hippocampal CA1 pyramidal neurons after transient global brain ischemia, reduced the lesion of the insulted brain hemisphere and improved the neurological behavior using *in vivo* rat model of focal ischemia/reperfusion (I/R) injury induced by middle cerebral artery occlusion (MCAO) (Bei *et al*., 2009). *Ginkgo biloba* extracts such as EGb-761 containing flavonoids, proanthocyanidins and terpenoids have been suggested to have a multitude of beneficial effects on CNS function, from enhancing cognitive function in dementia to facilitating recovery from acute forms of neural damage such as hypoxia/ischemia. *Ginkgolide* B, one of the major components of EGb-761 is also regarded as having neuro-protective effects in the CNS (Maclennan *et al*., 2002).

Anthocyanins are naturally occurring compounds that impart color to fruits, vegetables and plants. It is probably the most important group of visible plant pigments besides chlorophyll a part from imparting color to plants, anthocyanins have an array of health-promoting benefits, as it can protect against a variety of oxidants through a various number of mechanisms (Kong *et al*., 2003). Many claims have been made reference to the benefits to our health from eating a variety of fruits and vegetables, especially those with strong colors, such as carrots, apricots, blackcurrants, broccoli, purple grapes, and red wine. Anthocyanins have been shown to inhibit the growth of cultured human malignant cells and have demonstrated excellent anti-inflammatory and antioxidant properties (Pasqua *et al*., 2009). Anthocyanins could inhibit carcinogenic activity against multiple cancer cell types *in vitro* and tumor types *in vivo* (Wang and Stoner, 2008). Anthocyanins have been shown to inhibit the development of tumors induced in mice following the

subcutaneous injection of lung tumor cells (Ding *et al*., 2006). Anthocyanins from purple sweet potato improved cognitive performance as measured by passive avoidance tests in ethanol-treated mice, and also effectively inhibited lipid per-oxidation as measured by DPPH radicals in rat brain tissues (Cho *et al*., 2003). Incorporation of anthocyanin from elderberry extract by endothelial cells improved resistance to the damaging effects of ROS and protected against hydrogen peroxide induced loss in cell viability (Youdin *et al*., 2000). The dietary supplementation with a blueberry (*Vaccinium* spp.) extract originally composed of anthocyanins retarded age-related declines in aspects of neurological function, motor behavioral performance on the rod walking and accelerated tasks, and Morris water maze performance (Joseph *et al*., 1999).

2.5 Mangosteen

Mangosteen (*Garcinia mangostana* Linn.) is a tropical evergreen tree with leathery and glabrous leaves. The tree can attain 6-25 meters in height. It grows mainly in Southeast Asia, Southern India, and also in tropical South American countries such as Brazil. It is known as "the queen of fruits". The edible fruit aril is white, soft with slightly sour taste, and somewhat fibrous, with an inedible, deep reddish-purple colored rind when ripe (Ji *et al*., 2007).

The fruit rind (pericarp) of *G. mangostana* has been used in Southeast Asia traditional medicine for the treatment of skin infections wound, and diarrhea (Jung *et al*., 2006; Suksamrarn *et al*., 2003). The fruit rind of *G. mangostana* is the rich source of polyphenols (xanthone, tannin, flavonoid and anthocyanins) (Asai *et al*., 1995; Chen *et al*., 2008; Chomnawang *et al*., 2007; Cui *et al*., 2010; Ji *et al*., 2007;

Maisuthisakul *et al*., 2007; Yu *et al*., 2007; Zadernowski *et al*., 2009). These polyphenols are of plant secondary metabolites. The most common roles of secondary compounds in plants are ecological roles that govern interactions between plants and other organisms. Many secondary compounds are brightly colored pigments like anthocyanin that color flowers.

Plant secondary metabolites have also been reported to possess a wide range of pharmacological activities, including antimicrobial, anti inflammatory, anti-diabetic and AChE inhibitory activities (Eldeen *et al*., 2005). The fruit rind of *G. mangostana* contained four types of the xanthones: α-mangostin, β-mangostin, γ-mangostin, and gartanin (Mahabusarkam and Wiriyachitra, 1986). These compounds are the major secondary metabolites of *G. mangostana* which are classified into prenylated xanthone derivatives (Pedraza-Chaverri *et al*., 2009).

Young stage of *Garcinia mangostana* fruit generally contained larger amounts of tannin and lesser amount of α-mangostin (Figure 2.3) than that of the old stage. By high performance liquid chromatography (HPLC) method, the mature fruit rind extract contained α-mangostin (13.63 ± 0.06%, w/w) about 2 times higher than that of the young fruit rind extract (8.07 ± 0.11%, w/w) (Pothitirat *et al*., 2008).

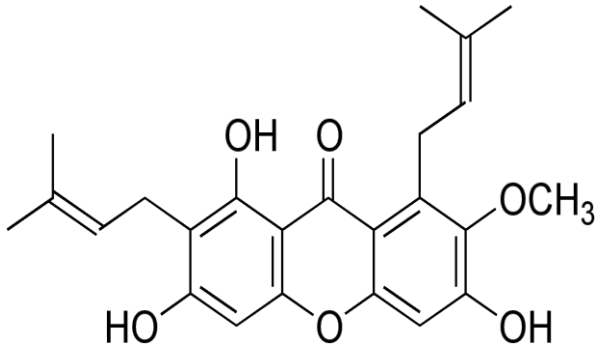

Figure 2.3 The structure of α-mangostin (Shibata *et al*., 2011).

Tannin can be found in *Garcinia mangostana* fruit hull (Pothitirat *et al*., 2009). Young stage of *G. mangostana* fruit generally contained larger amounts of tannin than that of the old stage. The contents of total tannins in young and mature fruit rind extracts of *G. mangostana* were 51.25 and 36.66 TAE·100g^{-1}, respectively (Pothitirat *et al*., 2009).

2.6 α-Mangostin

α-Mangostin, a xanthone derivative, is a class of plant polyphenols that can be found in the whole *G. mangostana* fruit (the fruit rind, edible arils and seed) (Moongkarndi *et al*., 2004). α-Mangostin purified from the fruit rind of *G. mangostana* exhibits a variety of biological activities, including antibacterial, anti-inflammatory and anticancer effects (Akao *et al*., 2008). The α-mangostin purified from the fruit rind of *G. mangostana* could prevent lipid peroxidation in rats treated with isoproterenol by reducing the activity of lactate dehydrogenase (LDH), creatine phosphokinase (CPK), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) and increased antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Devi Sampath and Vijayaraghavan, 2007). Furthermore, Chomnawang *et al*. (2007) found that the ethanol extract of *Garcinia mangostana* showed antioxidant activity with the IC_{50} of 6.13 μg·mL^{-1} as measured by inhibition of the formation of DPPH radicals. The extract of *G. mangostana* reduced the ROS production of polymorphonuclear leucocytes (PML) with 77.8% of superoxide anion inhibition ratio. Besides, the α-mangostins could decrease the human LDL oxidation induced by copper or peroxyl radical. α-Mangostins prolonged lag time of conjugated dienes at 234 nm in a dose-

dependent manner, decreased thiobarbituric reactive substances (TBARS) production, and decreased the α-tocopherol consumption induced by LDL oxidation (Williams *et al*., 1994). α-Mangostins and their synthetic derivatives prevented the decrease of the α-tocopherol consumption induced by LDL oxidation (Mahabusarakam *et al*., 2000). The *G. mangostana* extract significantly diminished intracellular ROS production on SKBR3 cell in the dose dependent manner as well as exposure time dependent manner, as measured by 2, 7-dichlorofluorescein diacetate (Moongkarndi *et al*., 2004). The four extracts (water, 50% ethanol, 95% ethanol and ethyl acetate) from fruit rind of *G. mangostana* showed antioxidant activity by the DPPH method and neuroprotective effect. Water and ethanolic (50%) extracts showed high antioxidant capacity (34.98 and 30.76 $\mu g \cdot mL^{-1}$, respectively). The antioxidant capacity of these extracts was tested on a neuroblastoma cell line (NG108-15) exposed to hydrogen peroxide (H_2O_2); both extracts at concentration of 50 $\mu g \cdot mL^{-1}$ exhibited neuroprotective activity. These results suggested that the water and 50% ethanol extracts from the fruit hull of *G. mangostana* may be potent neuroprotectants (Weecharangsan *et al*., 2006).

Structural modifications of α-mangostin could modify the antioxidant activity. For example, substitution of C-3 and C-6 with aminoethyl derivatives enhanced the antioxidant activity. The xanthone induced apoptosis in HL60 cells. α-Mangostin induced caspases 9 and 3 activation, loss of mitochondrial membrane potential, and release of ROS and cytochrome C. These results indicated that mitochondria play a pivotal role in induction of apoptosis by α-mangostin (Matsumoto *et al*., 2004). Theprenylated xanthones from the fruit rind of *G. mangostana* exhibited strong inhibitory effect against *Mycobacterium tuberculosis* with the minimum inhibitory

concentration value of 6.25 μg·mL^{-1} (Suksamrarn *et al*., 2003). Xanthones isolated from the fruit powder of *G. mangostana* showed HO·-scavenging activity (IC_{50} = 0.2 μg·mL^{-1}). All xanthones, except α-mangostin, were found to induce activity of quinone reductase (QR, phase II drug-metabolizing enzyme), using murine hepatoma cells (Hepa 1c1c7) *in vitro* (Chin *et al*., 1996). Mangiferin, a xanthone found in *Mangifara indica*, showed enhances recognition memory through a mechanism that might involve an increase in neurotropin and cytokine levels and an increase of supernatant level of nerve growth factor *in vitro* in human U138-MG glioblastoma cell (Pardo Andreu *et al*., 2010). Young stage of *G. mangostana* fruit generally contained larger amounts of α-mangostin, a xanthone isolated from *G. mangostana* fruit, than that of the old stage (Pothitirat *et al*., 2009). By high performance liquid chromatography (HPLC) method, the mature fruit rind extract contained α-mangostin (13.63 ± 0.06% w/w) about 2 times higher than that of the young fruit rind extract (8.07 ± 0.11% w/w) (Pothitirat *et al*., 2009). The yellowish excretion of the fruit hull of *G. mangostana*, a crystalline mixture consisting mainly α- and γ-mangostin showed an inhibitory effect on cAMP phospho-diesterase (Chairungsrilerd *et al*., 1996). α-Mangostin, a xanthone isolated from *G. mangostana* fruit, could reduce oxidative damage in rat brain tissue exposed to the toxic actions of a free radical generator (ferrous sulfate), an excitotoxic agent (quinolinate), and mitochondrial toxin (3-nitropropionate) (Márquez-Valadez *et al*., 2009).

Tannins are polyphenolic secondary compounds of plants which constitute a complex group of naturally occurring polymers, and a rigorous chemical definition is difficult. The term “tannin” was originally used to describe vegetable components that are responsible for converting animal hides into leather in the process of tanning by

forming stable complexes with skin collagen. Thus, tannins are considered to be polyphenolic metabolites of plants with a molecular weight larger than 500 and with the ability to precipitate gelatin and other proteins from solution (Bennick, 2002). Various chemical structures of tannins occurring in medicinal and food plants that are utilized world-wide showed several remarkable biological and pharmacological activities that are often very specific to certain tannin structures, and significant for human health. Tannin has been evaluated as antibacterial, antiviral, radical scavenging and complement modulating agent (Okuda, 2005). Tannin-containing plant extracts were used as astringents, against diarrhea, as diuretics, against stomach and duodenal tumors (Khanbabaee and Ree, 2001). Curcumin, a polyphenol extracted from the rhizome of *Curcuma longa*, is well known to have antioxidative, anti-cancerous and anti-inflammatory activities, and anti-ageing and neuroprotective potential effects.

Pedraza-Chaverri *et al*. (2008) ROS scavenging capacity and neuroprotective effect of α–mangostin against 3-nitropropionic acid in cerebellar granule neurons (Pedraza-Chaverrí *et al*., 2009), the natural xanthonoid α–mangostin reduces oxidative damage in rat brain tissue (Márquez-Valadez *et al*., 2009), renoprotection by α-mangostin which was related to the attenuation in renal oxidative/nitrosative stress induced by cisplatin nephrotoxicity (Pérez-Rojas *et al*., 2009), and effects of α-mangostin on mitochondrial energetic metabolism (Martínez-Abundis *et al*., 2010). Furthermore, Reyes-Fermín *et al*. (2012) found that α-mangostin and curcumin showed neuroprotective effect against iodoacetate-induced cell death.

The α–mangostin is one of the best dietary supplements, could be an effective nutraceutical compound for the preventive therapy of neurodegenerative diseases. α-Mangostin, a polyphenolic xanthone derivative from *G. mangostana*, attenuates

β-amyloid oligomers-induced neurotoxicity by inhibiting amyloid aggregation (Wang *et al*., 2012). The pretreatment of α–mangostin ameliorated the neuronal death in primary cultures of cerebellar granule neurons induced by iodoacetate in a concentration-dependent way, which was associated with an amelioration of reactive oxygen species formation (Reyes-Fermin *et al*., 2012).

Several researchers predicted that α-mangostin may cross the blood-brain barrier and achieve enough bioavailability to elicit a protective response in the brain being an effective nutraceutical compound for preventive therapy of neurodegenerative diseases (Reyes-Fermín *et al*., 2012) and α-mangostin, the derivatives may have better blood brain barrier penetration (Yoon *et al*., 2013). Nontamart *et al*. (2013) found that the crude extract from the fruit rind of *G. mangostana* exhibited memory enhancing and acetylcholinesterase inhibiting activities in normal adult and normal aging rats. Furthermore, Pedraza-Chaverrí *et al*. (2009) demonstrated ROS scavenging capacity and neuroprotective effect of α-mangostin against 3-nitropropionic acid in cerebellar granule neurons.

2.7 Morris Water Maze Test

The Morris Water Maze (MWM) is one of the most widely used tests to measure hippocampal-dependent spatial-based learning and memory. This test is affected by aging, hormonal changes, and amnesia producing as well as cognition enhancing drugs as well as genetic manipulations of genes associated with cognition. Various protocols exist for these tests, which are used depending on a plethora of variables. Some of the protocols may be too diffuse/easy and others too intense/difficult for the animal to learn. Variables include age, strain, experimental manipulation, or

predisposition to stress, therefore a careful study of these variables is necessary before carrying out the test. Test specification: The rodent is placed in a pool of water where it must use and remember visual cues located in the room to find a platform hidden underneath the surface of the water. The task is carried out across days to determine learning. Distance swam, latency to reach the platform, and swim speed are common measures of this test. The capacity of the animal to retrieve and retain information learned or flexibility to purge and re-learn new strategies can be determined using a probe trial & reversal trial. In the probe trial, the platform is taken out and the animals are allowed to swim in the pool. Time spent in the region that previously contained the platform, crossings over the platform area, and time to reach platform location is measured. The reversal trial is identical to the training trials but in this case the platform is switch to the opposite region of the pool, thus the animal has to have the cognitive flexibility necessary to re-learn the new location. A cued version of this task can also be used to measure none spatial strategies as well as visual acuity by rendering the platform visible (Morris, 1981).

2.8 Vitamin E

Vitamin E is a fat-soluble compound that includes both tocopherols and tocotrienols. Of the many different forms of vitamin E, γ-Tocopherol can be found in corn oil, soybean oil, margarine, and dressings. α-tocopherol, the most biologically active form of vitamin E, is the second-most common form of vitamin E. This variant can be found most abundantly in wheat germ oil, sunflower, and safflower oils. The various tocopherol forms rather than α- tocopherol alone may be important in the vitamin E protective association with AD by the administration of 4 cognitive tests

and clinical evaluations for AD (Morris *et al*., 2005)**.** Vitamin E is function as natural antioxidant scavenging free radicals in cell membranes and protecting unsaturated fatty acids from lipid peroxidation (Meydani, 1995). In addition, the combination of vitamine E (10, 25 and 50 μg/rat) with nicotine (0.1 μg/rat) or pilocarpine (0.5 μg/rat) increased memory retention using a step-through passive avoidance paradigm (Eidi *et al*., 2006). Aged 24-month-old male Sprague-Dawley rats treated for 4–5 months with daily i.p. injections of α-tocopherol (200 mg/kg) can improve cognitive function during aging (Socci *et al*., 1995). Vitamin E supplementation affected learning behavior and protect against the deterioration in passive avoidance response in aging rats (Ichitani *et al*., 1992). Vitamin E improved cognitive performance in aged animals and prevented the oxidative damage induced by β-amyloid in cell. Vitamin E can delay or prevent a clinical diagnosis of AD in elderly persons with mild cognitive impairment (Grundman, 2000).

2.9 Donepezil

DPZ is the major drug currently used for enhancing memory function in patients with AD, an action ascribed to the elevation of central cholinergic neurotransmission. DPZ, also known as Aricept or E2020, is one of the new generation cholinesterase inhibitor designed for the symptomatic treatment of mild to moderate AD by enhancing cognitive function in patients (Gupta and Dekundy, 2005). Although donepezil has some side effects which are generally tolerable and it is a selective inhibitor of AChE compared to earlier cholinesterase inhibitors such as rivastigmine which can inhibit both AChE and butyrylcholinesterase (BChE) (Sun *et al*., 2012), it however, has comparative advantages over the earlier designed cholinesterase

inhibitors by being more easily absorbed after oral administration, better tolerated and causes less complications (Gupta and Dekundy, 2005). Donepezil binds and reversibly inactivates the cholinesterases, thus inhibiting hydrolysis of acetylcholine, resulting in increases in acetylcholine concentrations at cholinergic synapses. The precise mechanism of action of donepezil in patients with AD is not fully understood. Certainly AD involves a substantial loss of the elements of the cholinergic system and it is generally accepted that the symptoms of AD are related to this cholinergic deficit, particularly in the cerebral cortex and other areas of the brain. It is noted that the hippocampal formation plays an important role in the processes of control of attention, memory and learning. Just the severity of the loss of cholinergic neurons of the central nervous system has been found to correlate with the severity of cognitive impairment.

In addition to its actions as an acetylcholinesterase inhibitor, DPZ has been found to act as a potent agonist of the σ1 receptor (K_i = 14.6 nM), and has been shown to produce specific antiamnestic effects in animals mainly *via* this action. In this study, we therefore investigated the possible influence of caffeine on the anticholinesterase and antioxidant properties of donepezil *in vitro* and *in vivo* in rat brain to elucidate the potential or otherwise of this food-drug interaction as it pertains to AD management. Previous study showed the effect of DPZ on memory impairment and on biomarkers of oxidative stress induced by scopolamine (SCOP) and lipopolysaccharide in mice. However, there are indications that DPZ may protect neurons against injury through the prevention of free radical-mediated neuroinflammation that has been implicated in the pathology of AD (Umukoro *et al*., 2014).

2.10 Scopolamine

Scopolamine (SCOP), known by the name levo-duboisine and hyoscine, sold as Scopoderm, is a tropane alkaloid drug that has anticholinergic properties and muscarinic antagonist effects. It is among the secondary metabolites of plants from Solanaceae (nightshade) family of plants, such as belladonna, brugmansia, henbane, mandrake, and datura. Scopolamine exerts its effects by acting as a competitive antagonist at muscarinic acetylcholine receptors, specifically M_1 receptors; it is thus classified as an anti-cholinergic, anti-muscarinic drug. Scopolamine has legitimate medical applications in very minute doses. As an example, in the treatment of motion sickness, the dose, gradually released from a transdermal patch, is only 330 microgrammes (μg) per day (Neurosoup, 2013). Scopolamine can be administered orally, subcutaneously, ophthalmically andintravenously, as well as *via* a transdermal patch (White, 2007). The transdermal patch (*e.g.*, Transderm Scōp) for prevention of nausea and motion sickness employs scopolamine base, and is effective for up to three days (Transdermscop, 2013). Scopolamine provides a suitable pharmacological model of the memory defects associated with cortical or sub-cortical dementias. Scopolamine clearly produces deficits on some measures of anterograde memory, anti-cholinergic drugs adequately mimic the full range of memory impairments observed in cortical or sub-cortical dementia (Beatty *et al*., 1986). Oh *et al*. (2009) showed that *Nelumbo nucifera* semen extract (1 $g \cdot kg^{-1}$) can improve memory on rats with scopolamine (1 $mg \cdot kg^{-1}$)-induced amnesia by inhibiting AChE activity. Scopolamine, an acetylcholine receptor-deregulating compound, has been used to create AD models for studies into the pathophysiological mechanisms of AD and drug development (Hasselmann, 2014). The relatively low doses of certain muscarinic acetylcholine-receptor

antagonists were found to induce transient cognitive deficits in young human volunteers that resembled those observed in elderly subjects (Drachman *et al*., 1974). Scopolamine has been employed in many studies to understand, identify, and characterize therapeutic targets for AD. Scopolamine-induced dementia is associated with impairments in memory and cognitive function, as seen in patients with AD. Ebert and Kirch (1988) found that scopolamine could alter certain features of the human electroencephalogram (*e.g*., delta, theta, alpha, and beta activities) in a fashion that mimics some of the changes observed in patients with AD. Scopolamine (0.2 mg) impaired new learning in the elderly as measured by Selective Reminding Test (SRT) (P<0.04) and SRT delayed recall (Zemishlany and Thorn, 1991). Interestingly, scopolamine appears to negatively affect cognitive performance to a greater extent in elderly subjects than in younger subjects (Flicker *et al*., 1992), and it impaired subjects with AD more dramatically than nondemented elderly subjects (Sunderland *et al*., 1974). Similarly, aged rodents displayed cognitive impairments in many learning and memory tasks (Ingram *et al*., 1994) and were more sensitive to the disruptive effects of scopolamine than young rats (Gallagher *et al*., 1974). Scopolamine also provides a suitable pharmacological model of the oxidative stress. Scopolamine treatment generates reactive oxygen species (ROS) (Tao *et al*., 2014). Oxidative stress-induced cell death is an important cause of neurodegeneration in this AD model.

CHAPTER III

EFFECTS OF α-MANGOSTIN ON THE ANTIOXIDANT AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES *IN VITRO* IN RELATION TO THE TREATMENT OF ALZHEIMER'S DISEASE

3.1 Abstract

Alzheimer's disease (AD) is a progressive neurodegerative disease clinically characterized by impairment of learning and memory. The effective therapeutic options for the treatment of AD are limited and thus there is a demand for new drugs. The crude extract from the fruit rind of *Garcinia mangostana* (Linn.) has been found that enhanced learning and memory. The present study aimed to investigate the effects of α-mangostin and donepezil on antioxidant properties and acetylcholine esterase inhibitory activities *in vitro*. The results of antioxidant activities showed that 100 - 600 $\mu g \cdot mL^{-1}$ α-mangostin and donepezil gave FRAP values within the 20 - 410 μmol $Fe^{2+} \cdot L^{-1}$. The concentrations of α-mangostin and donepezil required to inhibit 50% (IC_{50}) of ABTS were 21.52 ± 3.45 and 14.53 ± 1.86 $\mu g \cdot mL^{-1}$, respectively and DPPH were 38.12 ± 8.36 and 29.44 ± 5.13 $\mu g \cdot mL^{-1}$, respectively. The acetylcholinesterase inhibitory activity of α-mangostin and donepezil were measured by Ellman's and NA-FB method. By Ellman's method, the concentrations of α-mangostin and donepezil required to inhibit 50% of acetylcholine esterase activity (IC_{50}) were 64.23 ± 0.22 and

32.46 ± 0.14, respectively. At the same concentration, the acetylcholinesterase inhibitory activity of donepezil was stronger than α-mangostin with the same pattern of inhibitory action as their reference standard. By NA-FB method, there was no significant difference in the acetylcholinesterase inhibitory activity of α-mangostin and donepezil when compared to eserine at the same concentration (50 $\mu g \cdot mL^{-1}$). These findings suggested that the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil exhibited the potent antioxidant properties and acetylcholinesterase inhibitory activities *in vitro* and, thus, may be useful for the treatment of AD.

3.2 Introduction

Alzheimer's disease (AD), a major cause of mortality in elderly people, is a progressive neurodegenerative disorder characterized by a gradual loss of memory, cognition and behavioral abnormalities. The pathological hallmarks of AD are profound loss of cholinergic neurons, senile plaque consisting of Aβ protein, and neurofibrillary tangles of microtubule-associated protein tau (Serrano-Pozo *et al*., 2011). Although there has been tremendous progress in understanding the etiology and pathogenesis of AD, effective drugs remain limited. Extensive loss of cholinergic neurons, particularly in the basal forebrain is a prominent feature observed in AD patients that is accompanied by deficiency of acetylcholine (ACh), a neurotransmitter found in the synapses of the cerebral cortex (Drachman *et al*., 1974; Gallagher and Colombo, 1995). The loss of cholinergic neurons and the associated decrease in levels of ACh has been found to correlate well with the cognitive impairment seen in AD patients (Francis *et al*., 1999). Therefore, elevating ACh by inhibiting

acetylcholinesterase (AChE), which is involved in the breakdown of ACh, appears to improve the symptoms of cognitive deficit in AD and serves as an important strategy in the development of drugs. To date, only three cholinesterase inhibitors, donepezil, galantamine, rivastigmine, and memantine, have been approved by the US Food and Drug Administration to treat AD. These drugs have ameliorated symptoms and improved the functioning of patients with AD, but none can completely restrict or reverse the progression of AD (Khoury *et al*., 2017).

A substantial body of evidence implicates oxidative stress in the etiology and pathogenesis of AD. It has been shown that the reactive oxygen species (ROS) and other free radicals, which are formed and accumulated during oxidative stress as a result of an imbalance between their production and removal by the antioxidant system, induce cellular and molecular abnormalities in sporadic AD (Huang *et al*., 2016). Although the exact mechanisms underlying these deleterious effects remain unclear, it is known that oxidative stress occurs before the formation of neurofibrillary tangles and senile plaques, both of which are hallmarks of AD. It has been further demonstrated that Aβ protein, the major component of senile plaque in the brain of AD patients, causes an increase in free radical production in neuronal cells, leading to oxidative stress and cell death (Foster *et al*., 1994). Therefore, antioxidants have been suggested as therapies to prevent, delay or ameliorate the pathological changes underlying the progression of AD (Eidi *et al*., 2006; Gray *et al*., 2008; Grundman, 2000). Recently, there has been increasing interest in the natural antioxidants contained in the medicinal plants, which are candidates to prevent oxidative damage and cognitive deficit (Bei *et al*., 2009; Benamar *et al*., 2010; Chattipakorn *et al*., 2007; Cho *et al*., 2003; Devi *et al*., 2007; Eldeen *et al*., 2005; Howes and Houghton, 2003).

Recent studies indicate that oxidative stress and free radical generation have been implicated as major etiological factors in the pathogenesis of AD. The free radicals generated in turn induce the lipid peroxidation, production of free carbonyls, formation of advanced glycation end products (AGEs), and DNA damage, ultimately resulting in neurodegeneration. In addition to that, it has been verified that oxidative stress can induce amyloidogenic processing of beta-amyloid precursor protein (β-APP), resulting in accumulation of potentially neurotoxic Aβ species. One of the earliest pathological events in AD is thought to be the dysfunction and loss of basal forebrain cholinergic neurons and their cortical projections. In general, acetylcholinesterase (AChE), a serine hydrolase that is primarily associated with cholinergic neurons and catalyses the hydrolysis of esters of choline that is associated with supporting glial cells and specific cholinergic nerve tracts. The AChE is highly efficient since they are able to cleave more than 10,000 molecules of acetylcholine (ACh) per second and produce acetate and choline rapidly. Cholinergic neurons produce ACh by the action of enzyme choline acetyltransferase (ChAT), which gets concentrated in seminal vesicles and released from the presynaptic cell following depolarization. Under diseased conditions, the activity of AChE is high, resulting in scarcity in the levels of ACh, which ultimately results in the halt of neurotransmission. Furthermore, it has been previously demonstrated that AChE promotes Aβ aggregation and has been found to be associated with neuritic plaque formation and neurofibrillary tangles, which are the major neuropathological features of AD. Hence, in the current scenario, inhibitors of AChE have become the major targets for the development of therapeutic strategies for AD. The AChE inhibitor enhances the cholinergic neurotransmission by prolonging the time in which ACh molecules remain in the synaptic cleft. A preliminary study has

shown that *G. mangostana* has 1,1-diphenyl-2-picryl hydrazyl radical scavenging activity (Ngawhirunpat *et al*., 2010). Nontamart *et al*. (2013) found that the aqueous ethanol extract from the fruit rind of *G. mangostana* demonstrated antioxidant and memory enhancing activities possibly by exhibiting free radical scavenging and acetylcholinesterase inhibitory effects. The mechanism of action of *G. mangostana* could be attributed to a combination of cholinergic modulation and antioxidant effects. Although *G. mangostana* showed an anti-AD capability which has important medicinal values for the treatment of AD, no studies have yet examined its phenolic compound, α-mangostin. Therefore, the objective of this study was to evaluate the inhibition of AChE activity and antioxidant properties of α-mangostin in order to treat AD.

3.3 Materials and methods

3.3.1 Chemicals

α-Mangostin from the fruit rind of *Garcinia mangostana* (Linn.) in form of yellow crystals at purity (HPLC) 99.35% was purchased from Indofine Ltd. (Hillsborough, NJ, USA), Methanol (CH_3OH, Carlo Erba Reagents, France), 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS), potassium ferric cyanide, trichloroacetic acid, ferric chloride, acetylcholinesterase (AChE) type VI-S from an electric eel, Tris-HCl [Tris(hydroxy methyl) aminomethane hydrochloride], β-naphtyl acetate, 3,3'- dimethoxybiphenyl-4,4'-di (diazonium) zinc chloride (fast blue B salt), acetylthiocholine iodide (ATCI), 5,5'-dithiobis [2- nitrobenzoic acid] (DTNB), 2,2- diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), ascorbic acid (AA), quercetin (QCT) (Sigma-Aldrich, St. Louis, MO, USA).

3.3.2 Methods for determination of antioxidant activities *in vitro*

3.3.2.1 $ABTS^{\bullet+}$ radical scavenging activity

Several studies have indicated that Aβ induces apoptosis and neuronal cell death by producing ROS, which leads to the peroxidation of membrane lipids and oxidative stress. Radical cations are the reactive oxygen species causing lipid oxidation and enormous biological damage. $ABTS^{\bullet+}$ is a long-life time, nitrogen-centered radical cation and requires a short time in scavenging reaction, so it is extensively used to evaluate scavenging activities of antioxidants (Miller and Rice-Evans, 1997). In the present study, the assay was conducted in 0.1 $mol \cdot L^{-1}$ phosphate buffer, pH 7.4 and monitored at 10 min. To determine the radical scavenging capacity, the effect of α-mangostin on radical cations generated in an ABTS-$K_2S_2O_8$ system was analyzed by evaluating the degree of $ABTS^{\bullet+}$ degradation (Figure 3.1). All samples scavenged the radical cation generated in the reaction and enhanced degradation of $ABTS^{\bullet+}$. The radical scavenging activity of all samples was significant. The ABTS-persulfate method was performed according to colorimetric method described by Thaipong *et al*. (2006) and Gan *et al*. (2010). Briefly, $ABTS^{\bullet+}$ was produced by reacting ABTS stock solutions (7 $mmol \cdot L^{-1}$) and potassium persulfate (2.45 $mmol \cdot L^{-1}$) in water and the mixture was maintained in the dark for 16 h before use. Fifty µL of α-mangostin, donepezil, or standard (quercetin, QCT) solution at different concentrations was mixed with 2.5 mL of ABTS in phosphate buffer (0.1 $mol \cdot L^{-1}$, pH 7.4) with an absorbance of 0.75 ± 0.02 at 420 nm, which produced between 20−80% inhibition of the blank absorbance. The solution was incubated at 30°C for 10 min and absorbance at 420 nm was read (Figure 3.1). $ABTS^{\bullet+}$ radical scavenging ability (%) was calculated by using Equation 3.1.

Equation 1. Inhibition (%) = $[1 - (A_{sample}/A_{blank})] \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Test compound concentration providing 50 % inhibition (IC_{50}, expressed in $\mu g \cdot mL^{-1}$) was calculated from the graph plotting inhibition percentage against extract concentration. Synthetic antioxidant quercetin (QCT) was used as a reference standard and all tests were carried out in triplicate.

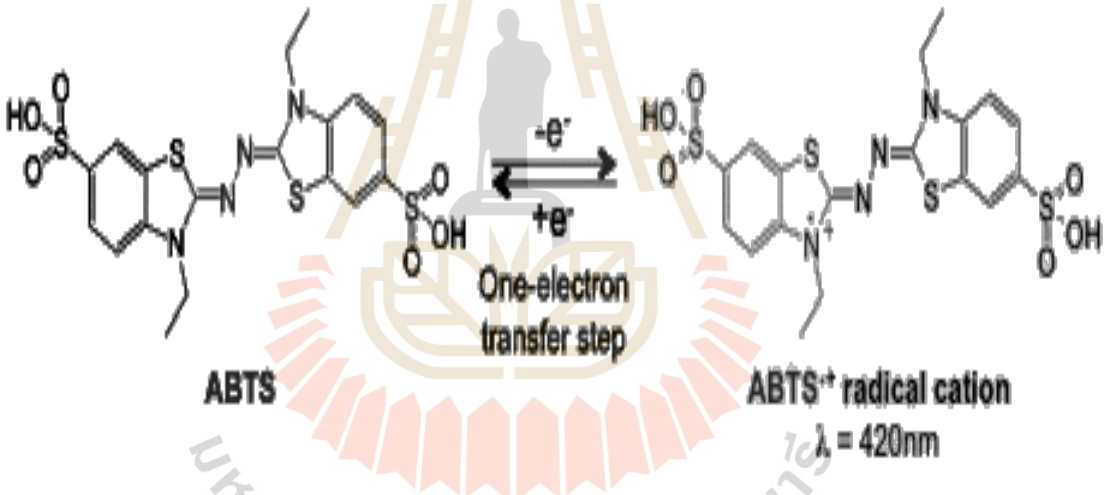

Figure 3.1 Chemical reaction of $ABTS^{\bullet+}$ scavenging activity.

3.3.4 Determination of DPPH radical scavenging activity

Radical scavenging activities are very important to prevent the deleterious role of free radicals in AD. The stable DPPH radical scavenging model is a widely used method to evaluate the free radical scavenging ability of various samples, including plant extracts, because of the relatively short time required for analysis. DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize

in the presence of antioxidants that can be quantitatively measured from the change in absorbance; the percentage of scavenging activity is then calculated (Kedare and Singh, 2011). The hydrogen atom or electron donation ability of α-mangostin was measured from the bleaching of purple colored methanol solution of DPPH. The DPPH radical-scavenging effect was evaluated following the procedure described in a previous study (Blois, 1958) with minor modifications. Briefly, aliquots 0.5 mL solution of α-mangostin, donepezil or standard (synthetic antioxidant butylated hydroxytoluene, BHT) solution at different concentration was mixed with 3 mL of 0.5 mmol·L^{-1} DPPH radical in methanol solution and incubated at room temperature for 45 min in a dark place to complete the reaction. The absorbance of the solution was measured at 519 nm (Figure 3.2). DPPH free radical scavenging activity was calculated by using Equation 3.1 and expressed as a half maximal inhibitory concentration (IC_{50}) value.

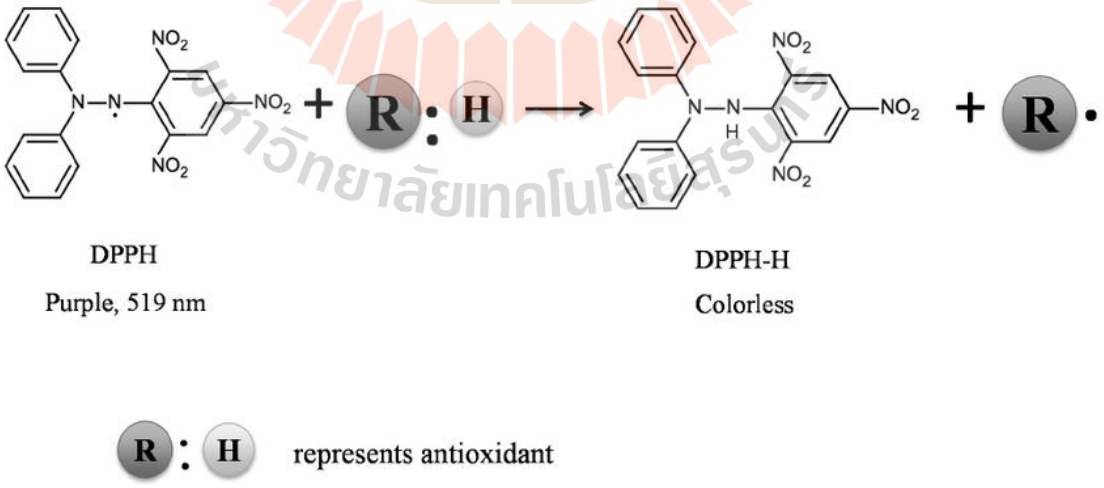

Figure 3.2 Chemical reaction of DPPH scavenging activity.

3.3.2.3 Determination of ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) of a compound is a significant indicator of its potential antioxidant activity because of its ability to break the free radical chain through donation of a hydrogen atom. The reducing capacity of the different samples was evaluated which is based on the reduction of the ferric (Fe^{3+}) - cyanide complex to the ferrous (Fe^{2+}) form by donating an electron using the method described by Oyaizu (1986) with minor modification. Various concentrations of α-mangostin, donepezil, or standard (ascorbic acid, AA) solutions (1 mL) were mixed with 2.5 mL potassium phosphate buffer (0.1 $mol \cdot L^{-1}$, pH 7.4) and 2.5 mL potassium ferric cyanide [$K_3Fe(CN)_6$, 1%]. After heating for 20 min at 50°C, 2.5 mL trichloroacetic acid (TCA, 10%) solution was added. The total mixture was centrifuged at 3,300 g for 10 min. Next, 2.5 mL supernatant solution was withdrawn from the mixture and mixed with 2.5 mL distilled water and 0.5 mL ferric (Fe^{3+}) chloride [$FeCl_3$, 0.1% (v/v)] solution. The absorbance of the solution was measured at 593 nm (Figure 3.3).

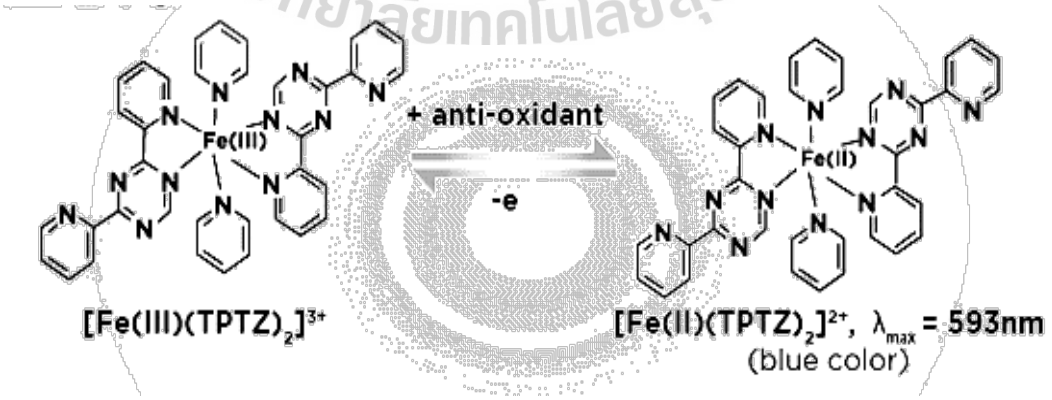

Figure 3.3 Chemical reaction of ferric reducing antioxidant power (FRAP).

3.3.3 Determination of acetylcholinesterase inhibitory activity *in vitro*

3.3.3.1 Ellman's assay

The acetylcholinesterase (AChE) inhibitory assay was measured according to the colorimetric method described by Ellman *et al*. (1961) and Ingkaninan *et al*. (2003) with minor modifications. Briefly, 25 µL of α-mangostin, donepezil, or standard (eserine) solution was mixed with 25 µL of 0.22 $U \cdot mL^{-1}$ AChE enzyme solution and incubated at 37°C for 15 min. Absorbance was read immediately after adding Ellman's reaction mixture [25 µL of 1.5 $mmol \cdot L^{-1}$ acetylthiocholine iodide (ATCI), 125 µL of 3 $mmol \cdot L^{-1}$ 5,5′-dithio bis-2-nitrobenzoic acid (DTNB), and 25 µL of 0.1 $mol \cdot L^{-1}$ phosphate buffer (pH 7.4)] to the above reaction mixture. The enzyme hydrolyzes the substrate ATCI to thiocholine and acetic acid. Thiocholine is allowed to react with DTNB, and this reaction resulted in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity (Figure 3.4).

Reading was repeated for 10 min at 2 min intervals for calculating the velocities of the reactions. Donepezil in 0.1 $mol \cdot L^{-1}$ phosphate buffer (pH 7.4), was used as the standard control in this study under the same experimental condition. The percentage inhibition of AChE activity was calculated using Equation 3.1 and expressed as IC_{50} values.

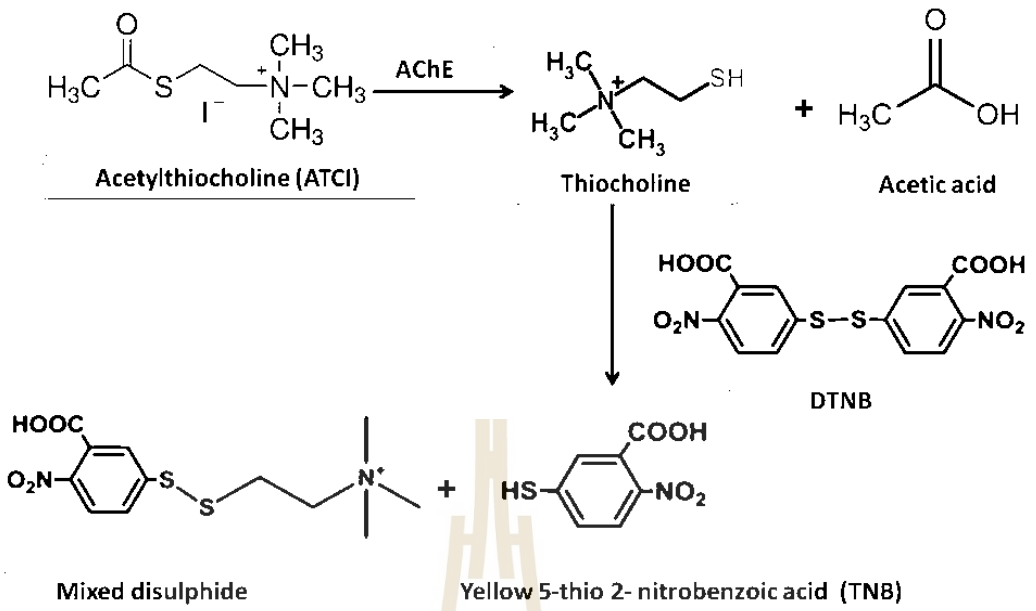

Figure 3.4 Chemical mechanism of Ellman's method (Ali-Shtayeh *et al*., 2014).

3.3.3.2 Beta-naphthyl acetate- fast blue B assay

The AChE inhibitory activity was performed using β-naphthyl acetate- fast blue B (NA-FB) assay described by Ali-Shtayeh *et al*. (2014) with minor modifications. Briefly, 10 µL of α-mangostin, donepezil, or standard (eserine) at different concentration was mixed with 200 µL of 3.33 U·mL^{-1} AChE enzyme solution and incubated at 37°C for 15 min. The mixture mixed with 50 µL of 0.25 mg·mL^{-1} β-naphthyl acetate dissolved in methanol and incubated at 4°C for 40 min. The enzyme hydrolyzes the substrate β-naphthyl acetate to naphthol and acetate. Naphthol is allowed to react with fast blue B. This reaction resulted in the development of a stable purple color (Figure 3.5). The color intensity of the product at 600 nm was read immediately after adding 10 µL of 2.5 mg·mL^{-1} fast blue B dissolved in water to the above reaction mixture. Donepezil in 0.1 mol·L^{-1} phosphate buffer (pH 7.4), was used as the standard control in this study under the same experimental condition. The percentage of inhibition of AChE activity was calculated using Equation 3.1.

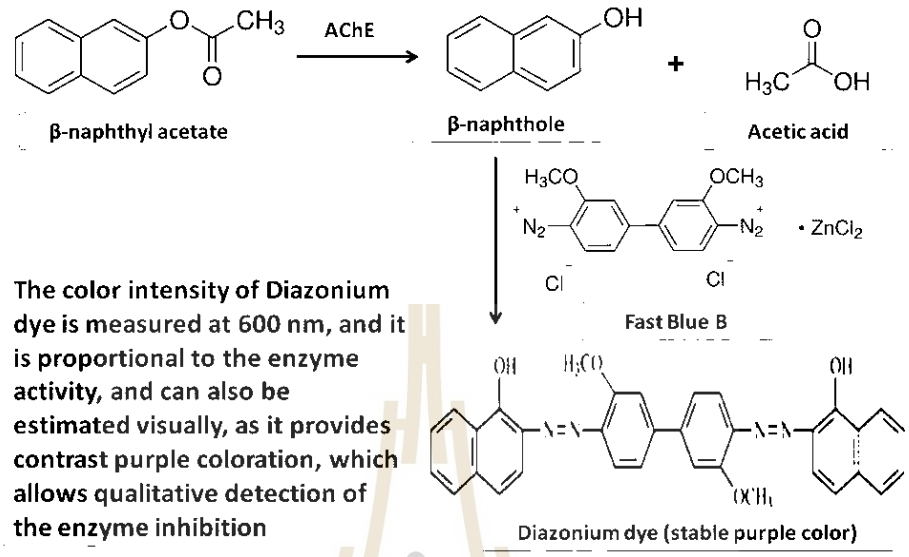

Figure 3.5 AChE activity Assay using β-naphtyl acetate and fast blue B salt (NA-FB) (Ali-Shtayeh *et al*., 2014).

3.4 Statistical analysis

All determinations were carried out in triplicate and the experimental data were presented as mean ± standard deviation (SD). The significant differences between treatment levels were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test (SigmaStat version 3.5). *P* value less than 0.05 ($P < 0.05$) was recognized statistically significant. All graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

3.5 Results

The present study demonstrated that both α-mangostin (α-MG) and donepezil (DPZ) had antioxidant and radical scavenging activities. Effect of α-mangostin on

$ABTS^{\bullet+}$ radical scavenging activity of α-mangostin and donepezil *in vitro* were shown in Figure 3.6. The percentage of scavenging ABTS radicals indicated that α-mangostin, donepezil, and quercetin (QCT) have potential scavenging activity with an IC_{50} value of 21.52 ± 3.45, 14.53 ± 1.86, and 26.15 ± 4.56 $\mu g \cdot mL^{-1}$, respectively. At the same concentration, ABTS radical scavenging activity of α-mangostin, donepezil, and reference standard was in the following order: QCT < α-MG < DPZ. The result of DPPH radical scavenging assays of α-mangostin is given in Figure 3.7. The percentage of scavenging DPPH radicals indicated that α-mangostin, donepezil, and BHT have potential scavenging activity with an IC_{50} value of 38.12 ± 8.36, 29.44 ± 5.13, and 43.16 ± 7.45 $\mu g \cdot mL^{-1}$, respectively. At the same concentration, DPPH radical scavenging activity of α-mangostin, donepezil, and reference standard was in the following order: BHT < α-MG < DPZ. In this study, all the concentrations showed considerable ferric reducing antioxidant power; 100 - 600 $mg \cdot mL^{-1}$ α-mangostin gave an FRAP value within the 20 - 410 μmol $Fe^{2+} \cdot L^{-1}$ (Figure 3.8). The α-mangostin showed a significant increase of the FRAP values in a concentration-dependent manner. At the same concentration, the reducing power of α-mangostin, donepezil, and reference standard was in the following order: AA < α-MG < DPZ. The results demonstrated that the radical scavenging activity of α-mangostin is stronger than donepezil when compared with their reference standards.

In the present study, the acetylcholinesterase inhibitory activity of α-mangostin and donepezil were measured by Ellman's and NA-FB method by using β-naphthyl acetate (NA) and acetylthiocholine iodide (ATChI) as substrate, respectively, and eserine was used as a positive control since serine has a strong acetylcholinesterase inhibitory activity. By Ellman's method, the concentrations of α-mangostin and

donepezil, and eserine required to inhibit 50% of acetylcholine esterase activity (IC_{50}) were 64.23 ± 0.22 and 32.46 ± 0.14, 100.67 ± 1.72 $\mu g \cdot mL^{-1}$, respectively. At the same concentration, the acetylcholinesterase inhibitory activity of donepezil exhibited stronger than α-mangostin with the same pattern of inhibitory action as eserine (Figure 3.9). By NA-FB method, there was no significant difference in the acetylcholinesterase inhibitory activity of α-mangostin and donepezil when compared to eserine at the same concentration (50 $\mu g \cdot mL^{-1}$) as shown in Figure 3.10.

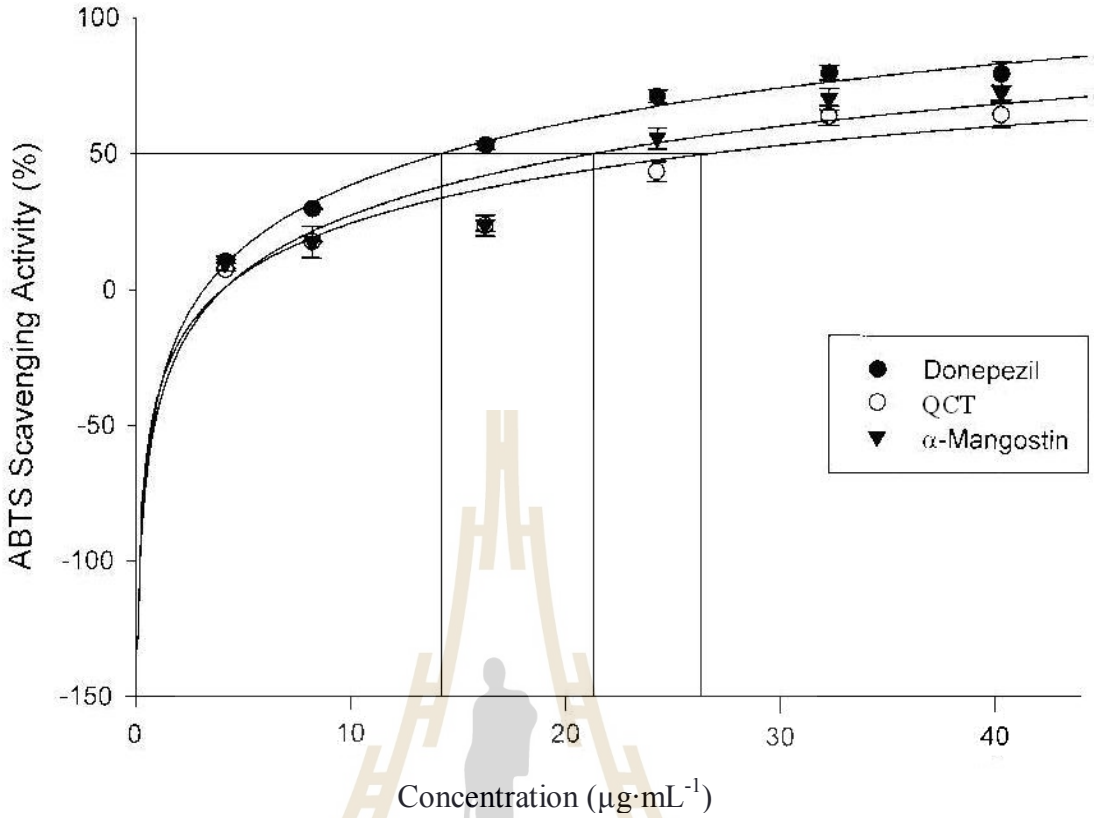

Figure 3.6 Effects of α-MG purified from the fruit rind of *G. mangostana*, DPZ, and the reference standard QCT on $ABTS^{\bullet+}$ radical scavenging activity *in vitro*. The results were expressed as mean ± S.D. of three independent experiments (n = 3). ▼, ●, and ○ represent fit curves of α-MG, DPZ, and QCT, respectively. DPZ, donepezil; α-MG, α-mangostin; QCT, quercetin; ABTS, 2,2'-azino-bis-3-ethylbenzthia zoline-6-sulphonate.

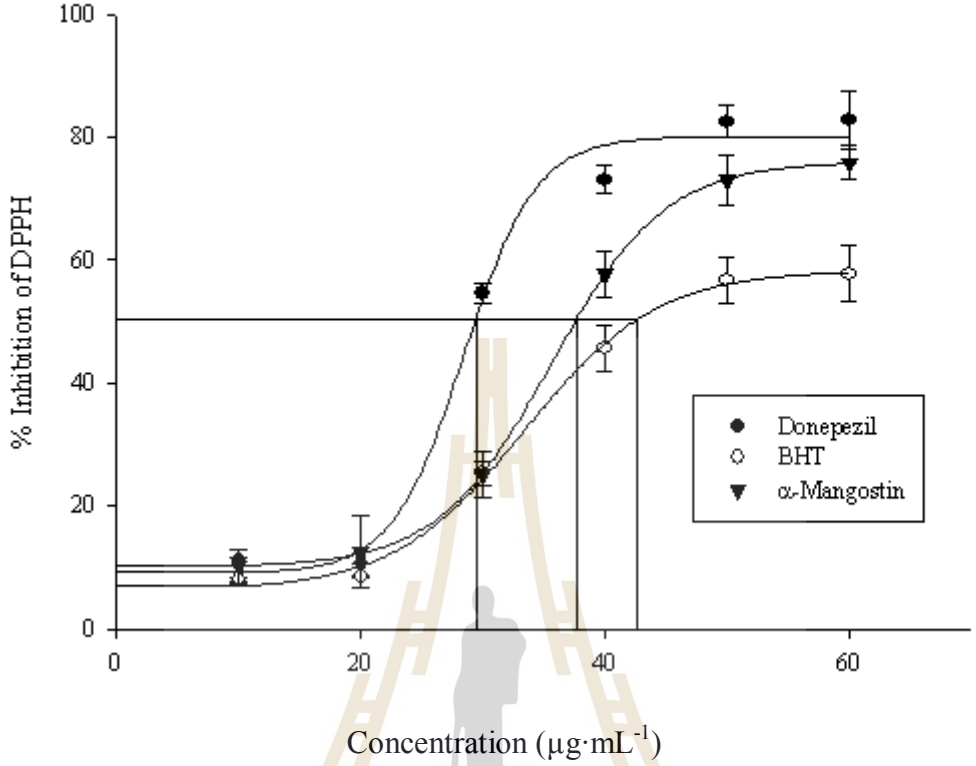

Figure 3.7 Effects of α-MG purified from the fruit rind of *G. mangostana*, DPZ, and the reference standard BHT on DPPH radical scavenging activity *in vitro*. The results were expressed as mean ± SD of three independent experiments (n = 3). ▼, ●, and ○ represent fit curves of α-MG, DPZ, and BHT, respectively. DPZ, donepezil; α-MG, α-mangostin; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

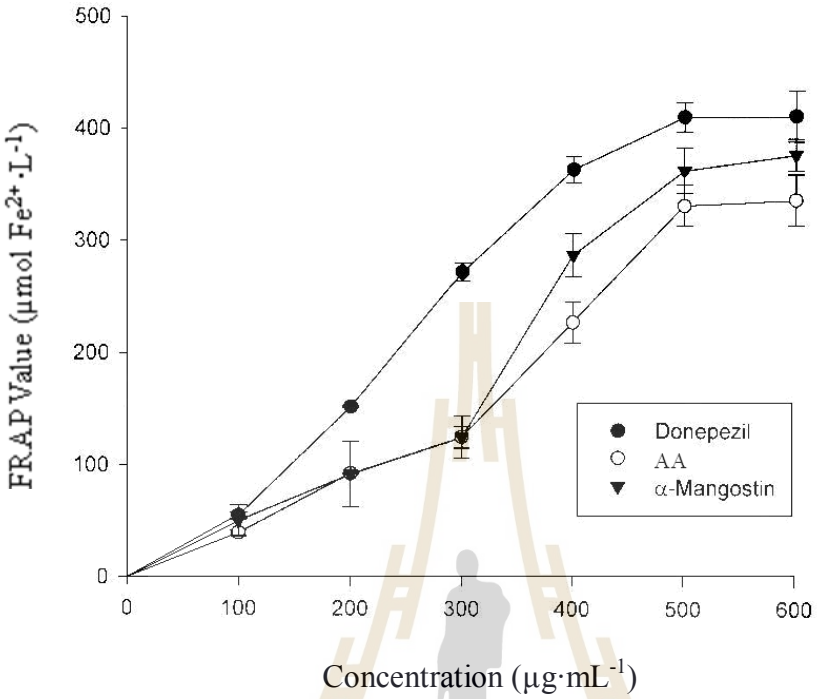

Figure 3.8 Effects of α-MG purified from the fruit rind of *G. mangostana*, DPZ, and the reference standard AA on FRAP *in vitro*. The results were expressed as mean ± SD of three independent experiments (n = 3). ▼, ●, and ○ represent curves of α-MG, DPZ, and AA, respectively. AA, ascorbic acid; FRAP, ferric reducing antioxidant power; DPZ, donepezil; α-MG, α-mangostin.

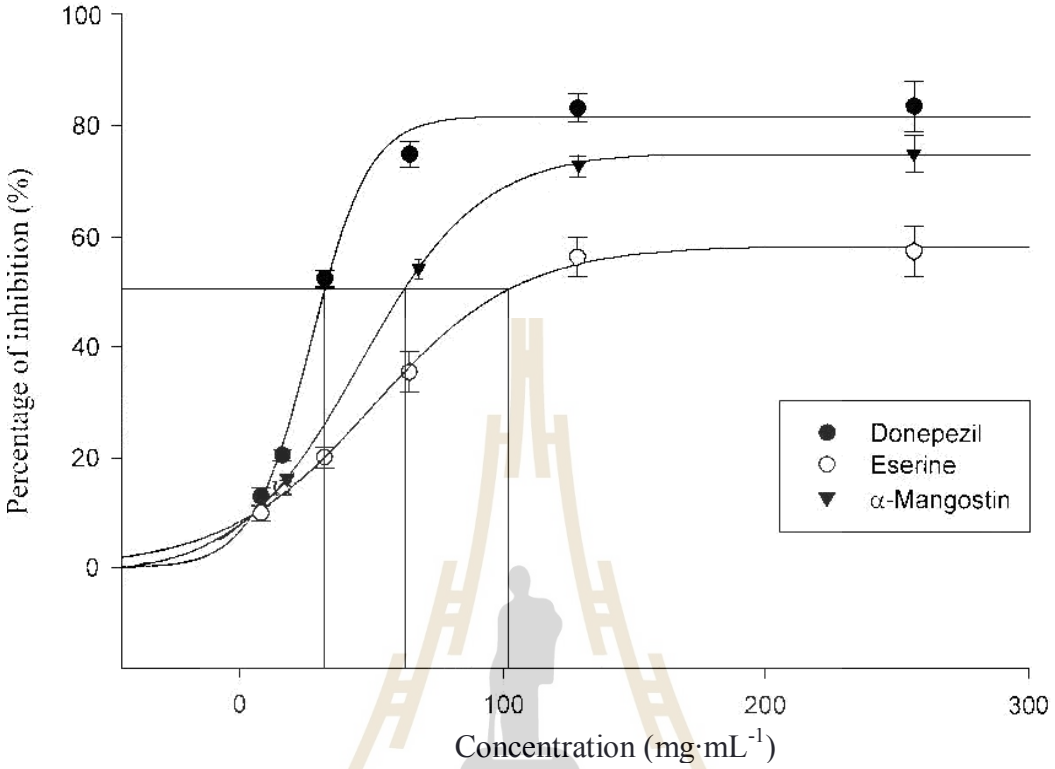

Figure 3.9 Effects of α-MG purified from the fruit rind of *G. mangostana*, DPZ, and reference standard eserine on acetylcholinesterase inhibitory activity *in vitro*. The acetylcholinesterase inhibitory activity was measured by the Ellman's method. The results were expressed as mean ± SD of three independent experiments (n = 3). ▼, ●, and ○ represent curves of α-MG, DPZ, and eserine, respectively. DPZ, donepezil; α-MG, α-mangostin.

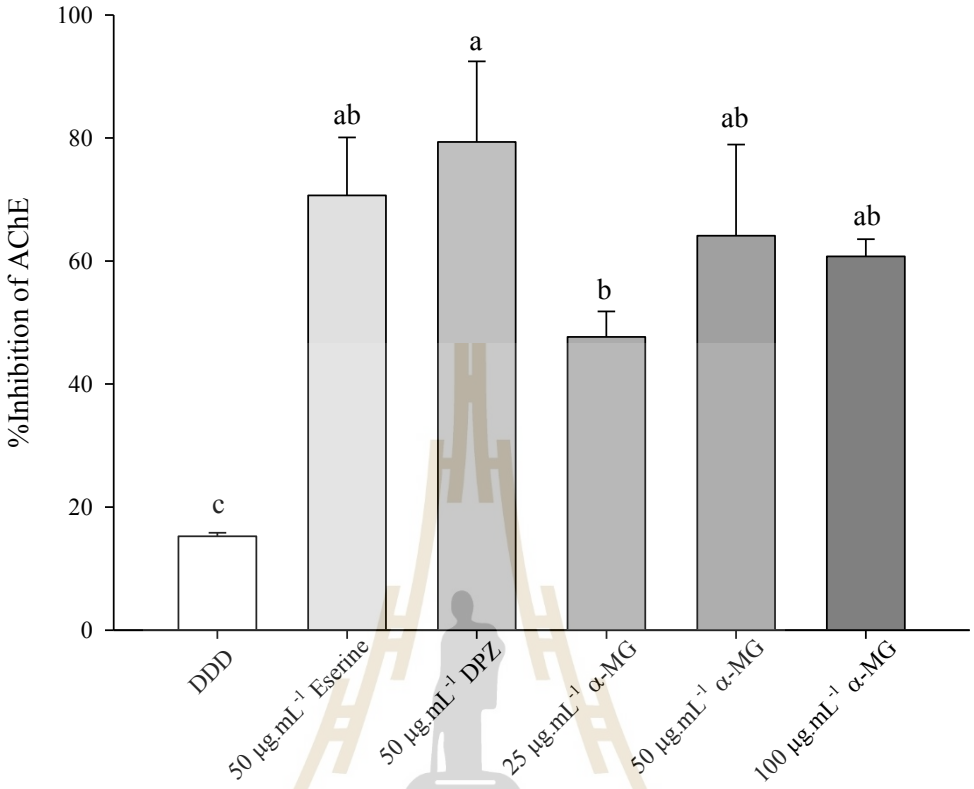

Figure 3.10 Effects of the α-MG purified from the fruit rind of *G. mangostana*, DPZ, and reference standard eserine on acetylcholinesterase inhibitory activity *in vitro*. The acetylcholinesterase inhibitory activity was measured by the NA-FB method. The results were expressed as mean ± SD of three independent experiments (n = 3). Different alphabets indicate significantly different (P<0.05). DDD, double distilled deionized water; DPZ, donepezil; α-MG, α-mangostin.

3.6 Discussion and conclusion

AD is a severe neurodegenerative disease and a leading cause of death among the elderly. Although a number of factors have been identified, oxidative stress and cholinergic dysfunction have been implicated as major contributing factors in the pathogenesis of AD. Therefore, it has been suggested that the compounds, either plant or plant-derived molecules, that modulate cholinesterase activity and multiple components of the oxidative stress pathway would be an effective candidates for potential drugs that restrict the development of AD (Hussain *et al*., 2018). *G. mangostana* has been reported to possess antioxidant, anti-tumor, anti-allergic, anti-inflammatory, anti-bacterial, anti-fungal, and anti-viral activities. The antioxidant potential evaluation of α-mangostin by several *in vitro* assays, including FRAP, DPPH and ABTS radical scavenging assay were performed. The α-mangostin showed radical antioxidant activities of all assays in a concentration-dependent manner (Figure 3.6, 3.7, and 3.8). Extensive evidence indicates that the ROS, which are formed during oxidative stress, induce cellular and molecular abnormalities in sporadic AD. Radical cations are the ROS and are highly toxic; they greatly contribute to oxidative stress and are known contributors to neuronal damage in AD. The antioxidant activity of the polyphenolic compound is believed to result from their redox properties, which play an important role in adsorbing and neutralizing free radicals. The mechanism of action of polyphenols is through the scavenging of free radicals. Previous findings indicate that the crude extract of *G. mangostana* also possesses AChE inhibitory properties and antioxidant activities (Nontamart *et al*., 2013). Although, it is now well accepted that inhibition of AChE activity can ameliorate the symptoms and improve the functioning of patients with AD, no studies have yet examined α-mangostin. Our results of this *in*

vitro study revealed that α-mangostin inhibited AChE in a dose-dependent manner (Figure 3.9). However, our results demonstrated the moderate AChE inhibitory activity of α-mangostin.

Numerous polyphenolic compounds have been isolated from plants that reduce oxidative stress and inhibit AChE. The free radicals generated in turn induce the formation of advanced glycation end products, nitration, lipid peroxidation adduction products, the carbonyl-modified neurofilament protein and free carbonyls, and also the activity of ChEs will be high, resulting in scarcity in the levels of ACh, ultimately resulting in neurodegeneration. The mechanisms of action of polyphenols in plant-derived natural compounds is through the antioxidant and acetylcholinesterase inhibitory activities *via* scavenging ROS and enhance the cholinergic neurotransmission by prolonging the time in which ACh molecules remain in the synaptic cleft. Donepezil is a commercial drug which used to improve cognition and behavior of people with Alzheimer's *via* inhibiting acetylcholinesterase enzyme. Our results indicated that α-mangostin is one of plant-derived natural compounds, which might contribute to antioxidant and acetylcholinesterase inhibitory activities by scavenging free radicals and inhibiting acetylcholinesterase, respectively (Figure 3.11).

The antioxidant properties and acetylcholine esterase inhibitory activities of α-mangostin purified from the fruit rind of *G. mangostana* were supported by the presence of a phenolic core structure (Figure 2.3). Polyphenols are the most abundant antioxidants in the plant kingdom, and it is claimed they have neuroprotective effect. The antioxidant activity of the polyphenolic compound is believed to result from their redox properties, which play an important role in adsorbing and neutralizing free radicals. Polyphenols are the most ubiquitous groups of plant secondary metabolites and have good

antioxidant potential. The mechanism of action of polyphenols in plant-derived natural compounds is through the scavenging activities of free radicals. Numerous polyphenolic compounds have been isolated from plants that exhibited the antioxidant properties and acetylcholine esterase inhibitory activities.

In conclusion, the results suggested that α-mangostin, a major xanthone derivative in *G. mangostana* rind, effectively inhibited AChE activity *in vitro* and multiple components of the oxidative stress pathway that contribute to Alzheimer's pathology. As a result, α-mangostin might have the potential to be an effective and safe treatment for AD.

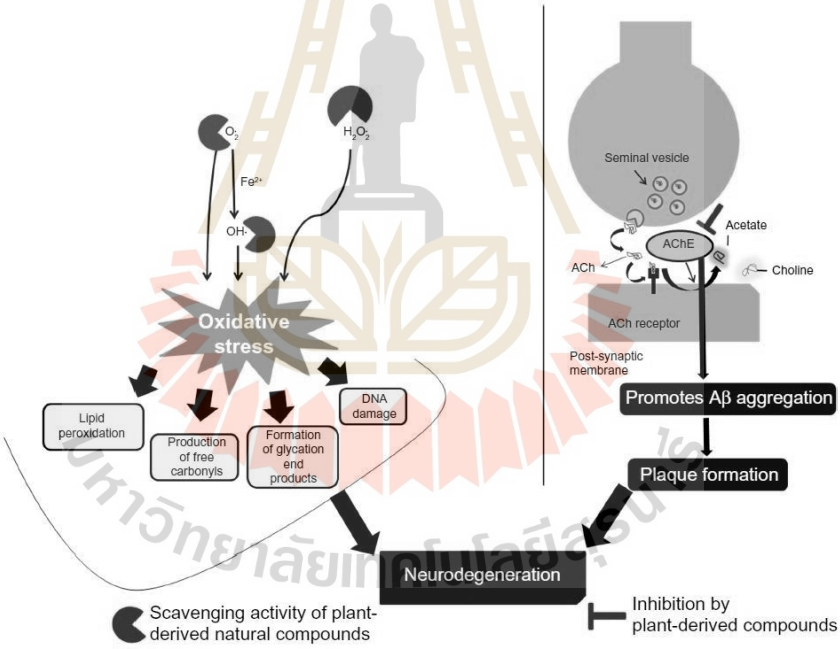

Figure 3.11 Role of oxidative stress and acetylcholinesterase in neurodegeneration (Syad and Devi, 2014).

CHAPTER IV

EFFECTS OF α-MANGOSTIN ON LEARNING AND MEMORY PERFORMANCE IN SCOPOLAMINE-INDUCED AMNESIC RATS

4.1 Abstract

Cholinergic neurons, particularly in the basal forebrain, are markedly depleted in Alzheimer's disease (AD) which is associated with cognitive deficits. Muscarinic antagonist scopolamine (SCOP)-induced cognitive deficits is commonly used as a model for AD. The crude extract from the fruit rind of *Garcinia mangostana* (Linn.) was recently reported to improve spatial memory in SCOP-induced amnesic rats. α-Mangostin (α-MG) is a prenylated xanthone derivative from the fruit rind of *G. mangostana*. The effects of α-MG on learning and memory performance were thus investigated in SCOP-induced amnesic rats. Eight groups (n = 8 each) of 8-weeks-old male Wistar rats were i.p. injected with normal saline solution (1 $mL \cdot kg^{-1}$), donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$; positive control), α-MG (50 or 100 $mg \cdot mL^{-1} \cdot kg^{-1}$) followed by i.p. injected with SCOP (2 $mg \cdot mL^{-1} \cdot kg^{-1}$) or normal saline solution (1 $mL \cdot kg^{-1}$) an hour later. Thirty minutes later, the learning and memory performance were assessed using Morris water maze test. All rats received four trials per day. These procedures were repeated for 7 days. On day 7, all rats were tested in the probe trial session.

We found that donepezil and α-MG (50 and 100 $mg \cdot kg^{-1}$) given to rats before SCOP administration could ameliorate adverse effects of SCOP by significantly decreasing time to find platform on training session, increasing time spent, and number of entries into the target quadrant in probe trial session on training day 7 of Morris water maze test when compared to their vehicle control group. These findings indicated that donepezil and α-MG could improve spatial memory impairment. Pretreatment with donepezil and α-MG provided the memory enhancing effects against SCOP-induced memory deficits and cognitive impairment.

4.2 Introduction

The frequent causes of dementia include AD. Cognition deficits produced by cholinergic antagonism mimic the cognitive symptomology of AD. Scopolamine, a muscarinic receptor antagonist, is reported to impair long term potentiation (LTP), and frequently used as amnesic agent for evaluation of antiamnesic effect of new drugs. Considering the adverse effects of synthetic drugs, there is search for natural remedies which are safe and effective. The World Health Organization (WHO) estimates that 80% of the world's population presently uses herbal medicine for some aspects of primary health care. Therefore, natural products may provide a new source of beneficial neuropsychotropic drugs provided they are adequately tested and their mechanisms are properly deciphered. Galantamine, a cholinesterase inhibitor and isolated from *Galanthus nivalis* and *Lycoris radiate*, is useful for treatment of mild to moderate AD. *Centella asiatica* is also reported to improve memory and promote the neuronal dendritic growth in hippocampus (Mohandas *et al*., 2009).

Garcinia mangostana extract has been reported to improve the memory in adult and aged male Wistar rats. It improves the performance of rats in Morris water maze. Moreover, *Garcinia mangostana* extract also provides protection from scopolamine-induced cognition deficit (Nontamart *et al*., 2013). α-Mangostin is the prominent constituent of the crude *Garcinia mangostana* extract. We thus examined the antiamnesic effects of α-mangostin isolated from the fruit rind of *Garcinia mangostana* on learning and memory performance in scopolamine-induced amnesic rats and found that the α-mangostin isolated from the fruit rind of *Garcinia mangostana* is useful for the treatment of AD.

4.3 Materials and methods

4.3.1 Chemicals

α-Mangostin from the fruit rind of *Garcinia mangostana* (Linn.) in form of yellow crystals at purity 99.35% (HPLC) was purchased from Indofine (Hills-borough, NJ, USA, Cat. No.: M-002, CAS Number: [6147-11-11], Lot. No.: 97072) since November, 2013. Donepezil hydrochloride at purity ≥ 98% (HPLC) and scopolamine hydrochloride at purity 90% (HPLC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2 Drug solutions

2% Tween 80 (200 mL):

Preparation: mixed 4 mL of Tween 80 (BDH Ltd., UK) with DDD water and then adjusted the final volume to 200 mL.

4 mg·mL^{-1} Donepezil (2 mL): Stock solution

Preparation: 8 mg donepezil hydrochloride (Sigma-Aldrich; St. Louis, MO, USA) was dissolved in 2% Tween 80 and then adjusted the final volume to 2 mL.

2 mg·mL^{-1} Donepezil (2 mL):

Preparation: mixed 1 mL of 4 mg·mL^{-1} donepezil with 2% Tween 80 and then adjusted the final volume to 2 mL.

2 mg·mL^{-1} scopolamine (8 mL): Stock solution

Preparation: 50 mg·mL^{-1} scopolamine (1 g of scopolamine) was dissolved in 20 mL 2% Tween 80.

Preparation: mixed 0.32 mL of 50 mg·mL^{-1} scopolamine with normal saline solution (0.9% NaCl, Otsuka, Ltd, Thailand) and then adjusted the final volume to 8 mL.

500 mg·mL^{-1} α-Mangostin (500α-MG) (30 mL): Stock solution

Preparation: 2,000 mg of α-MG was dissolved in 4 mL of 2% Tween 80.

100 mg·mL^{-1} α-Mangostin (100α-MG) (20 mL):

Preparation: mixed 4 mL of α-MG500 with 18 mL 2% Tween 80.

50 mg·mL^{-1} α-Mangostin (50α-MG) (20 mL):

Preparation: mixed 10 mL of α-MG100 with 10 mL of 2% Tween 80.

Ice cold normal saline solution (0.9% NaCl) (4000 mL):

Preparation: dissolved 32.4 g of sodium chloride in deionized distilled (DD) water and then adjusted the final volume to 4000 mL.

Note: - α-MG is the α-mangostin purified from the fruit rind of *G. mangostana*.

- Donepezil is function as reversible acetylcholinesterase inhibitor. Its main therapeutic use is in the palliative treatment of Alzheimer's disease (AD) (Bryson and Benfield, 1997). Donepezil was dissolved in normal saline solution (0.85% NaCl) and used at the dose of 2 $mg \cdot kg^{-1}$ (1 $mL \cdot kg^{-1}$, i.p.) as positive control (Jayarajan *et al*., 2013).

- Scopolamine dissolved in normal saline solution (0.85% NaCl) and used at the concentration of 2 $mg \cdot kg^{-1}$ (1 $mL \cdot kg^{-1}$, i.p.) to induce dementia in rats (Umukoro *et al*., 2013). Scopolamine is function as a muscarinic receptor (M_1) antagonist that can induce amnesia (Agrawal *et al*., 2008; Eidi *et al*., 2006; Oh *et al*., 2009).

4.3.3 Animals

Experiments were carried out on adult male Wistar rats (8 weeks old) weighing 250 ± 50 grams which were housed two per cage and maintained under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1°C) with free access to food and water. Animals were obtained from the Institutional Animal Care for laboratory animal research of the Center for Scientific and Technological Equipment (CSTE), Suranaree University of Technology, were used throughout this work. Animals were allowed to acclimate to their new environment for at least 3 days after arrival before initiation of the experiment. All the protocols were conducted under permit of Suranaree University of Technology's the Institutional Animal Care and Use Committee (IACUC) in ensuring the ethical and sensitive care and use of animals in research.

4.3.4 Experimental design

Eight weeks old male Wistar rats were randomly assigned to eight groups of eight animals each (n = 8). For consecutive 7 days, all treatments were daily given *via* the intraperitoneal route (1 $mL \cdot kg^{-1}$, i.p.) as follows:

Group 1; served as vehicle-control group. Rats were received 1% (v/v) Tween 80 followed by NSS (0.9% NaCl, 1 $mL^{-1} \cdot kg^{-1}$) an hour later.

Group 2; served as positive-control group. Rats were received standard donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$) followed by NSS (0.9% NaCl, 1 $mL^{-1} \cdot kg^{-1}$) an hour later.

Group 3; served as low dose α-mangostin group. Rats were received α-mangostin (50 $mg \cdot mL^{-1} \cdot kg^{-1}$) followed by NSS (0.9% NaCl, 1 $mL^{-1} \cdot kg^{-1}$) an hour later.

Group 4; served as high dose α-mangostin group. Rats were received α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$) followed by NSS (0.9% NaCl, 1 $mL^{-1} \cdot kg^{-1}$) an hour later.

Group 5; served as vehicle-experimental group. Rats were received 1% (v/v) Tween 80 (1 $mL \cdot kg^{-1}$) followed by scopolamine (2 $mg \cdot mL^{-1} \cdot kg^{-1}$) an hour later.

Group 6; served as positive-experimental group. Rats were received standard donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$) followed by scopolamine (2 $mg \cdot mL^{-1} \cdot kg^{-1}$) an hour later.

Group 7; served as low dose experimental group. Rats were received α-mangostin (50 $mg \cdot mL^{-1} \cdot kg^{-1}$) followed by scopolamine (2 $mg \cdot mL^{-1} \cdot kg^{-1}$) an hour later.

Group 8; served as high dose experimental group. Rats were received α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$) followed by scopolamine (2 $mg \cdot mL^{-1} \cdot kg^{-1}$) an hour later.

Thirty minutes later, all rats were tested for their spatial memory using the Morris Water Maze test. The protocol consisted of 21 training trials (3 times per day for 7 days) and probe trial on the day 7 (Figure 4.1). Body weight was recorded every day.

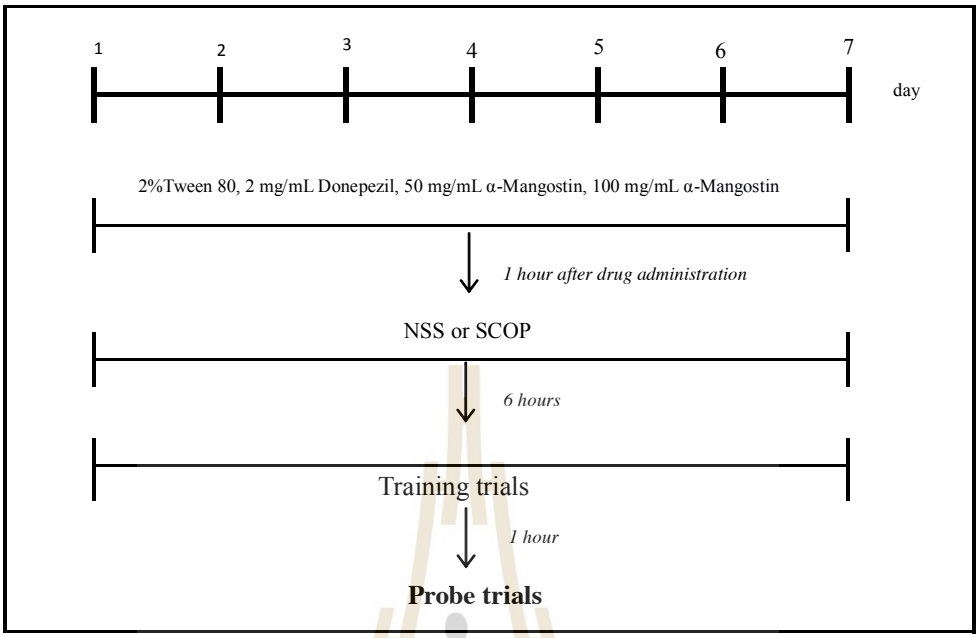

Figure 4.1 Schematic diagram showing protocol for Morris water maze test.

4.3.5 Morris Water Maze Test

The Morris water maze test developed by Richard Morris at the University of St Andrews in Scotland (Morris, 1981) is widely used to study spatial memory and learning in rodent. Briefly, animals were placed into open pool of water which contains an escape platform hidden below the water that is colored opaque with milk powder or non-toxic paint. Visual cues, such as colored shapes, are placed around the pool in plain sight of the animal. The animals were forced to swim around the pool in search of an exit and on subsequent trials the rat is able to locate the platform more rapidly.

Apparatus: The apparatus for Morris water maze test used in the present study consisted of large circular pool (2.3 m in diameter and 63 cm in height) which contains no internal cues, stimuli, markings on objects, but is surrounded by stable,

salient extra-maze cues. The pool was filled to a depth of 21.5 cm with water at temperature of 25 °C and made opaque with white non-toxic water paint (TOA, Co., Ltd, Thailand). The pool was divided into four quadrants of equal areas [Q_1, Q_2, Q_3 and Q_4 (with platform)] and surrounded by 4 extra maze distal visual cues of different shape on the wall of each quadrant. A white platform (19.5 cm in diameter and 28.5 cm in height) was submerged 2 cm below the water surface and placed in the center of Q_4 and it was located in the same position on every trials. A video camera (Sony Handycam, Japan) was placed above the centre of the pool to capture images of the swimming animal.

Training Trial: On the day before training trials, the animal was placed in the pool and allowed to swim for 60 s with the absent of the escape platform. During the seven training days, the animal was given three trials sessions each day with an intertribal interval of 60 s. The trial began when the animal was randomly placed in the water at one of the locations (Q_1, Q_2, and Q_3) with its head facing the wall of the pool and allowed 60 s to swim, search and climb up to the platform. Once the animal found the platform, rat was allowed to remain on the platform for 60 s. The escape latency to the platform (time to find platform) was recorded. If the animal did not locate the platform within 60 s, the animal was guided to the platform by experimenter and let the animal sit on platform for 15 s. If animal jumped off, guided it back. The repeated three trial training processes for all animals were performed consecutively. Once the animal had completed all three trials, dried it off with a towel.

Probe trial: After the completion of training trial on day 7, the experimenter conducted a probe trial in which the escape platform was removed from the pool. The animals were released from the quadrant opposite to quadrant where the platform was

located and allowed to swim for 60 s, after which the rat was taken out of the pool. Generally, a well-trained rat swam to the target quadrant of the pool and repeatedly acrossed the former location of the platform until starting to search elsewhere. The time spent in target quadrant and the numbers of entries into the target quadrant were recorded (Morris *et al*., 1982; Morris, 1981; 1984; 2008).

4.4 Statistical analysis

All determinations were carried out in triplicate and the experimental data were presented as mean ± standard error of mean (S.E.M.). The significant differences between treatment levels were statistically analyzed by one-way analysis of variance (ANOVA) and two way repeated measures ANOVA for time to find platform (sec) in Morris water maze test on training day 1 to day 7 followed by Newman-Keuls post hoc test (SigmaStat version 3.5). *P* value less than 0.05 ($P<0.05$) was recognized statistically significant. All graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

4.5 Results

4.5.1 Effects of α-mangostin on learning and memory performances in the scopolamine-induced amnesic rats

The effects of the α-mangostin purified from the fruit rind of *G. mangostana* and vitamin E on memory were examined in normal saline treated rats and scopolamine-induced amnesic rats using Morris water maze test. Time to find platform in training trials, time spent in target quadrant and numbers of entries into the target quadrant in probe trial were recorded. Rats received daily oral dose of vehicle (10% Tween 80, 1 $mL \cdot kg^{-1}$), donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$), or the α-mangostin (α-MG) purified from the fruit rind of *G. mangostana* at doses of 50 and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ followed by either normal saline solution (NSS, 1 $mL \cdot kg^{-1}$) or scopolamine (SCOP, 2 $mg \cdot mL^{-1} \cdot kg^{-1}$) for 7 days. In both normal saline treated rats and scopolamine-induced amnesic rats, all doses of α-MG and donepezil administration significantly decreased time to find platform on training day 4, 5, 6 and 7 when compared to day 1, ($P<0.05$, two way repeated measures ANOVA) (Figure 4.2).

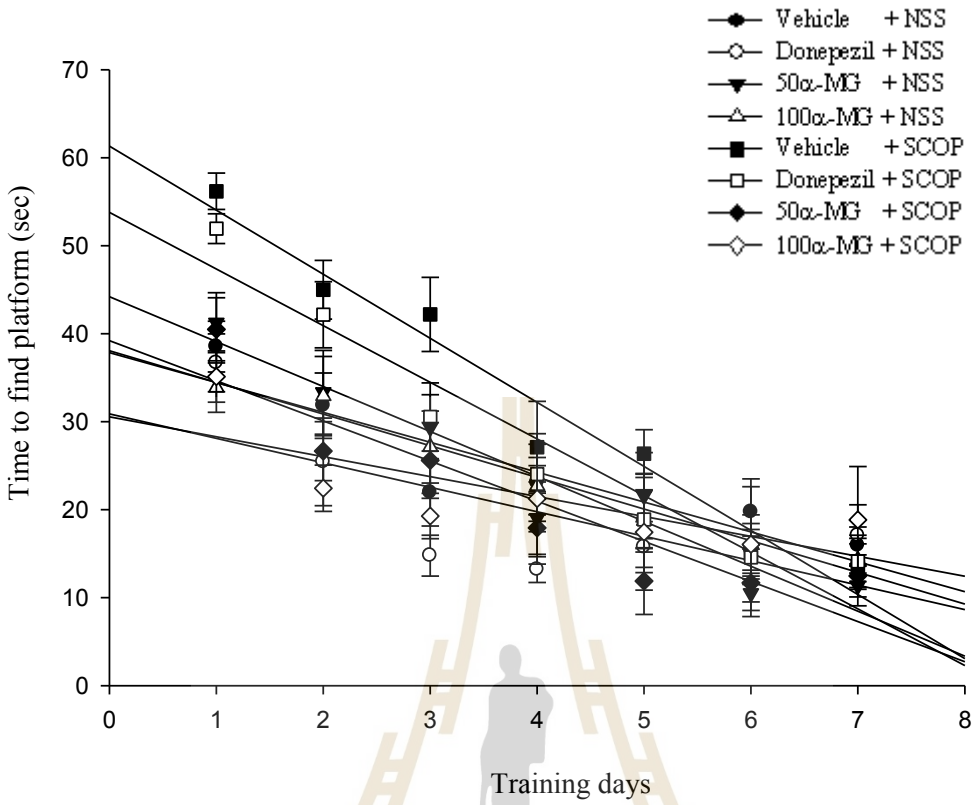

Figure 4.2 Effect of α-MG from the fruit rind of *G. mangostana* and DPZ on time to find the platform (sec) in Morris water maze test on day 1 to day 7 of training trials in NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

In scopolamine-induced amnesic rats, administration of vehicle + SCOP significantly increased time to find platform on day 7 when compared to vehicle + NSS (P<0.05, one way ANOVA, Figure 4.3). The results of scopolamine-induced amnesic rats showed that administration of 50α-MG + SCOP, 100α-MG + SCOP and donepezil + SCOP, significantly decreased time to find the platform on day 7 when compared to vehicle + SCOP (P<0.05, one way ANOVA, Figure 4.3). In normal saline-treated rats, 50α-MG and donepezil did not cause any change in time spent in the target quadrant and number of entries into the target quadrant, respectively (Figure 4.4 and 4.5). In scopolamine-induced amnesic rats, administration of vehicle + SCOP significantly decreased time spent in the target quadrant when compared to vehicle + NSS (P<0.05, one way ANOVA, Figure 4.4). In normal saline treated rats, 50α-MG + NSS significantly increased time spent in target quadrant when compared to vehicle + NSS (P<0.05, one way ANOVA, Figure 4.4). The results of scopolamine-induced amnesic rats showed that administration of 50α-MG + SCOP, 100α-MG + SCOP and donepezil + SCOP, significantly increased time spent in target quadrant when compared to vehicle + SCOP (P<0.05, one way ANOVA, Figure 4.4).

In scopolamine-induced amnesic rats, administration of vehicle + SCOP significantly decreased number of entries into target quadrant when compared to vehicle + NSS (P<0.05, one way ANOVA, Figure 4.5).

The results of scopolamine-induced amnesic rats showed that administration of 50α-MG + SCOP, 100α-MG + SCOP and donepezil + SCOP, significantly increased number of entries into target quadrant when compared to vehicle + SCOP (P<0.05, one way ANOVA, Figure 4.5).

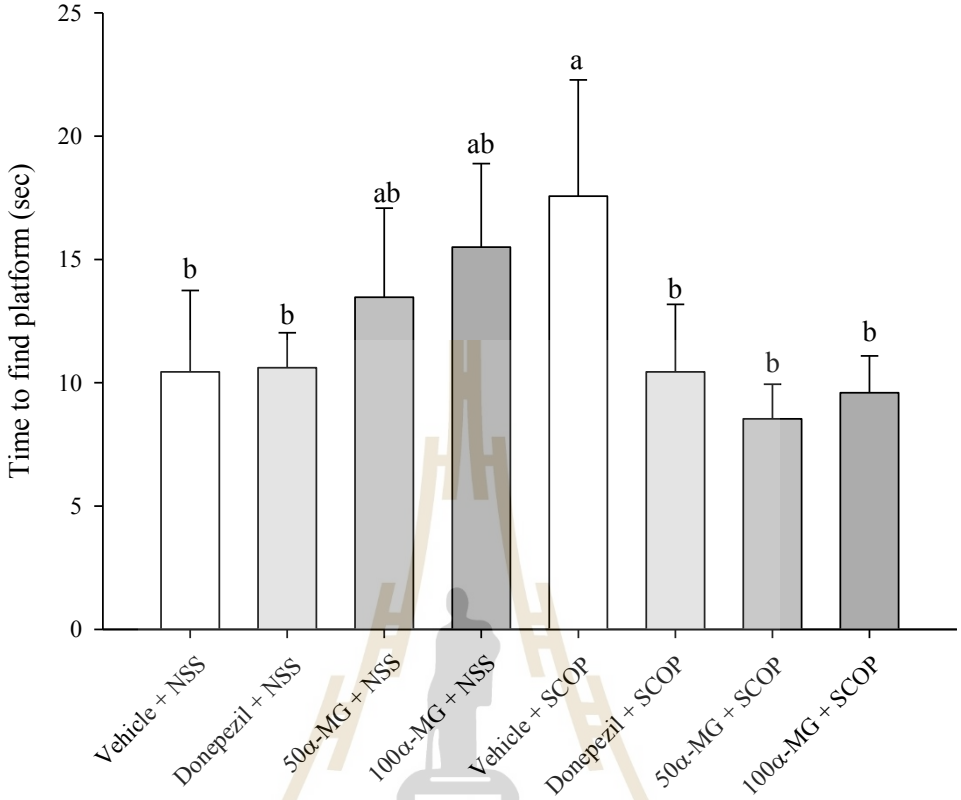

Figure 4.3 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on time to find the platform in Morris water maze test on day 7 of training trials in NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

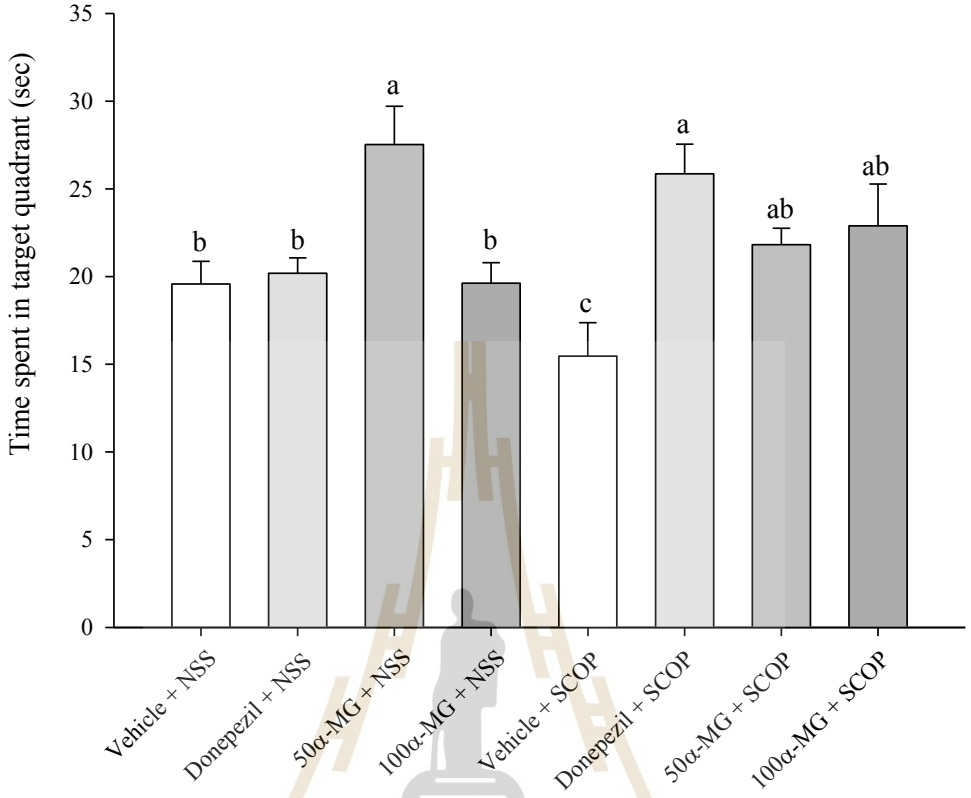

Figure 4.4 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on time spent in the target quadrant in Morris water maze test on probe trials of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

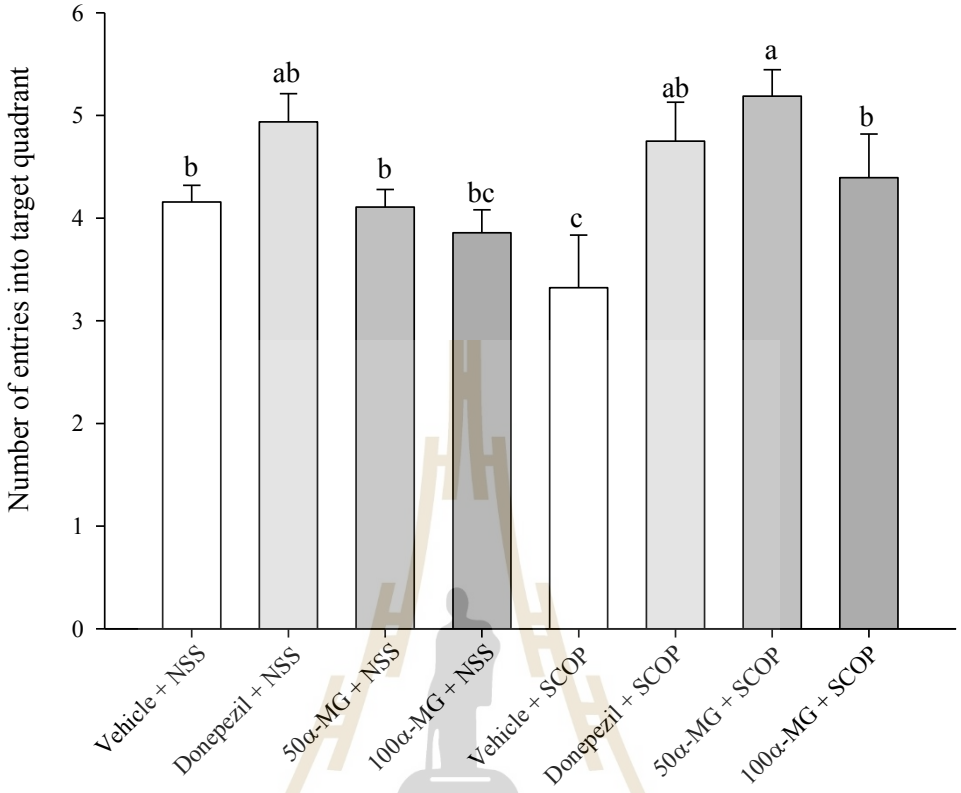

Figure 4.5 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on number of entries into the target quadrant in Morris water maze test on probe trials of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

3.6 Discussion and conclusion

Dementia is a symptom complex of continuous global weak of intellectual function. It becomes a major medical, economic, and social problem that is deterioration as the increasing number of elderly people. Currently, available drug therapies for Alzheimer's disease (AD) and other diseases that cause dementia consist primarily of acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine, Huperzine A), an N-methyl-D-aspartate receptor antagonist (memantine) and some neuroprotective agents (Olivares *et al*., 2012). These drugs cause side effects, thus an alternative bioactive compounds from plants and fruits with little or no side effects should be used to replace these drugs. Polyphenolic compounds may be one of candidates because of their memory enhancing properties. Polyphenols from many fruits and plants are known to possess cognitive enhancing effects and acetylcholinesterase inhibitory activity (Papandreou *et al*., 2009).

High amount of polyphenolic compound can be found in the crude extract from the fruit rind of *G. mangostana*. *G. mangostana* is a source of bioactive compounds with potential health-promoting activity. The crude extract from the fruit rind of *G. mangostana* possessed potent acetylcholinesterase inhibitory activity (Nontamart *et al*., 2013). Several studies revealed that the fruit rind of *G. mangostana* is the rich source of xanthone (α-mangostin, β-mangostin, γ-mangostin, and gartanin) (Asai *et al*., 1995; Chen *et al*., 2008; Chomnawang *et al*., 2007; Cui *et al*., 2010; Ji *et al*., 2007; Maisuthisakul *et al*., 2007; Yu *et al*., 2007; Zadernowski *et al*., 2009). Thus, the α-mangostin purified from the fruit rind of *G. mangostana* may possess biological activities related to polyphenolic compounds. The present study demonstrated for the first time that the α-mangostin purified from the fruit rind of *G. mangostana* could

enhance memory probably by inhibiting brain acetylcholine esterase activity in scopolamine-induced male Wistar rats.

The present study provided the first evidence that α-mangostin possessed memory enhancing effects in scopolamine-induced amnesic rats by decreasing time to find the platform and increasing time spent and number of entries into the target quadrant using Morris water maze test in similar manner to donepezil (Figure 4.3, 4.4, and 4.5).

Neuroprotective effects of the crude extract from the fruit rind of *G. mangostana* and vitamin E were demonstrated in the previous study. In both healthy adult and normal aging rats, subchronic administration of the crude extract from the fruit rind of *G. mangostana* could enhance spatial memory by increasing time spent in the target quadrant without changing in number of entries into the target quadrant using Morris water maze test (Nontamart *et al*., 2013). In the present study, intraperitoneal administration of the α-mangostin purified from the fruit rind of *G. mangostana* could enhance spatial memory by increasing both time spent in the target quadrant (Figure 4.4) and number of entries into the target quadrant (Figure 4.5). Cognitive dysfunction such as learning impairment and delayed amnesia are the most striking age-related changes observed in human being and animals (Foster *et al*., 1994; Gray *et al*., 2008).

The cholinergic system is responsible for the storage and retrieval of item in memory and it degradation correlates well with the severity of cognitive and memory impairment. Therefore, it has been suggested that elevation of the acetylcholine level might be helpful in to improve the symptoms of cognitive deficit (Gasparin *et al*., 1998). Many different theories have been offered regarding the cause of AD; a well-

established theory suggests that acetylcholine levels are too low in the brain of AD patients (Davies and Maloney 1976). Polyphenol-rich wild blueberry extracts exhibited a significant improvement in learning and memory tested by the passive avoidance behavioral test (Papandreou *et al*., 2009). Mukhergee *et al*. (2006) and Yang *et al*. (2008) reported that N. *nucifera* rhizome extract inhibited AChE activity and improved learning and memory by enhancing neurogenesis in the dentate gyrus of hippocampus.

The present study also demonstrated that α-mangostin and donepezil at any tested doses did not affect the animal behavior and health status and did not produce any plasma biochemical parameters abnormality of healthy adult male Wistar rats during the period of treatment for constitutive 7 days, suggesting that α-mangostin is medically safe for liver and kidneys of rats.

In conclusion, we demonstrated remarkable memory enhancing effects of α-mangostin in scopolamine-induced amnesic model (Changlek and Srisawat, 2015). Memory enhancing effects of α-mangostin may be a result of its antioxidant and cholinergic enzyme regulatory activity. Thus, pretreatment with α-mangostin may be an effective therapeutic option for the treatment of cognitive impairment. The α-mangostin, a plant-derived substance, is a phenolic compound which is beneficial for prevention of dementia.

CHAPTER V

EFFECTS OF α-MANGOSTIN ON PLASMA BIOCHEMICAL PARAMETERS AND BODY WEIGHT CHANGES IN SCOPOLAMINE-INDUCED AMNESIC RATS

5.1 Abstract

Mangosteen (*Garcinia mangostana* Linn.) is one of the most delicious and popular fruits in Thailand. Previous *in vitro* and *in vivo* studies have suggested that α-mangostin (α-MG), a prenylated xanthone derivative from the fruit rind of *G. mangostana*, is beneficial for health due to its biological activities such as antioxidant, antiviral and anticancer properties. The effects of α-MG have not been completely investigated. Therefore, the current study was performed to clarify the *in vivo* effects of the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil on plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) and body weight changes in scopolamine-induced amnesic rats. In this study, α-MG was purchased from Indofine (Hillsborough, NJ, USA) and dissolved in 2% Tween 80. Eight groups (n = 8, each) of 8-weeks-old male Wistar rats were daily injected with normal saline solution (1 $mL \cdot kg^{-1}$, i.p.), donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.; positive control), α-MG (50 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.), or α-MG (100 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) followed by daily injected with SCOP (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) or normal saline solution (1 $mL \cdot kg^{-1}$, i.p.) for constitutive 7 days. Body weight, food intake, and water intake of all rats was recorded daily.

At day 7, animals were terminated 6 hours after dosing. Blood samples, collected by cardiac puncture, were used for monitoring liver function and tissue damage by determination of the plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) measured by automated analyzer. In all instances, administration of all doses of α-MG showed no effect on behavior, food and water intake, growth or health status of these animals. After 7-day administration of all doses of α-MG, their body weight and LDH plasma level did not alter from those of the vehicle control. In NSS groups, plasma AST levels were decreased after administration of donepezil and the α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$). Scopolamine significantly increased plasma ALT level. In SCOP groups, plasma ALT level was decreased after administration of the α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$).

Furthermore, the plasma AST and ALT levels had no change after intraperitoneally administration of SCOP and NSS, respectively. The plasma LDH level had no change after intraperitoneally administration of both SCOP and NSS. In present study, no significant decrease in the plasma AST, and LDH levels following the α-mangostin and donepezil administration in SCOP-induced amnesic rats. The α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$) had anti-toxic effect which could suppress the increase plasma ALT level in scopolamine-induced amnesic rats. The α-mangostin purified from *G. mangostana* and donepezil had no toxicity on liver. Therefore, the α-mangostin purified from *G. mangostana* and donepezil are beneficial for anti-toxic effect. In conclusion, α-MG at the doses tested did not produce any significant undesirable effects in the experimental animals. Their livers which are the most sensitive organs to toxic factors were apparently normal and showed no signs of

dysfunction. Further studies are needed to evaluate pharmacologic mechanisms, long-term safety and chronic toxicity of α-MG on bodily functions.

5.2 Introduction

The liver is our body's most important organ after the heart, performing many important functions including metabolism, detoxification, and formation of important compounds including blood clotting factors. It also filters, regulates, and stores blood. Stress, poor diet, and over-medication are common problems in our "civilized" lifestyle. These may lead to stress and functional damage to the liver. As a result, "sluggish" liver has become a common ailment. It may affect memory, sleep, thyroid, body weight, and other body functions. Since the liver is the principal organ which removes the wastes, its failure to remove them efficiently often leads to their accumulation in the body and is a major cause of the above diseases. Liver health is related to brain health the brain cells are especially affected because the blood has to flow against gravity to reach them. In an article on Alzheimer disease (AD) in the January 1988 issue of the Mayo Clinic Health Letter, malfunction of the liver and kidneys was mentioned as one of the causes leading to AD. The brain is only 2% of our body weight, yet it needs 20% of our oxygen supply. If the toxin-loaded blood from a weak liver has limited capacity to carry oxygen, the brain cells are affected most.

The liver is the most important organ after the heart. It performs hundreds of functions including: Circulation: transfer of blood from portal to systemic circulation, activity of the liver's reticulo-endothelial system (kupffer cells) in the immune system. The liver stores and regulates the blood and is responsible for nourishing every cell in

our body. Every part of the body depends on blood from the liver for nourishment and sustenance.

Excretion: formation and secretion of bile for digestion and cleansing of blood; removal of ammonia from blood; excretion of substances filtered from the blood by the liver such as heavy metals or dyes.

Metabolism: carbohydrate, protein, lipid (fat), mineral and vitamin metabolism; manufacturing and storage of many nutrients such as glucose and vitamins; production of heat through metabolism.

Protection and detoxification: removal of foreign bodies from the blood (phagocytosis); detoxification by conjugation, methylation, oxidation and reduction. Production; formation of urea, serum albumin, glycogen and blood coagulating proteins such as prothrombin, fibrinogen and heparin; erythrocyte (red blood cells) destruction. Regulation of hormones: inactivation and elimination of hormones through the bile or urine. The liver also regulates body functions which affect emotional and mental activities. In a diseased condition, the liver's blood storage and regulatory functions are affected and bleeding or clots can result. When liver blood is deficient, nourishment to tendons and blood vessels is curtailed, the joints become stiff, and muscles become spasmodic and numb. Blood deficiency in the liver may even lead to stroke, dizziness, headaches, tinnitus, deafness, fainting or convulsion. If the liver is affected by stress or unhappy feelings, its vitality may be repressed and the sides hurt, and hiccups or hernia may develop. The bowels may become constipated and sleep may be disturbed causing nightmare or insomnia.

5.3 Materials and methods

The methodology of chemicals, drug solutions, animals, and experimental design is described in Materials and methods 4.3.1, 4.3.2, 4.3.3, and 4.3.4, respectively.

5.3.1 Blood collection

After last trials in Materials and methods 4.3.5, all rats were anesthetized with pentobarbital sodium (Nembutal, Ceva Santé Animale, France, 60 $mg \cdot kg^{-1}$, i.p.). Blood samples (3-4 mL from each rat) were collected *via* cardiac puncture into heparinized tubes. Heparinized blood was centrifuged at 2000 RCF (relative centrifugal force) for 5 minutes, obtained plasma was stored at -20 °C until further biochemical analysis.

5.3.2 Determination of plasma biochemical parameters

The plasma biochemical parameters [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH)] were determined by VITROS® 5600 Integrated System following the manufacturer's instruction (Ortho Clinical Diagnostics, New Jersey, United States).

5.4 Statistical analysis

All determinations were carried out in triplicate and the experimental data were presented as mean ± S.E.M. The significant differences between treatment levels were statistically analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test (SigmaStat version 3.5). *P* value less than 0.05 ($P<0.05$) was recognized statistically significant. All graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

5.5 Result

The results showed plasma biochemical parameters [creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH)] of scopolamine-induced amnesic rats intraperitoneally administration with the α-mangostin purified from the fruit rind of *Garcinia mangostana* (Linn.) and donepezil for constitutive 7 day.

In all instances, administration of all doses of α-MG showed no effect on behavior, food and water intake, growth or health status of these animals. After 7-day administration of all doses of α-MG, their body weight changes (Figure 5.1) and LDH plasma level did not alter from those of the vehicle control (5.5). In NSS groups, plasma AST levels were decreased after administration of donepezil and the α-mangostin (100 $mg \cdot m^{-1} \cdot kg^{-1}$) as shown in Figure 5.3. Scopolamine significantly increased plasma ALT level. In SCOP groups, plasma ALT level was decreased after administration of the α-mangostin (100 $mg \cdot m^{-1} \cdot kg^{-1}$) as shown in Figure 5.4. No plasma biochemical parameter exceeded the normal range (Figure 5.2).

No toxicological signs were found in all body weight changes and plasma biochemical parameters between all treatment groups. Intraperitoneally administration of the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil did not affect behavior of scopolamine-induced amnesic rats.

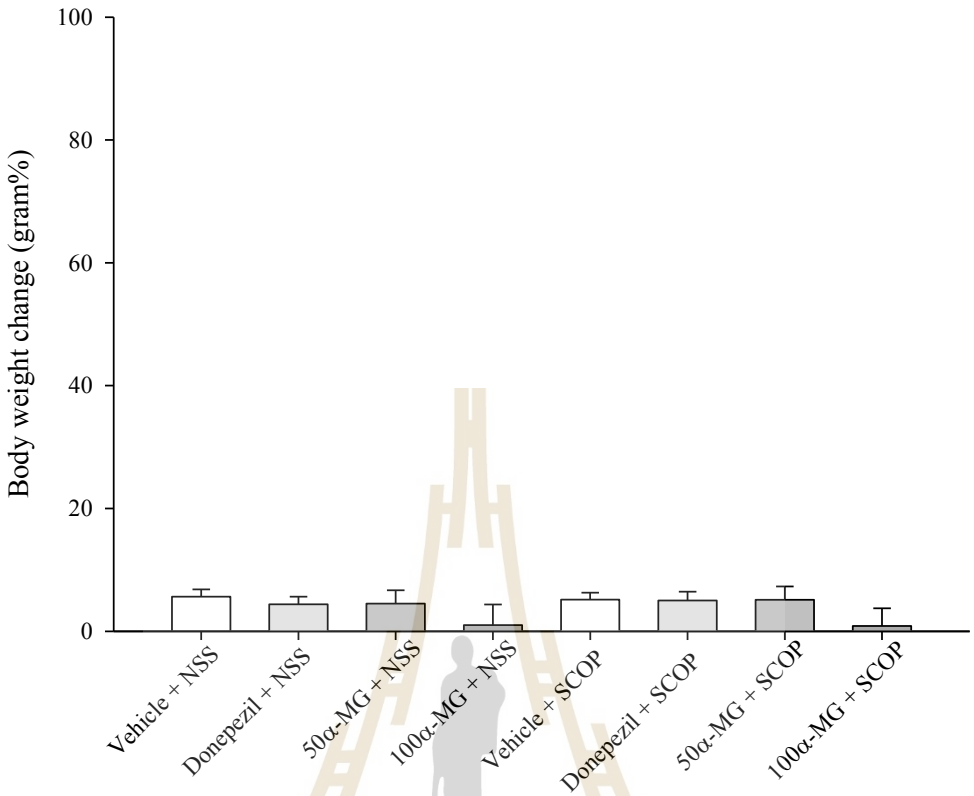

Figure 5.1 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on percentage of body weight changes in NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). There was no significant difference among NSS and SCOP groups. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

Figure 5.2 Clinical laboratory parameters for rats. Serum chemistry data for 8-17 weeks old male rats (Giknis and Clifford, 2008).

TEST	UNIT	N	MEAN	S.D.	RANGE
Aspartate aminotransferase	$U \cdot L^{-1}$	164	105	20	74-143
Alanine aminotransferase	$U \cdot L^{-1}$	164	28	7	18-45
Lactate dehydrogenase	$U \cdot L^{-1}$	10	1305	510	272-1965

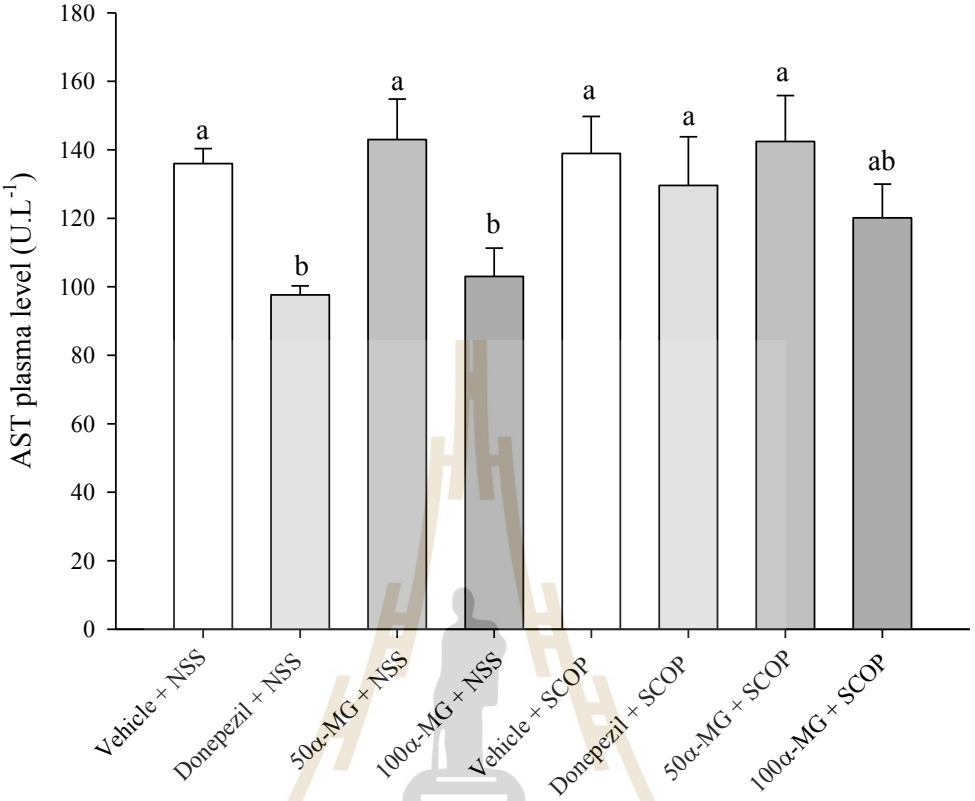

Figure 5.3 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on AST plasma level of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

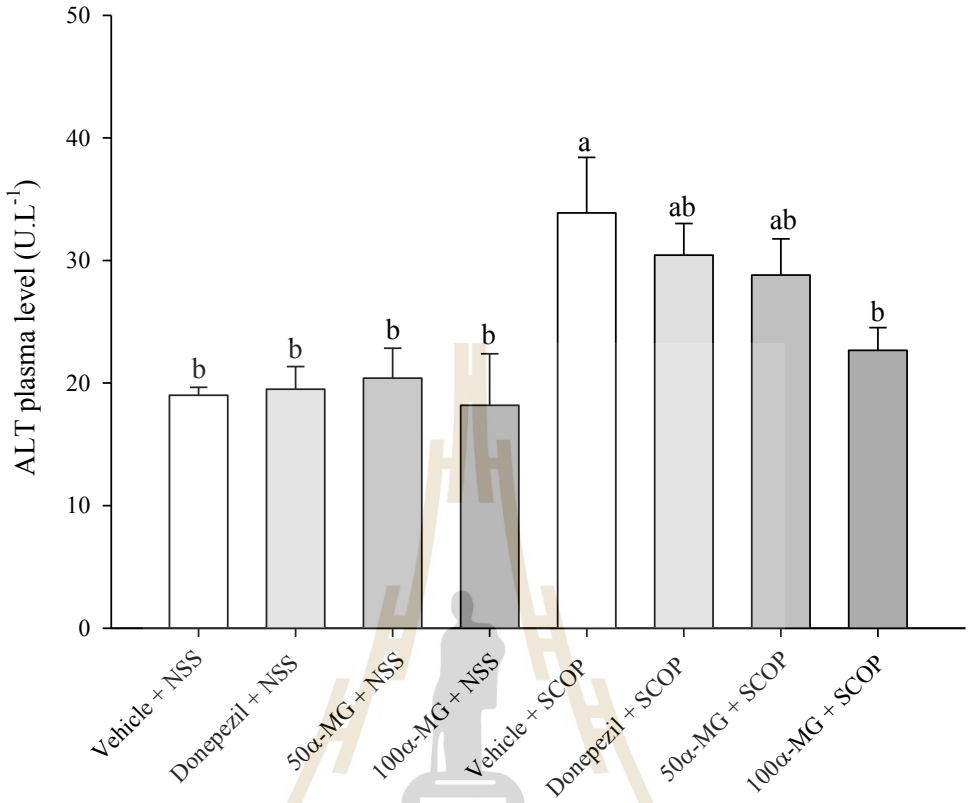

Figure 5.4 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on ALT plasma level of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

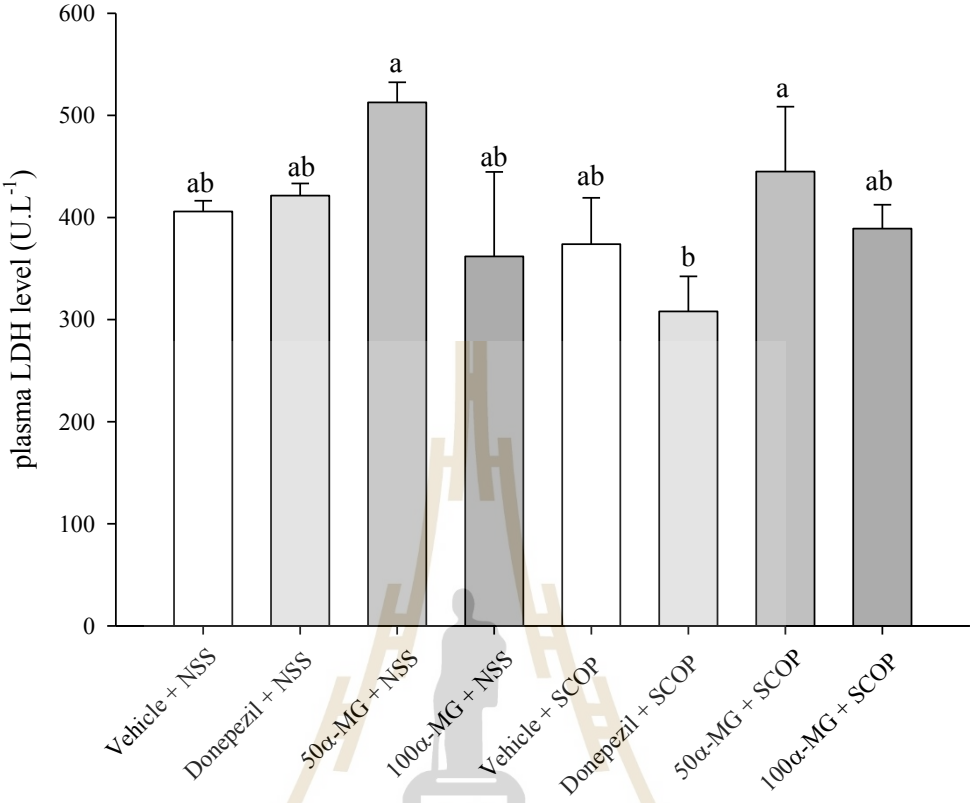

Figure 5.5 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on LDH plasma level of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

5.6 Discussion and conclusion

The present study was carried out in order to determine the effects of α-mangostin and donepezil on plasma AST, ALT, and LDH levels in the scopolamine-induced amnesic rats. The present study provided the first evidence of the ALT level induced by scopolamine that was significantly decreased by the α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$) when compared with the control group after 6 h. There was no significant difference in the AST and LDH levels of the scopolamine-induced amnesic rats either α-mangostin or donepezil when compared with scopolamine-treated group after 6 h. In the present study, the α-mangostin and donepezil could suppress the elevation of plasma AST level. However, the underlying mechanisms are needed to investigate. Both AST and ALT are enzymes produced by liver cells which were released when liver cell damaged (Patil, Somashekarappa, and Rajashekhar, 2011). The possible mechanism of the effect of α-mangostin on the reduction of ALT levels may be related to a reduction in plasma free fatty acid (FFA) levels, a reduction in FFA flux into the liver, and an increase in hepatic insulin sensitivity (Zelber-Sagi *et al*., 2006). The result of this study demonstrated that the α-mangostin and donepezil had no changed the levels of plasma AST and LDH. The previous studies declared that the levels of AST and ALT had no change after administration of the crude extract from the fruit rind of *G. mangostana* (Nontamart *et al*., 2013). In addition, the present findings showed that increased level of ALT in the scopolamine-induced amnesic rats was reduced by the α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$). Therefore, the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil are beneficial for anti-toxic effect and there is not toxicity on liver which could reduce AST level.

CHAPTER VI

EFFECTS OF α-MANGOSTIN ON CHOLINERGIC ENZYMES IN CEREBRAL CORTEX, HIPPOCAMPUS, AND BASAL FOREBRAIN IN SCOPOLAMINE-INDUCED AMNESIC RATS

6.1 Abstract

Acetylcholine (ACh), a neurotransmitter, plays a role in synaptic plasticity, including learning and memory. Hippocampal acetylcholine (ACh) plays a role in synaptic plasticity, including learning and memory. Cholinergic enzymes, particularly in the cerebral cortex, hippocampus, and basal forebrain, are markedly depleted in Alzheimer's disease (AD) which is associated with cognitive deficits. Muscarinic antagonist scopolamine (SCOP)-induced learning and memory impairment is commonly used as a model for AD. The extract from the fruit rind of *G. mangostana* (*Garcinia mangostana* L.) was recently reported to improve spatial memory in SCOP-induced amnesic rats. α-Mangostin (α-MG) is an aprenylated xanthone derivative from the fruit rind of *G. mangostana*. We examined whether α-mangostin (α-MG) improved activity *in vitro* or *in vivo* of cholinergic enzymes in rats induced by administration of scopolamine (SCOP), anticholinergic agent that blocks the activity of the muscarinic acetylcholine receptor which commonly used as a model for AD.

The Ellman's and NA-FB methods for investigating the acetylcholinesterase inhibitory activity *in vitro* were used. Eight groups (n = 8 each) of 8-weeks-old male Wistar rats were i.p. injected with normal saline solution (1 $mL \cdot kg^{-1}$), donepezil (2 $mg \cdot mL \cdot kg^{-1}$; positive control), α-MG (50 $mg \cdot mL \cdot kg^{-1}$), or α-MG (100 $mg \cdot mL \cdot kg^{-1}$) followed by i.p. injected with SCOP (2 $mg \cdot mL \cdot kg^{-1}$) or normal saline solution (1 $mL \cdot kg^{-1}$) an hour later. Six hours later, the animals were sacrificed and the brains were dissected on ice into 3 regions: cerebral cortex, hippocampus, and basal forebrain and homogenized for biochemical analysis to investigate the cholinergic enzyme activities *in vivo*. In the present study, the α-mangostin and donepezil exhibited the strong inhibition of acetylcholinesterase activity *in vitro* which appeared to be similar to that of the reference standard, eserine. The results showed that α-MG increased the activities *in vivo* of choline acetyltransferase (ChAT) whereas decreased the activity of acetylcholinesterase (AChE). In conclusion, α-MG has potential therapeutic value in alleviating SCOP-induced cognitive deficits in rat brain. Additionally, α-MG is one of the potent muscarinic agonists which its mechanism might be involved in regulating the acetylcholinesterase inhibitory and choline acetyltransferase enhancing activities and facilitating learning and memory and useful as a new therapy and an alternative supplement for treatment of dementia and neurodegenerative diseases.

6.2 Introduction

Dysfunction of cholinergic neurotransmission in the brain that causes cognitive decline has been reported in AD. The gradual death of cholinergic cells in AD is accompanied by loss of the acetylcholine (Vinutha *et al*., 2007). A cholinergic deficit has been shown to be associated with memory loss and the severity of AD. Loss of the

cholinergic markers, acetylcholinesterase (AChE) is neurological changes consistently found in the brains of AD patients (Court and Perry, 1991). Hence, it has been suggested that elevation of acetylcholine level might be helpful in attempt to improve the symptoms of cognitive deficit in AD. One of the most promising approaches for treatment of this disease is to enhance the acetylcholine level in the brain using acetylcholinesterase inhibitors (Chattipakorn *et al*., 2007; Ingkaninan *et al*., 2003; Vinutha *et al*., 2007).

The use of herbal remedies is an alternative treatment for AD. Many medicinal plants such as pomegranate, grape seed, curcumin, soy, *Ginkgo biloba*, and *Paeonia suffruticosa* have been reported as potential treatments for AD (Jung and Park, 2007; Lenta *et al*., 2007). These plants are good sources of polyphenols and other antioxidant components. Polyphenols found in plants and fruits (*Allanblackia monticola, Symphonia globulifera, Agrimonia pilosa, Salvia albimaculata, Salvia cyanescens, Pistacia atlantica, Pistacia lentiscus,* strawberry, walnut, rosemary, and soybean) could inhibit the activity of AChE (Benamar *et al*., 2010; Jung and Park, 2007; Lee *et al*., 2004; Lenta *et al*., 2007; Orhan *et al*., 2008; Orhan *et al*., 2007). Moreover, polyphenol extracts from green tea and wild blueberry could attenuate AChE activity (Kim *et al*., 2004; Papandreou *et al*., 2009). It is hope that α-mangostin found in the fruit rind of *G. mangostana* could prevent the onset of dementia by involving in the cholinergic system.

6.3 Materials and methods

The methodology of chemicals, drug solutions, animals, and experimental design is described in Materials and methods 4.3.1, 4.3.2, 4.3.3, and 4.3.4, respectively.

6.3.1 Preparation of tissue homogenates

After blood collection in Materials and methods 5.3.1, all rats were perfused with 225 ± 25 mL of ice-cold heparinized saline solution (0.9% NaCl) at a flow rate of 40 $mL \cdot min^{-1}$ using peristaltic pump (model SP 311, VELP Scientifica, Europe). The rats were perfused through the left ventricle of the heart. Immediately after starting the pump, the right atrium was cut to allow an escape route for the blood and perfusion fluid. After perfusion, rats were decapitated and brains were rapidly removed and placed on petri dish filled with ice. The cerebral cortex, hippocampus and basal forebrain were dissected out after localization according to the atlas of Paxinos and Watson (2009). The dissected brain structures were weighed, immediately frozen on dry ice and stored at -80 °C until homogenization. The tissue homogenates were prepared by modified methods of Chattipakorn *et al*. (2007) and Papandreou *et al*. (2009) as described in appendix A. Briefly, the brain tissues were homogenized using a sonicator (Ultrasonic, Sonic & Material, Inc., USA) with ice cold 10% of 0.1 M phosphate buffer (pH 7.4) containing 1% Triton-X 100 at 15,000 rpm for 15 min at 4°C.

6.3.2 Determination of protein content

6.3.2.1 Chemicals

Solution A [Copper sulfate ($CuSO_4 \cdot 5H_2O$), sodium tartate ($Na_2C_4H_4O_6 \cdot 2H_2O$), sodium azide], solution B [sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), sodium dodecyl sulfate (SDS: $C_{12}H_{25}NaO_4S$)], Folin-Ciocatteau reagent, and bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.3.2.2 Procedure

The method for measurement of protein content in brain homogenates was adapted from the method of Lowry *et al*. (1951). The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. Freshly mixed solution A and solution B (1:3). The homogenates were added to this mixed solution and incubated at room temperature for 60 minutes. The 2 N Folin-Ciocatteau reagent with DDD water (1:20) solution was added. Mixed and incubated at room temperature for 30 minutes. Read optical density (O.D) at 650 nm by Benchmark Plus Microplate Spectrophotometer (Benchmark Plus, BIO-RAD, Japan). Bovin serum albumin (BSA) was used as a standard protein. The blank samples contained the same mixture solution without the sample. Protein was determined by comparison of the values obtained from the standard curve.

Calculation: $\Delta_{A650\ nm}$ Standard = $A_{650\ nm}$ Standard - $A_{650\ nm}$ Blank

Plotted the $\Delta_{A650\ nm}$ standard against protein concentration on the standard graph

$$\Delta A_{650\ nm} \text{ Sample} = A_{650\ nm} \text{ Sample} - A_{650\ nm} \text{ Blank}$$

Determined the mg protein from the standard curve.

6.3.3 Determination of acetylcholinesterase (AChE) activity

6.3.3.1 Chemicals

Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4), sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4 \cdot H_2O$), 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB: $C_{14}H_8N_2O_8S_2$), and acetylthiocholine iodide (ATCI) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.3.3.2 Procedure

Determination of AChE activity was based on colorimetric method adapted from the method of Ellman *et al*. (1961), Chattipakorn *et al*. (2007), and Nakdook *et al*. (2010). The assay method is shown in appendix B. Briefly, the assay mixture consisted of 50 µl of brain homogenates, 25 µl of 0.1 M phosphate buffer (pH 7.4), 125 µl of 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma) and 25 µl of 1 mM acetylthiocholine iodide (ATCI, Sigma) was mixed well. The absorbance of the assay mixture was measured by Benchmark Plus microplate Spectrophotometer (Benchmark Plus, BIO-RAD, Japan) at wavelength of 405 nm and monitored over period of 6 min. All determinations were performed in triplicate. AChE activity was assessed by following formula:

$$R = [\Delta A / (1.36 \times 10^4)] \times [1 / (C_O \times \text{mg of protein})]$$

$$R = \Delta A / [1.36 \times 10^4 \times (50/225) \times \text{mg of protein}]$$

R = rate of enzyme activity (expressed as mole of acetylcholine iodide hydrolyzed/ minute/ mg of protein),

ΔA = the change in absorbance per minute,

C_O = original concentration of tissue ($mg \cdot mL^{-1}$)

Volume correction = 50/225

The extinction coefficient of the yellow product = 1.36×10^4 $M^{-1} \cdot cm^{-1}$

6.3.4 Determination of choline acetyltransferase (ChAT) activity

6.3.3.1 Chemicals

Choline chloride ($C_5H_{14}ClNO$), sodium chloride (NaCl), Ethylenediaminetetra acetic acid (EDTA: $C_{10}H_{16}N_2O_8$), dithioerythritol (DTE: $C_4H_{10}O_2S_2$), neostigmine bromide ($C_{12}H_{19}BrN_2O_2$), sodium arsenite ($NaAsO_2$), and 2,4-dithiopyrimidine ($C_4H_4N_2S_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.3.3.2 Procedure

ChAT activity will be determined spectrophotometrically using the method of Chao and Wolfgram (1972). In each test tube, phosphate buffer, choline chloride, sodium chloride, EDTA, dithioerythritol, neostigmine bromide, and distilled water were added along with acetyl CoA and creatine. The mixture was preincubated at 37 °C for 5 min and tissue homogenate was added. For the blank, boiled homogenate was added. This mixture was incubated at 37 °C for 20 min and boiled at 100 °C for 2 min, 800 µL of 2.5 $mmol \cdot L^{-1}$ sodium arsenite was added. The test tubes were centrifuged at 12,000 g for 5 min, and suspension was collected. 2,4-Dithiopyrimidine was added, and the tubes were allowed to stand for 15 min before being read spectrophotometrically at 342 nm (Lee *et al*., 2004). The choline acetyltransferase (ChAT) activity was expressed as $U \cdot g^{-1}$ of brain tissue protein.

6.4 Statistical analysis

All determinations were carried out in triplicate and the experimental data were presented as mean ± S.E.M. The significant differences between treatment levels were statistically analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test (SigmaStat version 3.5). *P* value less than 0.05 ($P<0.05$) was recognized statistically significant. All graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

6.5 Result

6.5.1 Effects of α-mangostin on acetylcholinesterase activity of cerebral cortex, hippocampus, and basal forebrain in the scopolamine-induced amnesic rats

The effects of the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil on the acetylcholinesterase inhibitory activity in the cerebral cortex, hippocampus, and basal forebrain were examined in normal saline treated rats and scopolamine-induced amnesic rats using Ellman's method.

The effects of daily administration of the α-mangostin purified from the fruit rind of *G. mangostana* (50 and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) and donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) for 7 days on AChE activity in the cerebral cortex, hippocampus, and basal forebrain of normal saline treated rats and scopolamine-induced amnesic rats were determined.

In scopolamine-induced amnesic rats, administration of vehicle + SCOP significantly increased AChE activity in cerebral cortex and hippocampus but not basal forebrain when compared to vehicle + NSS ($P<0.05$, one way ANOVA, Figure 6.1, 6.2, and 6.3).

In normal saline treated rats, α-MG50 + NSS significantly increased AChE activity in hippocampus when compared to vehicle + NSS ($P<0.05$, one way ANOVA, Figure 6.2). The results of scopolamine-induced amnesic rats showed that administration of α-MG50 + SCOP, but not α-MG100 + SCOP and donepezil + SCOP, significantly decreased AChE activity in cerebral cortex and hippocampus when compared to vehicle + SCOP ($P<0.05$, one way ANOVA, Figure 6.1 and 6.2).

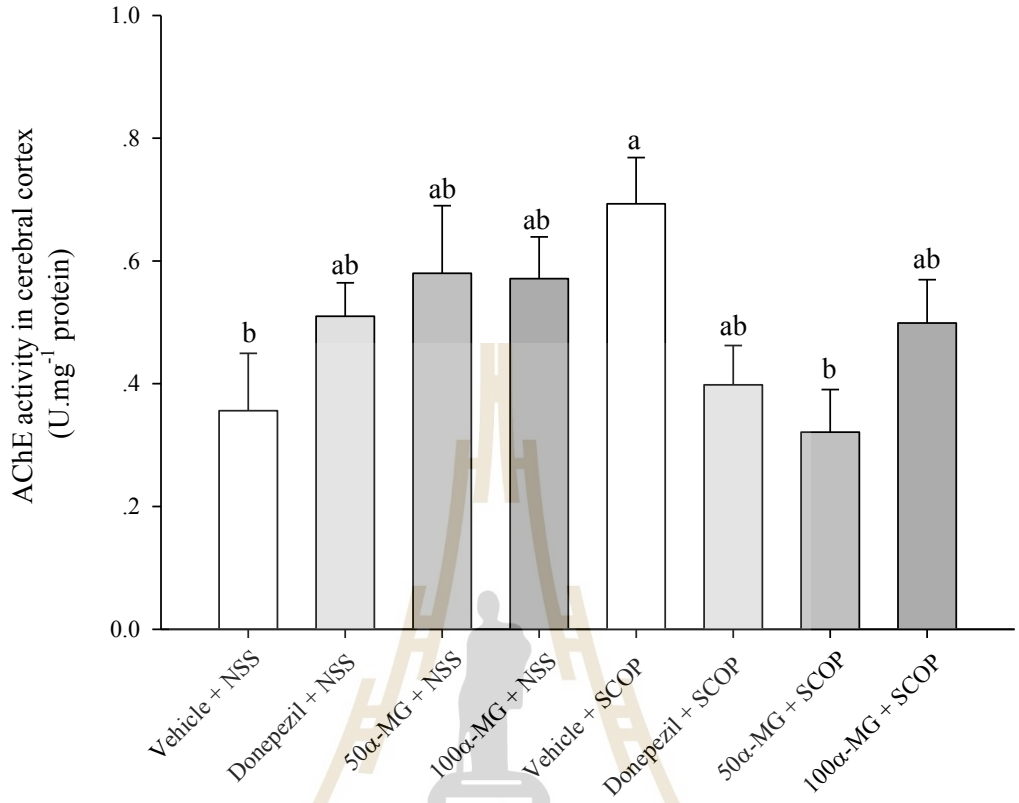

Figure 6.1 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on acetyl cholinesterase activity in cerebral cortex of NSS-treated and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

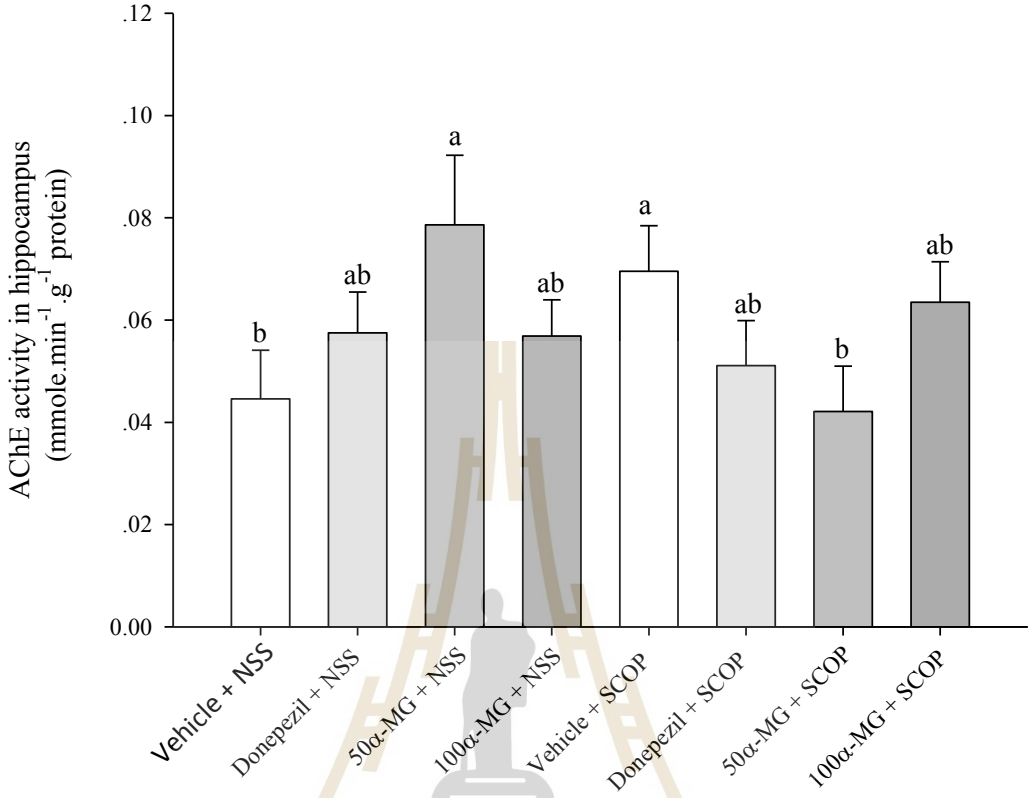

Figure 6.2 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on acetyl cholinesterase activity in hippocampus of NSS-treated and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

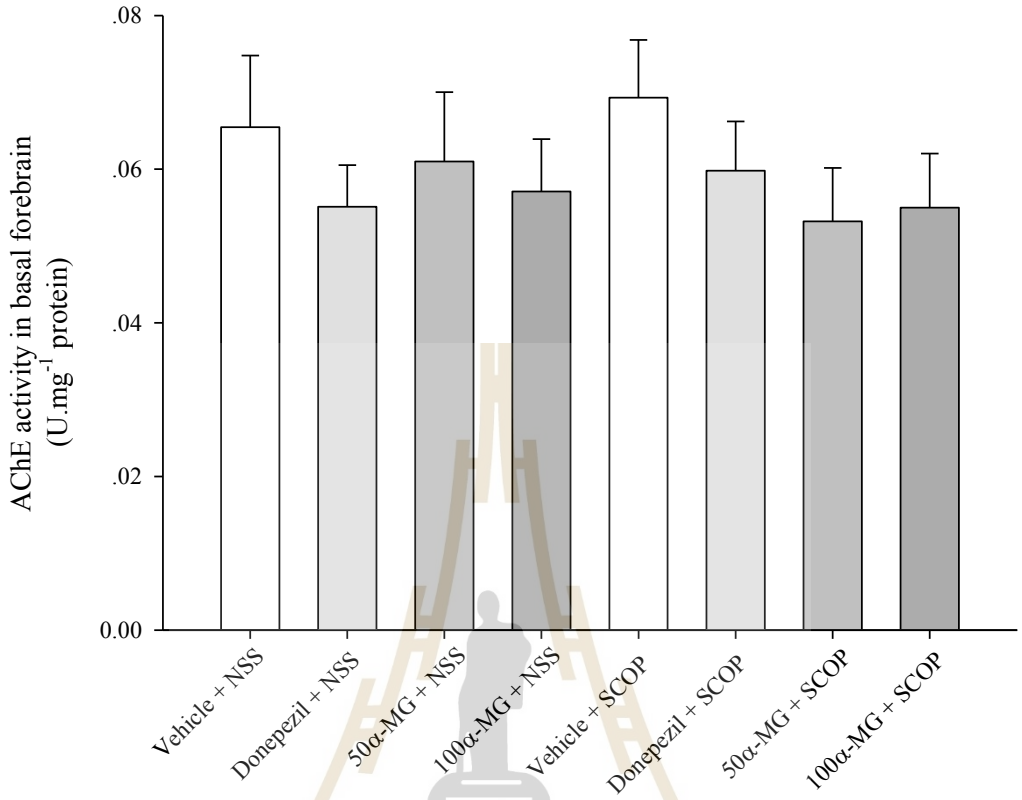

Figure 6.3 Effect of α-MG purified from the fruit rind of *G. mangostana* and donepezil on acetylcholinesterase activity in basal forebrain of NSS-treated and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). There was no significant difference among NSS and SCOP groups. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

6.5.2 Effects of α-mangostin on choline acetyltransferase activity of cerebral cortex, hippocampus, and basal forebrain in the scopolamine-induced amnesic rats

The effects of the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil on the choline acetyltransferase enhancing activity in the cerebral cortex, hippocampus, and basal forebrain were examined in normal saline treated rats and scopolamine-induced amnesic rats using Chao and Wolfgram assay.

The effects of daily administration of the α-mangostin purified from the fruit rind of *G. mangostana* (50 and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) and donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) for 7 days on ChAT activity in the cerebral cortex, hippocampus, and basal forebrain of normal saline treated rats and scopolamine-induced amnesic rats were determined.

In scopolamine-induced amnesic rats, administration of vehicle + SCOP significantly decreased ChAT activity in cerebral cortex and hippocampus but not basal forebrain when compared to vehicle + NSS ($P<0.05$, one way ANOVA, Figure 6.4, 6.5, and 6.6).

In normal saline treated rats, α-MG50 + NSS significantly increased ChAT activity in hippocampus when compared to vehicle + NSS ($P<0.05$, one way ANOVA, Figure 6.5). The results of scopolamine-induced amnesic rats showed that administration of α-MG50 + SCOP, but not α-MG100 + SCOP and donepezil + SCOP, significantly increased ChAT activity in cerebral cortex and hippocampus when compared to vehicle + SCOP ($P<0.05$, one way ANOVA, Figure 6.4 and 6.5).

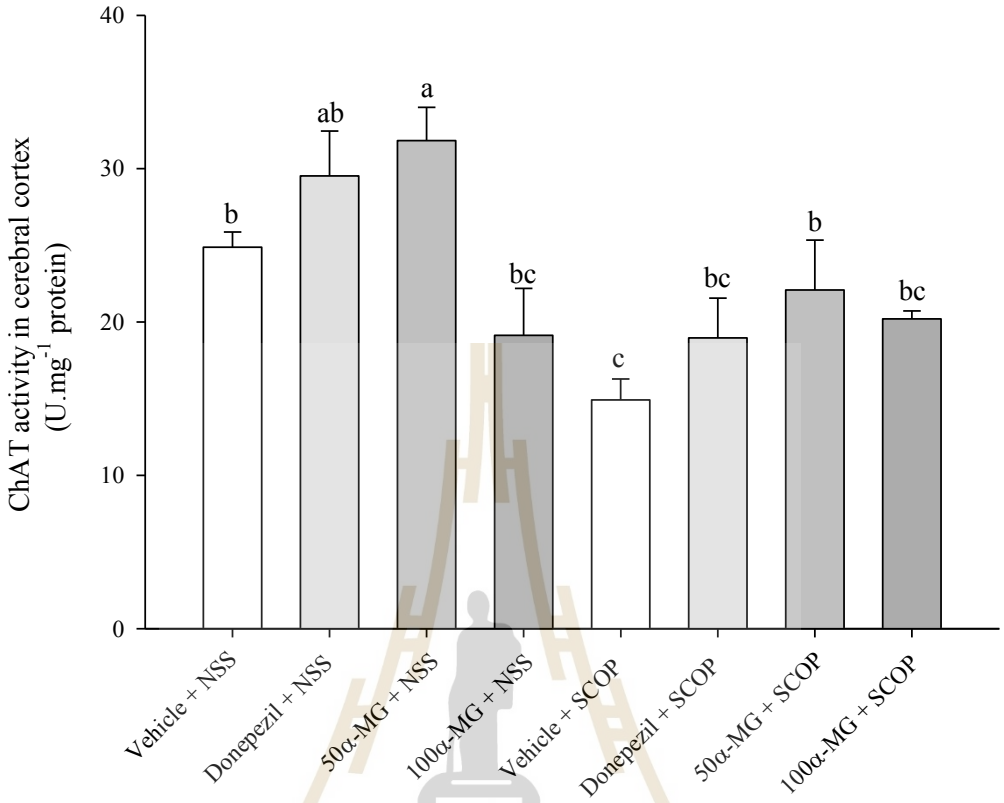

Figure 6.4 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on choline acetyl transferase activity in cerebral cortex of NSS-treated and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences among groups of rats (P<0.05). DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

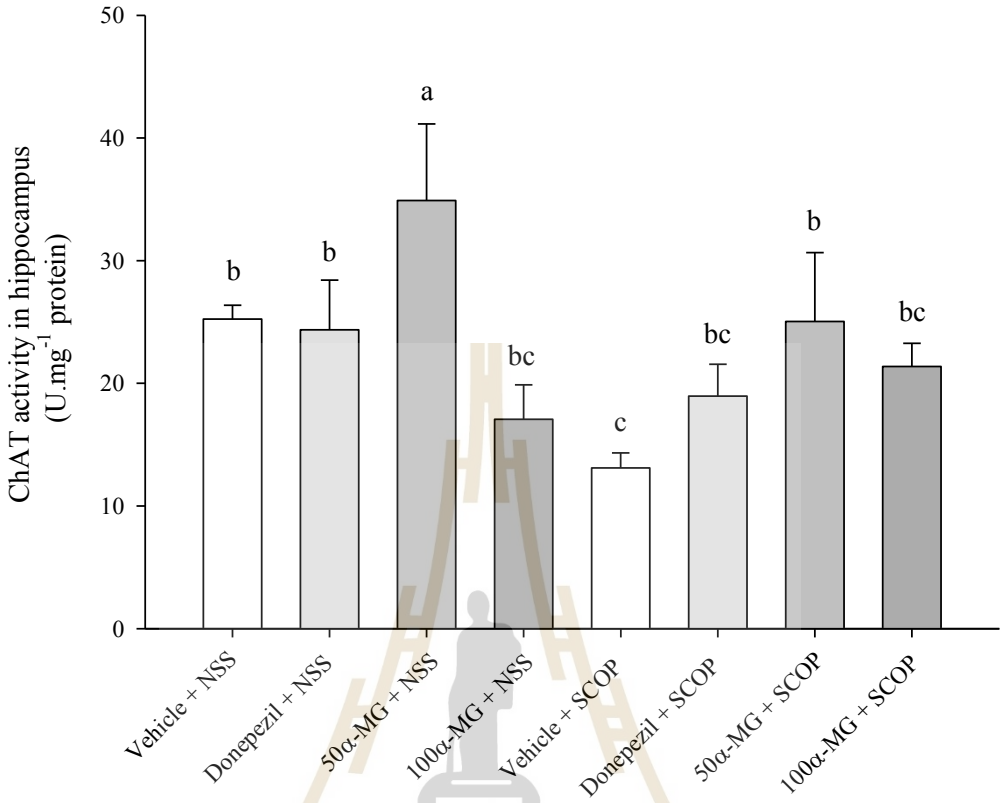

Figure 6.5 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on choline acetyl transferase activity in hippocampus of NSS-treated and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences among groups of rats (P<0.05). DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

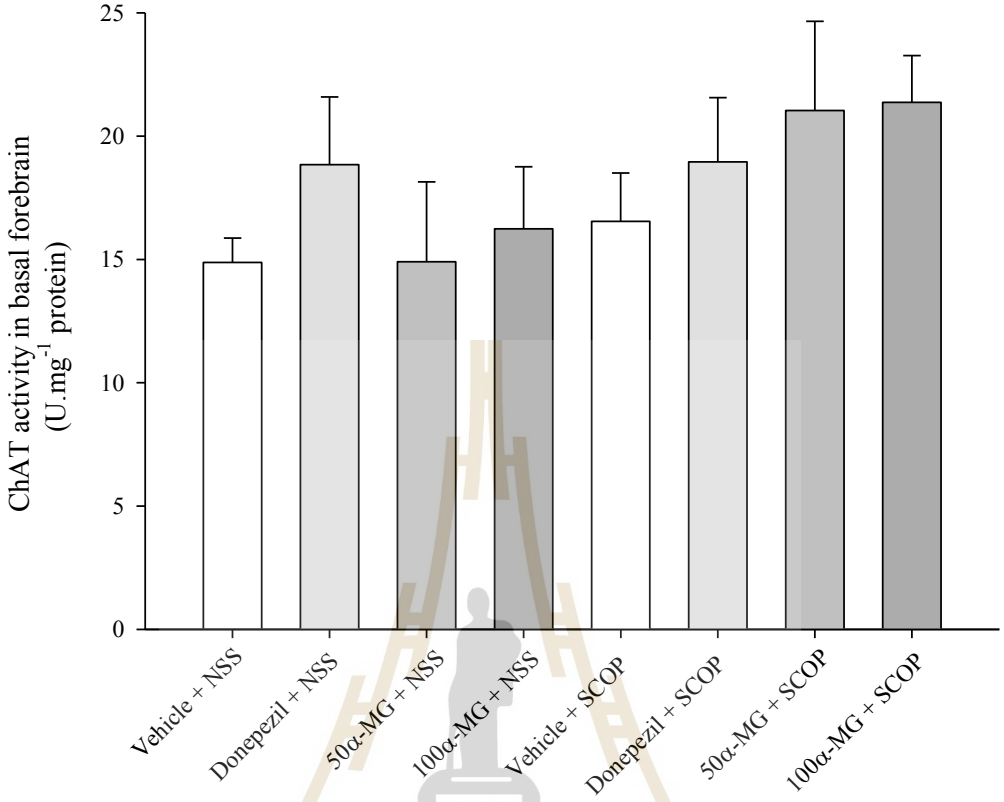

Figure 6.6 Effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on choline acetyl transferase activity in basal forebrain of NSS-treated and SCOP-induced amnesic rats. Data were expressed as means ± S.E.M. (n = 8). Different alphabets indicate significant differences among groups of rats (P<0.05). DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

6.5 Discussion and conclusion

Dementia is a symptom complex of continuous global weak of intellectual function. It becomes a major medical, economic, and social problem that is deterioration as the increasing number of elderly people. Degradation of the central cholinergic system resulting in low levels of acetylcholine is correlated well with severity of dementia. Currently, available drug therapies for AD and other diseases that cause dementia consist primarily of acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine, huperzine A), an N-methyl-D-aspartate receptor antagonist (memantine) and some neuroprotective agents (Olivares *et al*., 2012). These drugs cause side effects, thus an alternative bioactive compounds from plants and fruits with little or no side effects should be used to replace these drugs. Polyphenolic compounds from many fruits and plants are known to possess cognitive enhancing effects and acetylcholinesterase inhibitory activity (Papandreou *et al*., 2009). The α-mangostin purified from the fruit rind of *G. mangostana* may possess biological activities related to polyphenolic compounds. The present study demonstrated for the first time that the α-mangostin from the fruit rind of *G. mangostana* could enhance memory probably by inhibiting brain acetylcholinesterase activity and enhancing ChAT activity in scopoloamine-induced male Wistar rats in similar pattern to donepezil. These results were consistent with the results of AChE activity in cerebral cortex and hippocampus, but not basal forebrain. AChE activity in cerebral cortex and hippocampus was increased by the α-mangostin purified from the fruit rind of *G. mangostana* (50 $mg \cdot mL^{-1} \cdot kg^{-1}$) in normal saline treated rats, but was decreased in scopolamine-induced amnesic rats in similar manner to donepezil (Changlek and Srisawat, 2017).

Neuroprotective effects of the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil were demonstrated in the present study. In scopolamine-induced amnesic rats, daily intraperitoneal administration of the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil could inhibit AChE activity and enhance ChAT activity in cerebral cortex and hippocampus, but not basal forebrain. AChE activity in cerebral cortex and hippocampus were decreased by the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil in scopolamine-induced amnesic (Figure 6.1 and 6.2). ChAT activity in cerebral cortex and hippocampus, not basal forebrain was increased, while in basal forebrain was not changed after daily intraperitoneal administration of the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil as shown in Figure 6.2 and 6.3, respectively. These findings suggested that the antiamnesic effect of the α-mangostin purified from the fruit rind of *G. mangostana* could be due to its anti-acetylcholinesterase action in cerebral cortex and hippocampus of scopolamine-induced amnesia (Foster *et al*., 1994; Gray *et al*., 2008).

The cholinergic system is responsible for the storage and retrieval of item in memory and it degradation correlates well with the severity of cognitive and memory impairment. Therefore, it has been suggested that elevation of the acetylcholine level might be helpful in to improve the symptoms of cognitive deficit (Gasparin *et al*., 1998). Loss of cholinergic innervations, demonstrated by reduced choline acetyltransferase (ChAT) and elevated acetylcholinesterase (AChE) activity, is correlated with the degree of dementia (Zubenko *et al*., 1989). Many different theories have been offered regarding the cause of AD; a well-established theory suggests that acetylcholine levels are too low in the brain of AD patients (Davies and Maloney

1976). Therefore, one approach for treating AD is via the inhibition of AChE. Based on the cholinergic hypothesis, a defect in the cholinergic system is involved in AD (Francis *et al*., 1999). Polyphenols from plants and fruits can enhance memory by inhibiting AChE activity. The polyphenol-rich extract from lotus seed pods was found to inhibit AChE activity (Xu *et al*., 2009). Green tea extract containing polyphenols could inhibit AChE activity in scopolamine-induced amnesia mice (Kim *et al*., 2004). Polyphenol- rich wild blueberry extracts attenuating brain oxidative stress, increasing brain ascorbate and glutathione (GSH) levels, and decreasing AChE activity in mice whole brain homogenates, exhibited a significant improvement in learning and memory tested by the passive avoidance behavioral test (Papandreou *et al*., 2009). Mukhergee *et al*. (2006) and Yang *et al*. (2008) reported that N. *nucifera* rhizome extract inhibited AChE activity and improved learning and memory by enhancing neurogenesis in the dentate gyrus of hippocampus.

Previous study found that the crude extract from the fruit rind of *G. mangostana* possessed potent acetylcholinesterase inhibitory activity in the cerebral cortex, hippocampus, and basal forebrain of scopolamine-induced amnesic rats (Nontamart *et al*., 2013). The α-mangostin, a plant-derived substance, is one of the phenolic compounds found in fruit rind of *G. mangostana* may be responsible for those activities of the crude extract from the fruit rind of *G. mangostana*. The present study also provided the first evidence of the potent cholinergic enzyme regulatory effect of α-mangostin and donepezil both *in vitro* and *in vivo*. The α-mangostin and donepezil could suppress acetylcholinesterase and induce choline acetyltransferase activity both *in vitro* and *in vivo*. Regulation of these cholinergic enzyme activities by α-mangostin and donepezil in scopolamine-induced amnesic rats could suppress acetylcholine

cleavage and increase acetyl choline production, respectively, resulting in enhancing the cholinergic neurotransmission by prolonging the time in which acetylcholine molecules remain in the synaptic cleft. Regulation of these cholinergic enzymes by α-mangostin and donepezil is beneficial for prevention and treatment of dementia. The mechanisms of the cholinergic enzyme regulatory effect of α-mangostin and donepezil *in vitro* and *in vivo* are shown in Figure 6.7.

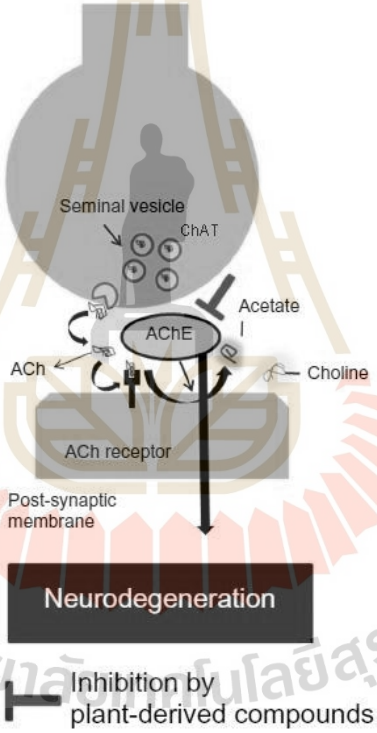

Figure 6.7 Mechanisms of the acetylcholinesterase inhibitory and choline acetyltransferase enhancing activities of plant-derived compounds. Abbreviations: ACh; acetylcholine, AChE; acetylcholinesterase enzyme, ChAT; choline acetyltransferase enzyme (Syad and Devi, 2014).

CHAPTER VII

EFFECTS OF α-MANGOSTIN ON APOPTOTIC CASPASE-3 ACTIVITY OF CEREBRAL CORTEX, HIPPOCAMPUS AND BASAL FOREBRAIN IN SCOPOLAMINE-INDUCED AMNESIC RATS

7.1 Abstract

The expression of apoptosis related proteins as active caspase-3, procaspase-9, and PARP were increased by scopolamine which plays a role in the pathogenesis of neurodegenerative diseases. Caspase-3, procaspase-9, and PARP served as markers for apoptosis were markedly increased in the brain which is associated with neurotoxicity. As such, inhibition of caspases is considered as a tool for prevention and compensation of various synaptic pathologies leading to cognitive deficit and Alzheimer's disease (AD) pathogenesis. The extract from the pericarp of *Garcinia mangostana* (L.) was previously reported to protect against cytotoxicity *in vitro* and improve spatial memory in scopolamine (SCOP)-induced amnesic rats. We therefore examined the ability of α-mangostin (α-MG), an aprenylated xanthone derivative from the extract, to attenuate SCOP-induced neurotoxicity in rat brains. Eight groups (n = 8 each) of 8-weeks-old male Wistar rats were i.p. injected with donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$), α-MG (50 $mg \cdot mL^{-1} \cdot kg^{-1}$), α-MG (100 $mg \cdot mL^{-1} \cdot kg^{-1}$), or vehicle (1 $mL \cdot kg^{-1}$) followed by SCOP (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) or NSS (1 $mL \cdot kg^{-1}$, i.p.) for 7 days.

Six hours after last dose of SCOP on day 7, the animals were sacrificed and three brain regions (basal forebrain, cerebral cortex, and hippocampus) were dissected and homogenized for biochemical analysis to investigate caspase-3 activity. No significant difference in was found between groups in NSS treatment in all studied brain regions. SCOP significantly elevated caspase-3 activity in all studied brain regions, compared to their respective NSS groups (P<0.05). Pretreatment of donepezil and α-MG (50 $mg \cdot mL^{-1} \cdot kg^{-1}$), but not α-MG (100 $mg \cdot mL^{-1} \cdot kg^{-1}$), significantly attenuated the increase of caspase-3 activity induced by SCOP in cerebral cortex and hippocampus (P<0.05), but not in basal forebrain. The findings demonstrated neuroprotective effect of α-MG against SCOP neurotoxicity. The present study is the first report of α-MG as a potential neuroprotective candidate, which its mechanism underlying this facilitating learning and memory might be involved in ameliorating scopolamine-induced neurotoxicity *via* activation and subsequent expression of apoptotic enzymes.

7.2 Introduction

Alzheimer's disease (AD), the leading cause of dementia in the aging population, is a neurodegenerative disease characterized by progressive deposition of amyloid β-peptide (Aβ) in senile plaques, aggregation of paired helical filament tau (PHFtau) in neurofibrillary tangles and neuritic plaques, progressive synaptic degeneration and neuron loss, and activation of astroglia and microglia. Among these pathological changes, synaptic loss has been described as the most highly correlated to severity of cognitive impairment. Synaptic degeneration in AD is characterized by dendritic spine loss, loss of axon terminals, and decreased expression of presynaptic and postsynaptic proteins. Elucidating the mechanisms by which synaptic

degeneration occurs is crucial to achieving a better understanding of AD dementia. Apoptosis-related caspases have been proposed to contribute significantly to progressive neuron death in AD. In cultured hippocampal neurons, Aβ causes caspase activation leading to apoptosis. Abnormal expression levels of initiator caspases 8 and 9 and effector caspases 3 and 6 have been reported in postmortem brain tissues from patients with AD. Additionally, the caspases have a role in amyloid precursor protein (APP) processing and the biogenesis of amyloidogenic Aβ peptide species. Both caspase-cleaved APP and activated caspase-9 have been found to be present in brains of AD patients but not in controls. Active caspases and the resulting cleaved caspase substrates, including fodrin, actin, tau, and APP, have been detected in postmortem AD brain tissue and AD animal models, which further supports the hypothesis that caspases contribute to AD neurodegeneration.

Previous studies found that scopolamine could induce apoptosis in the hippocampus of aged rats (Balaban *et al*., 2016). Moreover, induction of pro-apoptosis events *via* caspase-3 activation was induced by scopolamine (Jahanshahi *et al*., 2013).

Immunohistochemical analysis has previously demonstrated increased levels of active caspase-3 in hippocampal neuron somata and neurites of AD brains with a high degree of co-localization with neurofibrillary tangles and plaques. Caspase-3 also has been reported to be present in synapses in response to apoptotic insults, but its local effects at the synapse have not been described. To better understand the neurotoxicity associated with AD, we examined the activity of caspase-3 in the cerebral cortex, hippocampus, and basal forebrain of control and scopolamine-induced amnesic rats.

7.3 Materials and methods

The methodology of chemicals, drug solutions, animals, experimental design, preparation of tissue homogenates, and determination of protein content is described in Materials and methods 4.3.1, 4.3.2, 4.3.3, 4.3.4, 6.3.1, and 6.3.2, respectively.

7.3.1 Determination of caspase-3 activity

7.3.1.1 Chemicals

Tris hydrochloride (Tris-HCl: $C_4H_{11}NO_3$), sodium chloride (NaCl), Ethylenediaminetetraacetic acid (EDTA: $C_{10}H_{16}N_2O_8$), dithiothreitol (DTT: $C_4H_{10}O_2S_2$), Triton™ X-100 [$C_{14}H_{22}O(C_2H_4O)_n$(n=9-10)], and N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA: $C_{26}H_{34}N_6O_{13}$) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

7.3.1.2 Procedure

Caspase 3 activity assay was performed with the use of a colorimetric method (Filippova *et al*., 2004). Briefly, the rat brain homogenates were washed in caspase-3 lysis buffer containing 10 mM Tris-HCl, pH 7.4, 0.5M NaCl, 1 mM EDTA, 5 mM DTT, and 0.1% Triton™ X-100. The brain homogenates were incubated on ice for 15 min and centrifuged at 15,000 rpm for 15 min at 4 °C. The protein concentration was determined by the Lowry method and the caspase-3 activity in the supernatant was measured immediately. 50 g protein samples in 10 µl were added to 980 µl assay buffer. The reaction was initiated by adding 10 µl of 20 mM of the caspase-3 substrate Ac-DEVD-pNA. The tubes were covered and incubated at 37 °C overnight. Cleavage of the chromophore from the substrate was detected spectrophotometrically at a wavelength of 405 nm.

7.4 Statistical analysis

All determinations were carried out in triplicate and the experimental data were presented as mean ± S.E.M. The significant differences between treatment levels were statistically analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test (SigmaStat version 3.5). *P* value less than 0.05 ($P<0.05$) was recognized statistically significant. All graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

7.5 Result

The effects of the α-mangostin (α-MG) purified from the fruit rind of *G. mangostana* and donepezil on apoptotic caspase-3 activity were examined in normal saline treated rats and scopolamine (SCOP)-induced amnesic rats using colorimetric method (Filippova *et al*., 2004) with minor modification. The results showed that no significant difference was found between groups in NSS treatment in all studied brain regions. SCOP significantly elevated caspase-3 activity in all brain regions, compared to their respective NSS groups ($P<0.05$) as shown in Figure 7.1, 7.2, and 7.3. Pretreatment of donepezil and α-MG (50 $mg \cdot mL^{-1} \cdot kg^{-1}$), but not α-MG (100 $mg \cdot mL^{-1} \cdot kg^{-1}$), significantly attenuated the increase of caspase-3 activity induced by SCOP in cerebral cortex and hippocampus ($P<0.05$) as shown in Figure 7.1 and 7.2. In basal forebrain, all pretreatments had no effect on the increase of caspase-3 activity induced by SCOP (Figure 7.3).

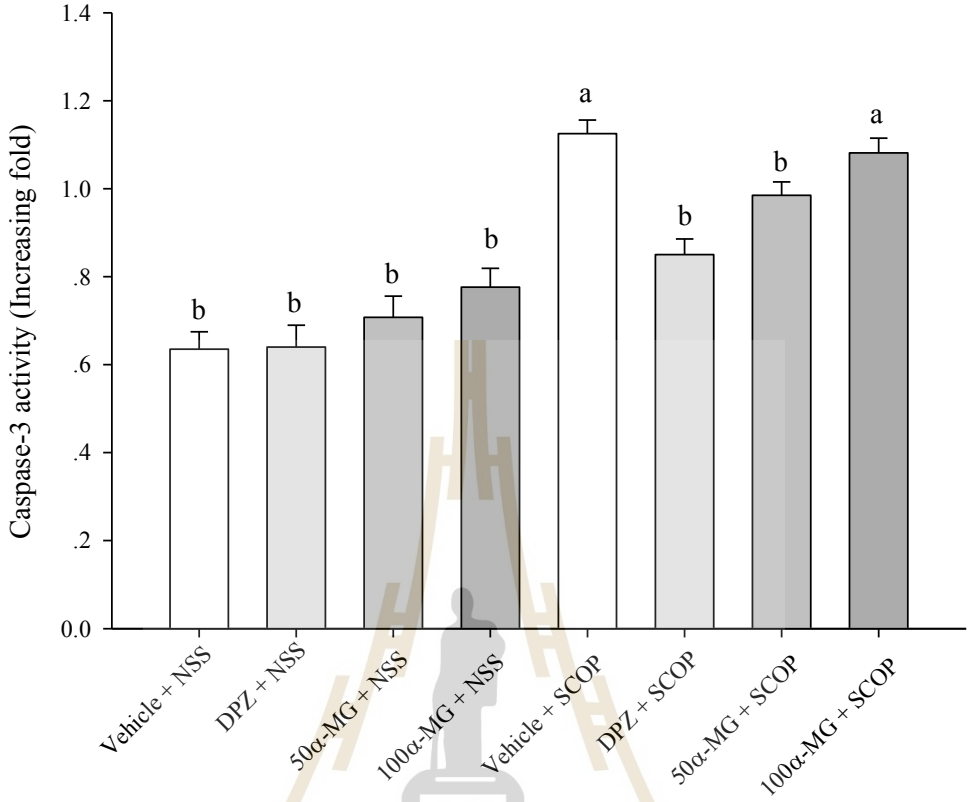

Figure 7.1 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on catalytic activity of caspase-3 enzyme in the cerebral cortex of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8) and are the average of three independent. Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

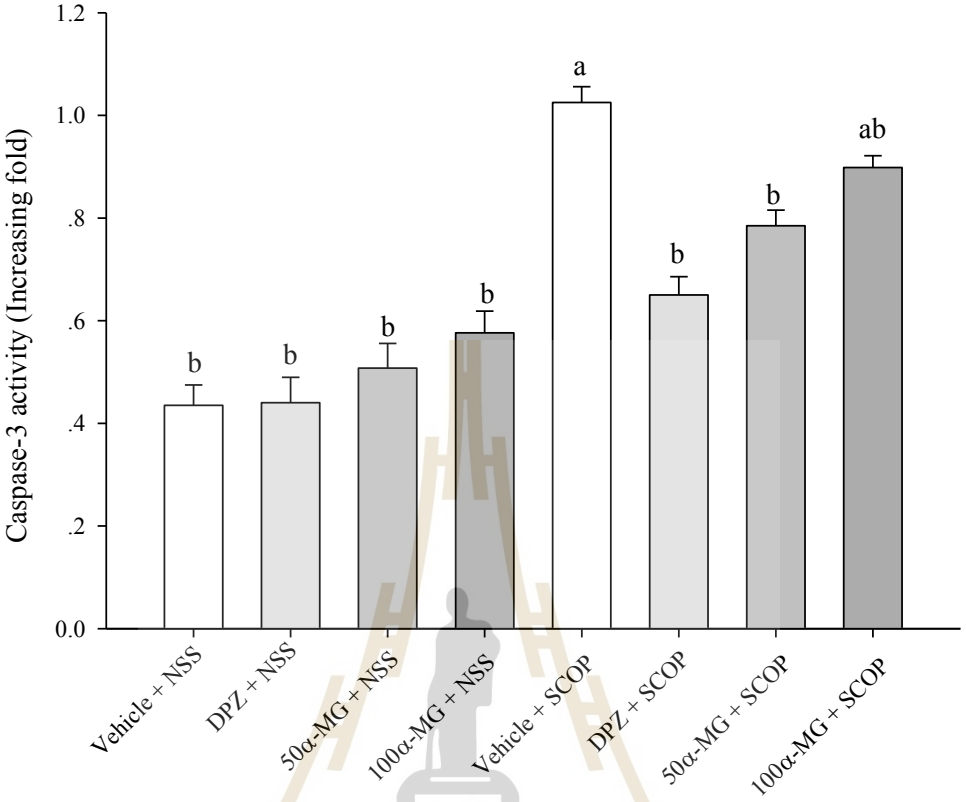

Figure 7.2 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on catalytic activity of caspase-3 enzyme in the hippocampus of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8) and are the average of three independent. Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

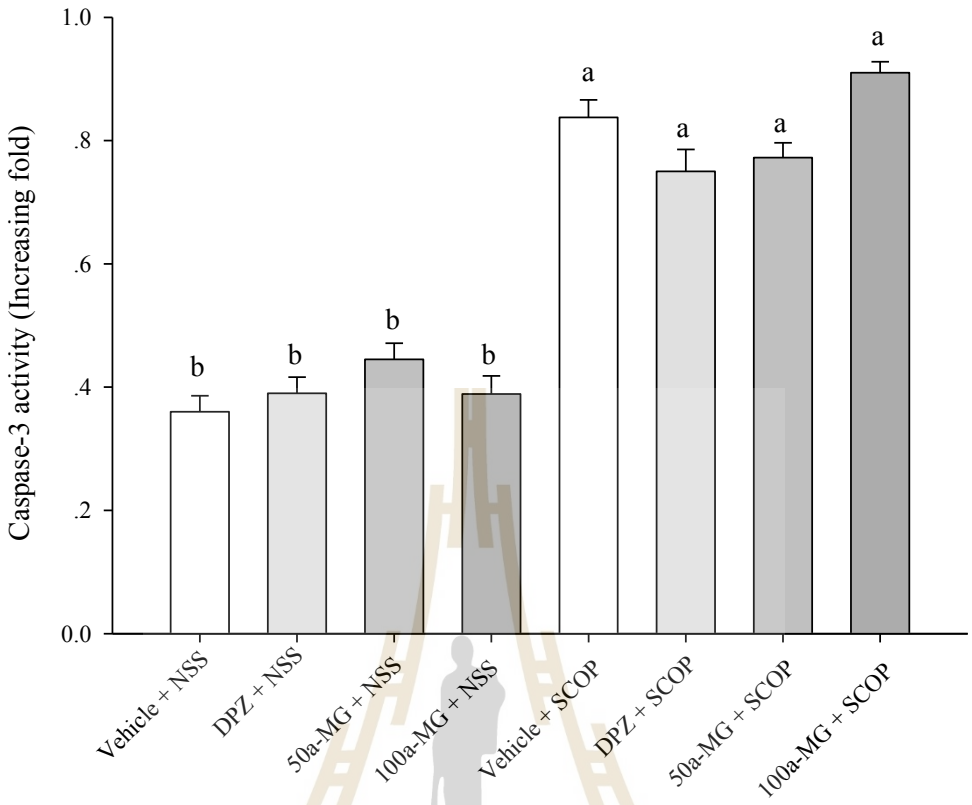

Figure 7.3 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on catalytic activity of caspase-3 enzyme in the basal forebrain of NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as means ± S.E.M. (n = 8) and are the average of three independent. Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

7.6 Discussion and conclusion

The previous study showed that scopolamine could induce cell death and DNA fragmentation by activating caspase-3-like protease activity in rat brains. The α-mangostin purified from the fruit rind of *G. mangostana* (50 $mg \cdot mL^{-1} \cdot kg^{-1}$) and donepezil effectively blocked the scopolamine-induced apoptosis. The caspase-3-like protease is a major component of the cellular machinery leading to scopolamine-induced cell death. Participation of the caspase-3-like protease in scopolamine induced cell death and DNA fragmentation had been demonstrated previously. The results suggested that treatment with α-mangostin and donepezil could suppress scopolamine-induced cell death and DNA fragmentation by inhibiting caspase-3 activity. Furthermore, α-mangostin and donepezil also prevented the stimulation of the caspase-3-like protease activity and PARP cleavage that occurs in scopolamine-induced apoptotic rats. PARP is one of the well studied physiological substrates of caspase-3. Our findings suggested that inhibition of the caspase-3-like protease activity may be a major mechanism by which α-mangostin and donepezil treatment protects rat brains from scopolamine-induced cell death. Caspases are cysteine proteases that contain cysteine in their catalytic domain. Site-directed mutagenesis studies have shown that replacement of cysteine in the active site with other amino acid abolishes the catalytic activity of caspases. Furthermore, the enzymatic activity of caspase-3 was lost when reactive thiol groups on the enzyme were modified chemically. It is interesting in this context that α-mangostin and donepezil might be capable of oxidizing thiol groups on proteins. We proposed, therefore, that at least one mechanism by which α-mangostin and donepezil treatment can prevent the caspase-3-like protease activation is the oxidation of critical cysteine residues on the caspase-3-

like protease. This hypothesis is consistent with our data using thiol reducing agents, Dithiothreitol (DTT). DTT prevented the inhibitory effect of α-mangostin and donepezil on caspase-3 activity in cleaving the substrate, Ac-DEVDpNA. The reducing agents also reversed the suppression by α-mangostin and donepezil of caspase-3-induced DNA fragmentation in rat brains. Internucleosomal DNA fragmentation, which has been long considered a biochemical hallmark of apoptosis, appears to occur at a late stage in many of the apoptotic events. There are accumulating lines of evidence that caspases play a pivotal role in the activation of nuclear DNA fragmentation. Caspases, especially caspase-3, have been shown to activate a caspase-activated DNase (CAD/DFF40) through cleaving its inhibitor (ICAD/DFF45). In our study, treatment with scopolamine resulted in the stimulation of internucleosomal DNA fragmentation, and the scopolamine-induced DNA fragmentation could be prevented by pretreatment of rats with α-mangostin and donepezil. This suggests that a caspase-3-like protease is associated with the mechanism that operates in the scopolamine induced DNA fragmentation. Further increases in oxidative stress and caspase 3 activity are suggestive of apoptosis in brain following systemic scopolamine treatment. The result demonstrated neuroprotective effect of α-MG against SCOP neurotoxicity. Our study suggests that first report about α-MG, a potential neuroprotective candidate, which its mechanism underlying this facilitating learning and memory might be involved in ameliorating scopolamine-induced neurotoxicity via activation and subsequent expression of apoptotic enzymes.

CHAPTER VIII

EFFECTS OF α-MANGOSTIN ON LIPID PEROXIDATION OF CEREBRAL CORTEX, HIPPOCAMPUS AND BASAL FOREBRAIN IN SCOPOLAMINE-INDUCED AMNESIC RATS

8.1 Abstract

G. mangostana has been used in traditional Thai medicine. Crude extract of its rind has been reported to have the antioxidant activity. However, the use of the plants is mainly based on local tradition without scientific knowledge. The present study aims to investigate the effects of its xanthone derivative, α-mangostin, and donepezil on the inhibition of lipid peroxidation reaction. The effect on inhibition of lipid peroxidation was determined by measuring the formation of TBARS (thiobarbituric acid reactive substances) in scopolamine-induced amnesic rats. In addition, α-mangostin and donepezil markedly exhibited inhibition of rat brain lipid peroxidation. The results showed that no significant difference was found between groups in NSS treatment in all studied brain regions. SCOP significantly elevated malondialdehyde (MDA) level in all brain regions, compared to their respective NSS groups (P<0.05). Pretreatment of donepezil and α-MG (50 $mg \cdot mL^{-1} \cdot kg^{-1}$), but not α-MG (100 $mg \cdot mL^{-1} \cdot kg^{-1}$), significantly attenuated the increase of MDA level induced by SCOP in cerebral cortex and hippocampus (P<0.05). In basal forebrain, all pretreatments had no effect on the increase of MDA level induced by SCOP.

The findings demonstrated neuroprotective effect of α-MG against SCOP-induced lipid peroxidation. The present study is the first report of α-MG as a potential neuroprotective candidate, which its mechanism underlying this facilitating learning and memory might be involved in ameliorating scopolamine-induced lipid peroxidation *via* activation and subsequent expression of apoptotic enzymes.

8.2 Introduction

It has been shown that the reactive oxygen species (ROS) and other free radicals, which are formed and accumulated during oxidative stress as a result of an imbalance between their production and removal by the antioxidant system, induce cellular and molecular abnormalities in old age related diseases. Although the exact mechanisms underlying these deleterious effects remain unclear, it is known that an increase in free radical production in neuronal cells, leading to oxidative stress and cell death. Therefore, antioxidants have been suggested as therapies to prevent, delay or ameliorate the pathological changes underlying the progression of old age related diseases. Recently, there has been increasing interest in the natural antioxidants contained in the medicinal plants, which are candidates to prevent oxidative damage. Recent studies indicate that oxidative stress and free radical generation have been implicated as major etiological factors in the pathogenesis of old age related diseases. The free radicals generated in turn induce the lipid peroxidation, production of free carbonyls, formation of advanced glycation end products (AGEs), and DNA damage, ultimately resulting in neurodegeneration.

Garcinia mangostana (*G. mangostana*), commonly known as mangosteen and belonging to the family Clusiaceae, is a fruit grown mainly in Southeast Asia. The medicinal properties of *G. mangostana* are well described in traditional Thai Traditional medicine, the fruit rind has been used to treat diarrhea, skin infection and wounds. The extract of the fruit rind has been reported to possess important pharmacological effects including antioxidant, anti-tumor, anti-allergic, anti-inflammatory, anti-bacterial, anti-fungal, anti-and anti-viral activities. The fruit rind of *G. mangostana* contained several types of polyphenolic compounds: anthocyanin, α-mangostin, β-mangostin, γ-mangostin, gartanin and mangostanol. Moreover, *G. mangostana* exhibited inhibition of lipid peroxidation. The aim of this study was to investigate the lipid peroxidation inhibition of α-mangostin purified from the fruit rind of *G. mangostana* by assay of malondialdehyde level using TBARS method.

8.3 Materials and methods

The methodology of chemicals, drug solutions, animals, experimental design, preparation of tissue homogenates, and determination of protein content is described in Materials and methods 4.3.1, 4.3.2, 4.3.3, 4.3.4, 6.3.1, and 6.3.2, respectively.

8.3.1 Determination of lipid peroxidation

8.3.1.1 Chemicals

Trichloroacetic acid (TCA: $C_2HCl_3O_2$), thiobarbituric acid (TBA: $C_4H_4N_2O_2S$), hydrochloric acid (HCl), and butylated hydroxytoluene (BHT: $C_{15}H_{24}O$) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

8.3.1.2 Procedure

Malondialdehyde (MDA), the parameter for lipid peroxidation, was measured using the method described by Buege and Aus (1978) with minor modifications. Briefly, 100 µL of samples of brain homogenate were incubated with either 100 µL TBARS (thiobarbituric acid reactive substance) solution including 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA), and 0.25 N HCl, was mixed thoroughly, or 100 µL TBARS solution containing the above together with 0.02% w/v butylated hydroxytoluene (BHT), was added. Samples were then mixed vigorously, heated for 15 min at 95 °C in the WiseBath® (Witeg Labortechnik GmbH, Wertheim, Germany). After cooling, followed by centrifugation at 3,000 × *g* for 10 min at 4 °C. The flocculent precipitate was removed. TBARSs in the resultant supernatant were determined by the Benchmark Plus™ Microplate Spectrophotometer (Bio-Rad Laboratories, Tokyo, Japan) at 532 nm using the extinction coefficient of 1.5×10^5 $M^{-1}cm^{-1}$. The TBARSs were compared with their control and they were expressed as $\mu mol \cdot g^{-1}$ of brain tissue protein.

8.4 Statistical analysis

All determinations were carried out in triplicate and the experimental data were presented as mean ± S.E.M. The significant differences between treatment levels were statistically analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test (SigmaStat version 3.5). *P* value less than 0.05 ($P<0.05$) was recognized statistically significant. All graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

8.5 Result

The effects of the α-mangostin (α-MG) purified from the fruit rind of *G. mangostana* and donepezil on lipid peroxidation were examined in normal saline treated rats and scopolamine (SCOP)-induced amnesic rats using colorimetric method (Filippova *et al*., 2004) with minor modification. The results showed that no significant difference was found between groups in NSS treatment in all studied brain regions. SCOP significantly elevated malondialdehyde (MDA) level in all brain regions, compared to their respective NSS groups ($P<0.05$) as shown in Figure 8.1, 8.2, and 8.3. Pretreatment of donepezil and α-MG (50 $mg \cdot mL^{-1} \cdot kg^{-1}$), but not α-MG (100 $mg \cdot mL^{-1} \cdot kg^{-1}$), significantly attenuated the increase of MDA level induced by SCOP in cerebral cortex and hippocampus ($P<0.05$) as shown in Figure 8.1 and 8.2. In basal forebrain, all pretreatments had no effect on the increase of MDA level induced by SCOP (Figure 8.3).

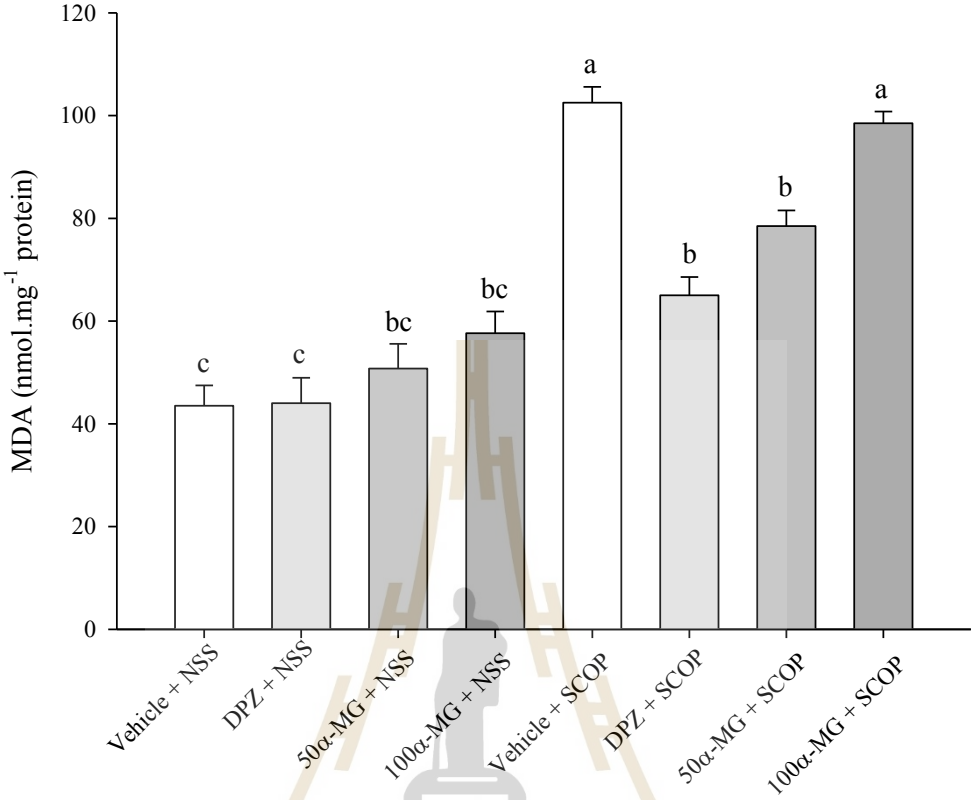

Figure 8.1 Inhibitory effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on LPO in the cerebral cortex of NSS-treated rats and SCOP-induced amnesic rats. LPO inhibitory activity was indicated by malondialdehyde (MDA) level, measured by the thiobarbituric acid reactive substance (TBARS) method. The results were expressed as mean ± S.E.M. (n = 8). Different alphabets indicate significant differences, P<0.05. Lipid peroxidation, LPO; DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

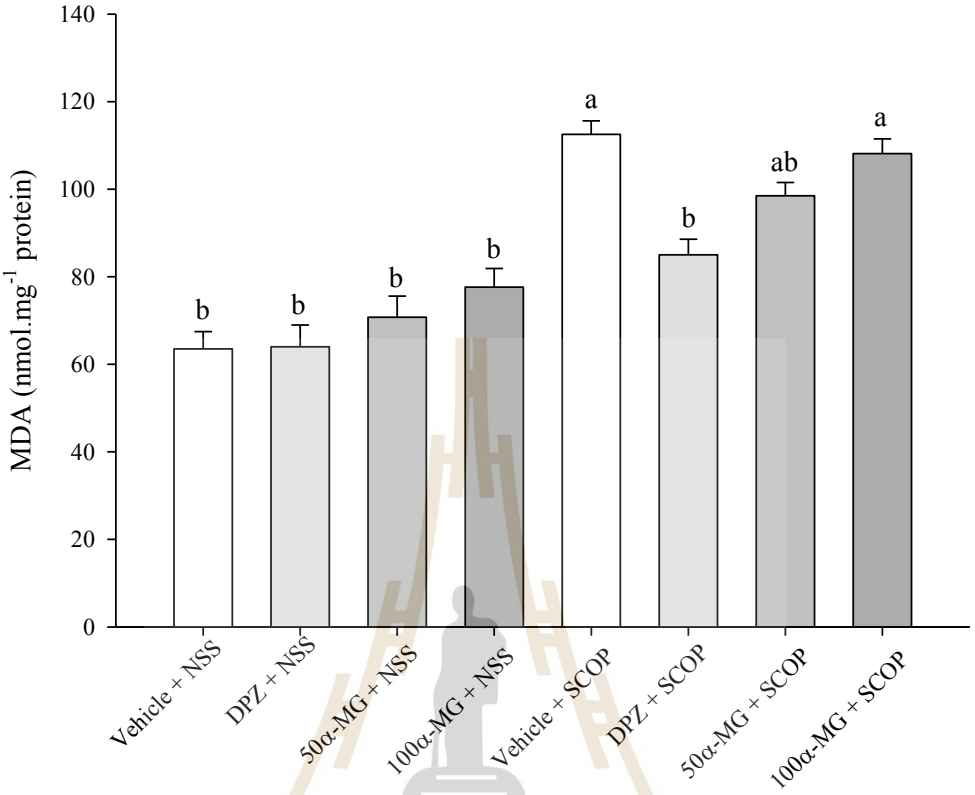

Figure 8.2 Inhibitory effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on LPO in the hippocampus of NSS-treated rats and SCOP-induced amnesic rats. LPO inhibitory activity was indicated by malondialdehyde (MDA) level, measured by the thiobarbituric acid reactive substance (TBARS) method. The results were expressed as mean ± S.E.M. (n = 8). Different alphabets indicate significant differences, P<0.05. Lipid peroxidation, LPO; DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

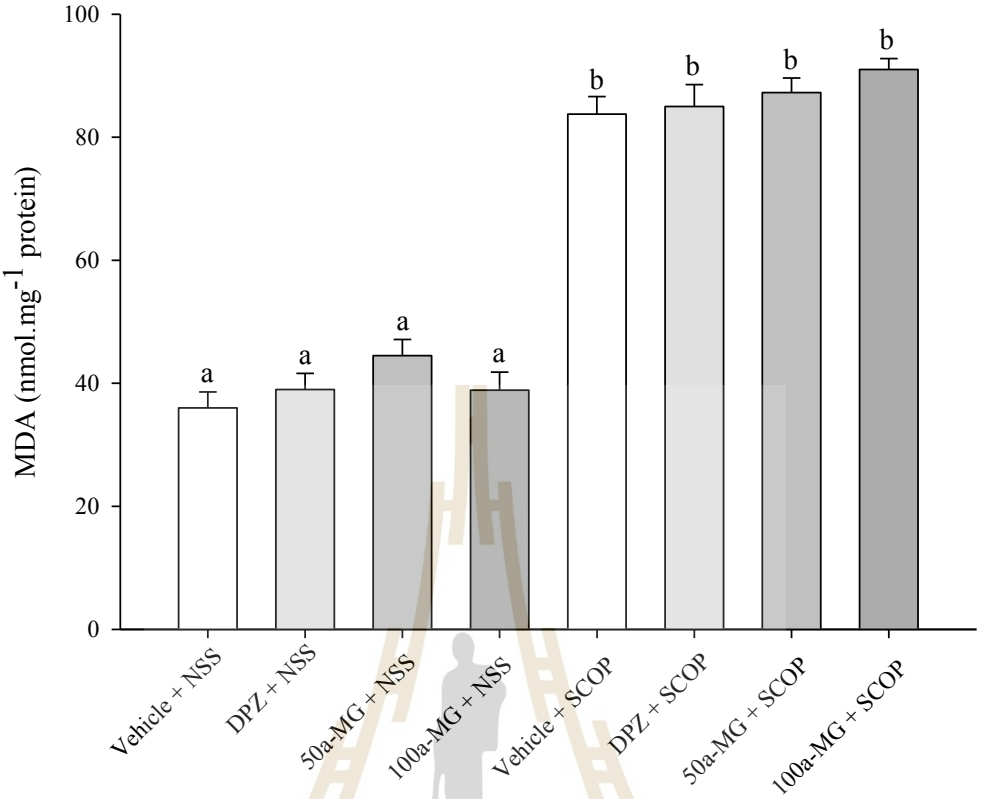

Figure 8.3 Inhibitory effect of α-MG purified from the fruit rind of *G. mangostana* and DPZ on LPO in the basal forebrain of NSS-treated rats and SCOP-induced amnesic rats. LPO inhibitory activity was indicated by malondialdehyde (MDA) level, measured by the thiobarbituric acid reactive substance (TBARS) method. The results were expressed as mean ± S.E.M. (n = 8). Different alphabets indicate significant differences, P<0.05. Lipid peroxidation, LPO; DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

8.6 Discussion and conclusion

AD is a severe neurodegenerative disease and a leading cause of death among the elderly. Although a number of factors have been identified, oxidative stress and cholinergic dysfunction have been implicated as major contributing factors in the pathogenesis of AD. Therefore, it has been suggested that the compounds, either plant or plant-derived molecules, that modulate cholinesterase activity and multiple components of the oxidative stress pathway would be an effective candidates for potential drugs that restrict the development of AD. *G. mangostana* has been reported to possess antioxidant, anti-tumor, anti-allergic, anti-inflammatory, anti-bacterial, anti-fungal, and anti-viral activities. Previous findings indicate that *G. mangostana* also possesses antioxidant activities. The antioxidant potential evaluation of α-mangostin by *in vivo* assay, using TBARS method for investigating malondialdehyde level as an indicator of lipid peroxidation. Extensive evidence indicates that the ROS, which are formed during oxidative stress, induce cellular and molecular abnormalities in sporadic AD. Radical cations are the ROS and are highly toxic; they greatly contribute to oxidative stress and are known contributors to neuronal damage in AD. The results revealed that α-mangostin possessed potential inhibitory activity against lipid peroxidation. Lipid peroxidation is the mechanism by which lipids are attacked by ROS to form a carbon radical that reacts with oxygen, resulting in a peroxyl radical and thus generating lipid peroxides. Due to the high lipid content and unusually high concentration of poly-unsaturated fatty acids that are particularly susceptible to oxidation, the brain is an important target of oxidative stress. The brains of individuals with AD have increased levels of lipid peroxidation products, such as 4-hydroxynonenal or 2-propenal, and enhanced lipid peroxidation has been detected in

the cerebrospinal fluid and plasma of individuals with AD. Lipid peroxidation products can be measured by using thiobarbituric acid. The results of an *in vivo* assay revealed that, induced by scopolamine, α-mangostin inhibited lipid peroxidation of rat brain. The antioxidant activities of α-mangostin were supported by the presence of a phenolic core structure. Polyphenols are the most abundant antioxidants in the plant kingdom, and it is claimed they have neuroprotective effect. The antioxidant activity of the polyphenolic compound is believed to result from their redox properties, which play an important role in adsorbing and neutralizing free radicals. Polyphenols are the most ubiquitous groups of plant secondary metabolites and have good antioxidant potential. The mechanism of action of polyphenols in plant-derived natural compounds is through the scavenging activities of free radicals generating in turn induce the lipid peroxidation, production of free carbonyls, formation of advanced glycation end products (AGEs), and DNA damage, ultimately resulting in neurodegeneration. Numerous polyphenolic compounds have been isolated from plants that reduce oxidative stress. Our results indicate that α-mangostin is a plant-derived natural compound, which may exhibit inhibitory activity against lipid peroxidation (Figure 8.4). In conclusion, the study found that α-mangostin, a major xanthone derivative in *Garcinia mangostana* (Linn.) rind, markedly inhibits multiple components of the oxidative stress pathway such as lipid peroxidation that can contribute to AD. As a result, α-mangostin might have the potential to be an effective and safe treatment for neurodegenerative disease.

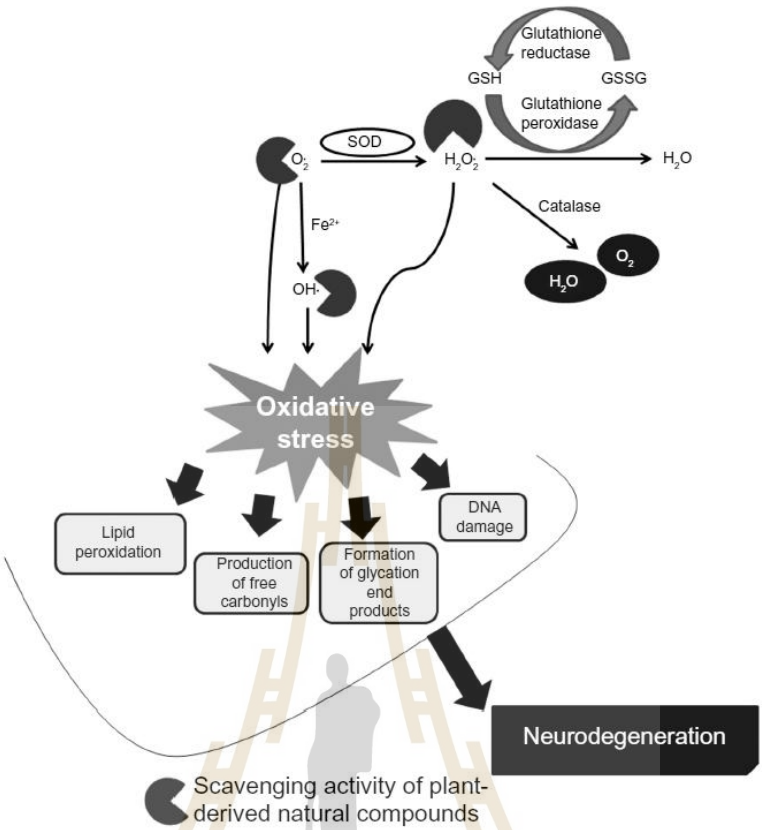

Figure 8.4. Role of plant-derived natural compounds in neurodegeneration.

CHAPTER IX

EFFECTS OF α-MANGOSTIN ON FOS EXPRESSION IN THE CHOLINERGIC NEURONS OF THE BRAIN AREAS REGULATING LEARNING AND MEMORY IN SCOPOLAMINE-INDUCED AMNESIC RATS

9.1 Abstract

There is no evidence to suggest neuroprotective effects of α-mangostin and donepezil against learning and memory impairment through central effects, especially for the cerebral cortex and hippocampus. The cerebral cortex and hippocampus are major brain regions that play an importance role in regulation of learning and memory *via* central cholinergic system of acetylcholine synthesis and cleavage. Hence, this study investigated the effects of α-mangostin and donepezil on Fos expression of cholinergic neurons in the brain areas involving a regulation of learning and memory which are the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHip) potent brain areas in the cerebral cortex and the hippocampus, respectively. Sixty-four rats were divided into eight groups of eight rats. Rats were given repeated dose for constitutive 7 days of α-mangostin (50 or 100 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.), donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.), or 2% Tween 80 (1 $mL \cdot kg^{-1}$). An hour later, animals were treated with scopolamine (5 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.). Six hours later, brains were removed and cerebral

cortex and hippocampus were assessed for expression of Fos in cholinergic neurons, a marker for neuronal activation, using immunohistochemistry for Fos+/ChAT+.

The present findings demonstrated the first evidence that α-mangostin (50 $mg \cdot mL^{-1}$) and donepezil could induce Fos expression of cholinergic neurons in the dHip of NSS-treated cerebral cortex but not in mPFC of NSS-treated hippocampus when compared to the vehicle control ($P<0.05$). In scopolamine-induced amnesic rats, donepezil, but not α-mangostin could significantly induced Fos expression of cholinergic neurons in mPFC and dHip when compared to the vehicle control ($P<0.05$). α-Mangostin (50 $mg \cdot mL^{-1}$) and donepezil may involve in regulation of learning and memory through neuronal activation of cholinergic neurons in the mPFC of cerebral cortex and dHip of hippocampus in both NSS-treated rats and scopolamine-induced amnesic rats.

9.2 Introduction

Alzheimer's disease (AD) is an irreversible, progressive disorder in which brain cells (neurons) deteriorate, resulting in the loss of cognitive functions, primarily memory, judgment and reasoning, movement coordination and pattern recognition. In advanced stages of the disease, all memory and mental functioning may be lost. The condition predominantly affects the cerebral cortex and hippocampus, which lose mass and shrink (atrophy) as the disease advances (Perl, 2010). The cerebral cortex is an extremely convoluted and complicated structure associated with the "higher" functions of the mind—thought, reasoning, sensation, and motion. The hippocampus plays a crucial role in learning and in processing various forms of information as long-term memory. Damage to the hippocampus produces global amnesia (Hilverman *et*

al., 2017). Acetylcholine is necessary for cognitive function. The significant factor in AD is the greatly reduced presence of acetylcholine in the cerebral cortex which commonly found in the brains of elderly people, they appear in the cerebral cortex of AD patients. Surrounding the protein are fragments of deteriorating neurons, especially those that produce acetylcholine (ACh), a neurotransmitter essential for processing memory and learning. Certain association areas in the human brain retain elevated levels of aerobic glycolysis in adulthood related to cognitive functions such as the medial prefrontal cortex, which is associated with working memory, and the hippocampus. The first lesions characteristic of AD appear in poorly myelinated limbic neurons in system areas related to memory and learning, such as the hippocampus and the association cortex. In addition, subcortical neuron loss occurs in the nucleus basalis of Meynert and the locus coeruleus, impairing the cholinergic transmitter system in the neocortex (Hanyu *et al*., 2007; Teipel *et al*., 2011).

Understanding the neural mechanisms underlying the regulation of learning and memory by the central cholinergic system is becoming important in neurodegenerative research because the neuronal system originating from various cerebrocortical and hippocampal nuclei regulates learning and memory in rodents and highlight current understanding of cholinergic system in these processes (Deiana *et al*., 2011). Many cerebrocortical and hippocampal brain areas are important in the regulation of learning and memory, including the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHip) (Davidson *et al*., 2009; Euston *et al*., 2012; Preston *et al*., 2013).

The cerebral cortex and hippocampus receive cholinergic innervations mainly from the basal forebrain neurons. ACh released from these neurons affects the ability of brain cells to transmit information to one another, and also encourages plasticity, or

learning. Thus, damage to the basal forebrain can reduce the amount of acetylcholine in the brain and impair learning and memory. These cholinergic neurons project to the mPFC and dHip (Paul *et al*., 2015; Placzek *et al*., 2009). Scopolamine could decrease the number of cholinergic neurons producing ACh in the mPFC and dHip of the rat cerebral cortex and hippocampus, respectively. The effect of donepezil on Fos expression in rat cerebral cortex and hippocampus was demonstrated. Expression of the immediate early gene *c-fos* and its protein product Fos has been extensively used to map stimulus-evoked functional activity in the brain (Nikolaev *et al*., 2002). Therefore, Fos has been used as neuronal activation marker that can be identified by immunohistochemistry to be located in the nuclei of neurons (Bullitt, 1990). Administration of scopolamine increases the activity marker Fos in the mPFC, including the infralimbic (IL) and prelimbic (PrL) subregions (Navarria *et al*., 2015).

Mangosteen (*Garcinia mangostana* Linn.) is a medicinal plant which crude extract of its fruit rind consists of xanthonoids, such as α-mangostin, and other phytochemicals which has potential to prevent dementia (Nontamart *et al*., 2013). However, there is no evidence about effects of α-mangostin on the brain areas which involve learning and memory. Therefore, this study was focused on the effect of α-mangostin on Fos expression, a marker for neuronal activation, of the cholinergic neurons in the mPFC and dHip in SCOP-induced amnesic rats.

9.3 Material and methods

The methodology of chemicals, drug solutions, animals, and experimental design is described in Materials and methods 4.3.1, 4.3.2, 4.3.3, and 4.3.4, respectively.

9.3.1 Brain fixation

9.3.1.1 Chemical solutions

0.9% Normal saline solution

0.9% Normal saline solution was prepared by adding 9 g of sodium chloride (Sigma-Aldrich, St. Louis, MO, USA) to 900 mL of deionized distilled (DI) water. This solution was adjusted volume to 1000 mL with DI water in a volumetric flask.

Heparinised saline solution

Heparinised saline (5 $IU \cdot mL^{-1}$) solution was prepared by adding 1 mL of a 5000 $units \cdot mL^{-1}$ heparin solution (LEO Phamaceutical product, Ballerup, Denmark) to 999 mL of 0.9% normal saline solution.

1 M Phosphate buffer solution (pH 7.4)

1 M Phosphate buffer solution (pH 7.4) was prepared by adding 106.5 g of di-sodium hydrogen phosphate anhydrous (Na_2HPO_4; BDH Ltd., UK) and 39.68 g of sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4 \cdot H_2O$; BDH) to 800 mL of double deionized distilled (DDD) water. This solution was stirred on a hot plate magnetic stirrer (VELP Scientifica, Europe) at 50-60 ºC for 1 h, left to be cool down to room temperature and then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH. This solution was adjusted volume to 1000 mL with DDD water in a volumetric flask.

0.1 M Phosphate buffer solution (pH 7.4)

0.1 M Phosphate buffer solution (pH 7.4) was prepared by adding 100 mL of 1 M phosphate buffer solution (pH 7.4) to 900 mL of DI water. This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH. 5 M Sodium chloride solution 5 M Sodium chloride solution was prepared by adding 292.2 g of sodium chloride (NaCl;

Sigma) to 600 mL of DDD water. This solution was then adjusted volume to 1000 mL with DDD water in volumetric flask.

4% Paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4)

4% Paraformaldehyde solution was prepared by adding 40 g of paraformaldehyde (Acros Organics, New Jersey, USA) to 900 mL of 0.1 M phosphate buffer solution (pH 7.4). This solution was stirred on a hot plate magnetic stirrer (VELP Scientifica, Europe) at 150-200 °C for 1-2 h, left to be cool down to room temperature and then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH. This solution was adjusted volume to 1000 mL with DI water in a volumetric flask.

30% Sucrose in 4% PFA in 0.1 M phosphate buffer (pH 7.4)

30% Sucrose in 4% PFA in 0.1 M phosphate buffer solution was prepared by adding 30 g of sucrose ($C_{12}H_{22}O_{11}$; Ajax Finechem Pty Ltd., Australia) to 60 mL of 4% PFA in 0.1 M phosphate buffer. This solution was then adjusted volume to 100 mL with 4% PFA in 0.1 M phosphate buffer in volumetric flask (pH 7.4). This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

30% Sucrose in 0.1 M phosphate buffer (pH 7.4)

30% Sucrose in 0.1 M phosphate buffer solution was prepared by adding 30 g of sucrose to 60 mL of 0.1 M phosphate buffer. This solution was then adjusted volume to 100 mL with 0.1 M phosphate buffer in volumetric flask (pH 7.4). This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

9.3.1.2 Procedure

After all treatments, all animals were anesthetized with pentobarbital sodium (Nembutal, Ceva Sante Amimale, Libourne, France) at a dose of 60 mg/kg (i.p.). The rats

were perfused through the left ventricle of the heart with 225 ± 25 mL of ice-cold heparinized saline solution (0.9% NaCl) at a flow rate of 40 mL·min^{-1} using peristaltic pump (model SP 311, VELP Scientifica, Europe). Immediately after starting the pump, the right atrium was cut to allow an escape route for the blood and perfusion fluid. After the atrium effluent was clear, the rats were perfused with 325 ± 25 mL of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at a flow rate of 40 mL·min^{-1} to fix the brain. After that, the brain were removed and soaked in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. Brains were then transferred into 30% sucrose in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight or until the brain sank at 4 °C. The brains were then soaked in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 °C for further 24 h. After that, the brains were covered with powdered dry ice until frozen and then stored at -20 °C until cryosectioned.

9.3.2 Brain cryosectioning

9.3.2.1 Chemical solutions

Anti-freeze cryoprotectant solution

Anti-freeze cryoprotectant solution was prepared by mixing 30 mL of 0.1 M phosphate buffer (pH 7.4), 30 g of sucrose, and 30 mL of ethanediol ($C_2H_6O_2$; Ajax Finechem) and then 1 g of polyvinylpyrrolidone (C_6H_9NO, PVP-40; Sigma) was added. This solution was adjusted volume to 100 mL with 0.1 M phosphate buffer (pH 7.4) water in volumetric flask. This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

9.3.2.2 Procedure

The brains regions of the dorsal hippocampus (dHip) in hippocampus (Figure 9.1) and the medial prefrontal cortex (mPFC) in cerebral cortex (Figure 9.2) were identified according to the rat brain stereotaxic atlas of Paxinos and Watson (2009). Frozen brains were coronally sectioned (30 μm thickness) through the levels of the mPFC (3.20 to 2.20 mm from bregma) and dHip (-2.30 to -3.80 mm from bregma) using a cryostat (Microm HM 525, Microm International GmbH., Germany). Cryosections were then floated in an anti-freeze cryoprotectant solution at -20 °C until further use.

Figure 9.1 Abbreviations of the subregions of the mPFC and dHip involving learning and memory. Nomenclature and abbreviations are from stereotaxic atlas of the rat brain (Paxinos and Watson, 2009).

Abbreviation		Full name
mPFC	=	Medial prefrontal cortex
Cg1	=	Cingulate cortex, area 1
IL	=	Infralimbic cortex
PrL	=	Prelimbic cortex
dHip	=	Dorsal hippocampus
CA1	=	Field CA1 of hippocampus
CA3	=	Field CA3 of hippocampus
DG	=	Dentate gyrus

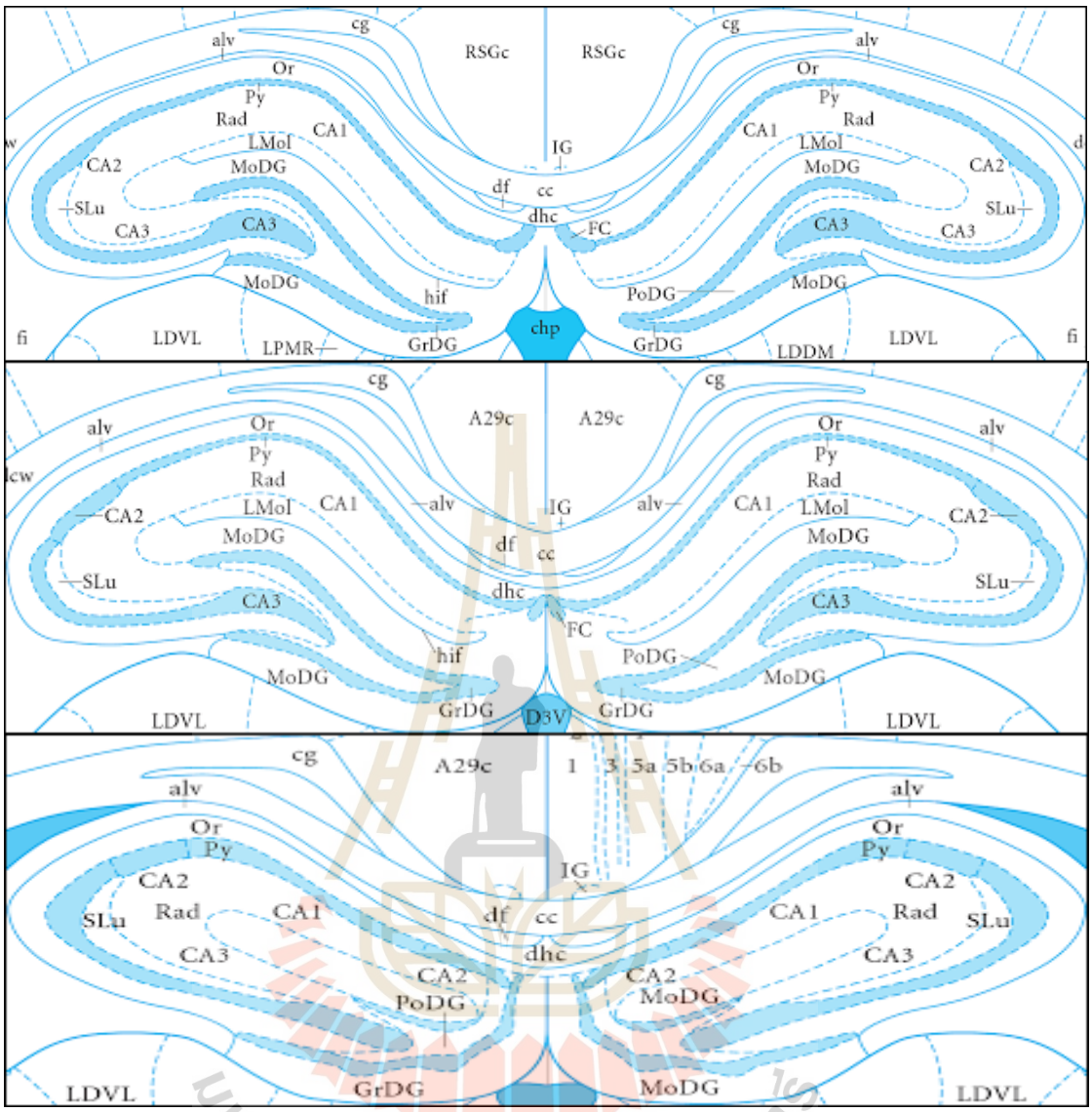

Figure 9.2 Schematic diagrams of coronal sections illustrating the brains regions of the dorsal hippocampus (CA1, CA3, and DG). The dHip (-1.92 to -3.24 mm from bregma). Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Figure 9.1.

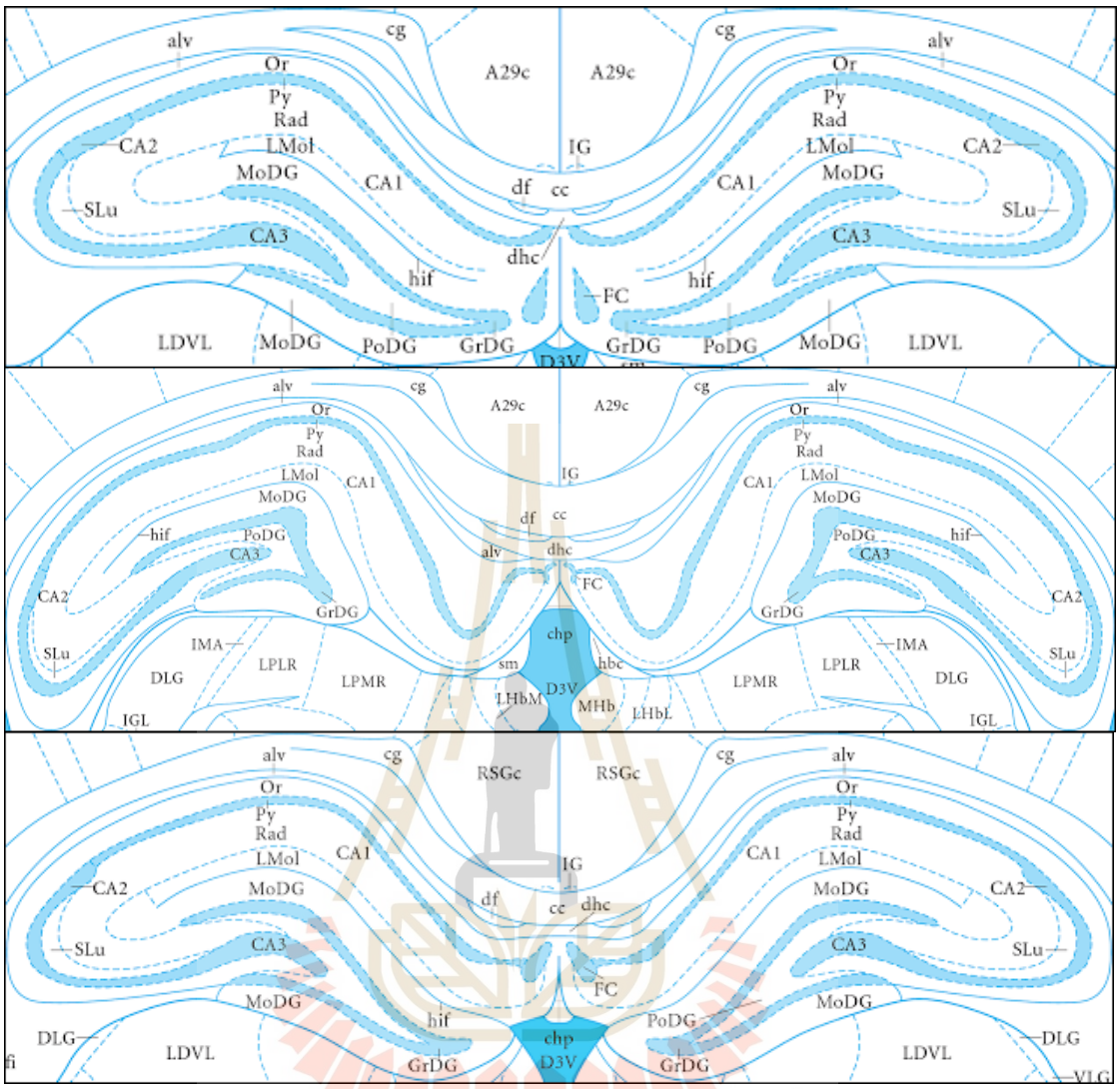

Figure 9.2 Schematic diagrams of coronal sections illustrating the brains regions of the dorsal hippocampus (CA1, CA3, and DG). The dHip (-1.92 to -3.24 mm from bregma). Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Figure 9.1.

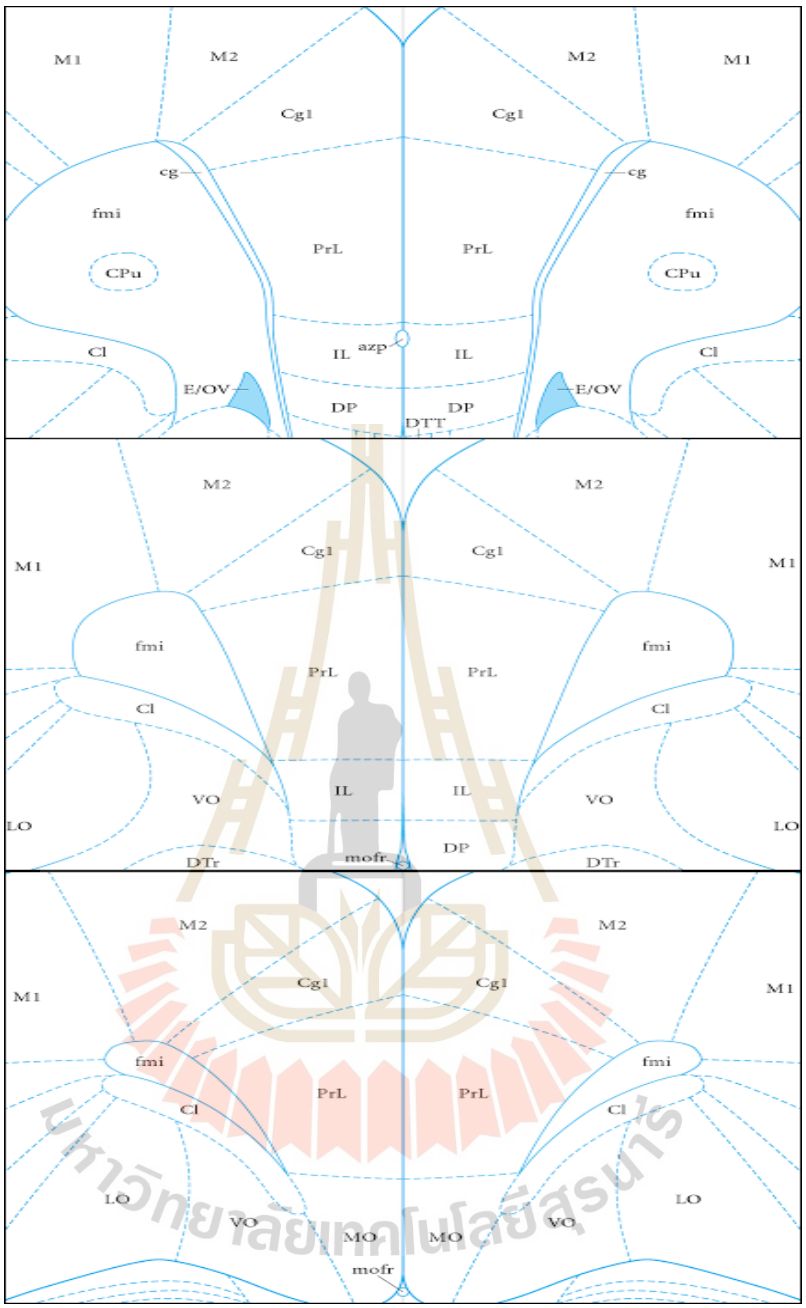

Figure 9.3 Schematic diagrams of coronal sections illustrating the brains subregions of the medial prefrontal cortex (Cg1, IL, and PrL). The mPFC (4.20 to 2.52 mm from bregma). Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Figure 9.1.

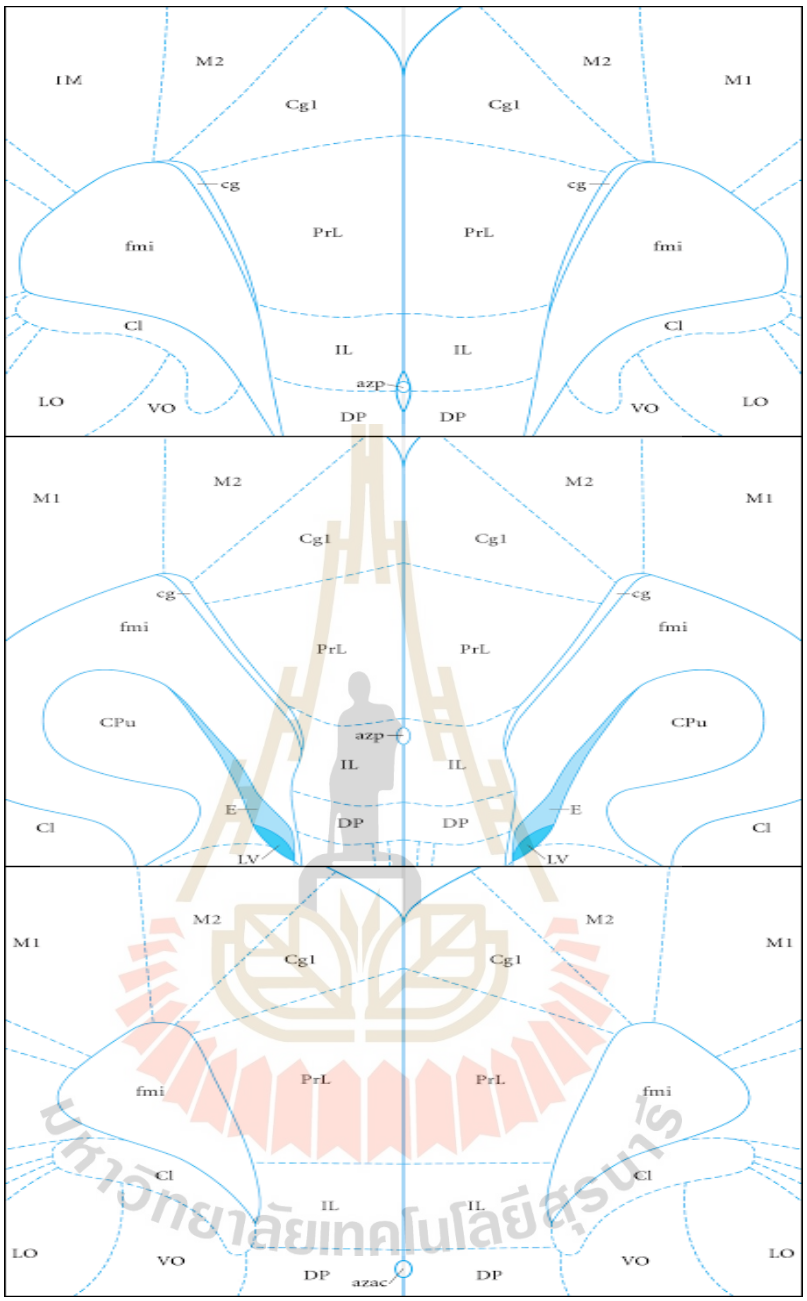

Figure 9.3 Schematic diagrams of coronal sections illustrating the brains subregions of the medial prefrontal cortex (Cg1, IL, and PrL). The mPFC (4.20 to 2.52 mm from bregma). Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Figure 9.1.

9.3.3 Fos immunohistochemistry

9.3.3.1 Chemical solution

1 M Phosphate buffer solution (pH 7.4)

1 M Phosphate buffer solution (pH 7.4) was prepared by adding 106.5 g of di-sodium hydrogen phosphate anhydrous (Na_2HPO_4; BDH Ltd., UK) and 39.68 g of sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4 \cdot H_2O$; BDH) to 800 mL of double deionized distilled (DDD) water. This solution was stirred on a hot plate magnetic stirrer (VELP Scientifica, Europe) at 50-60 °C for 1 hour, left to be cool down to room temperature and then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH. This solution was adjusted volume to 1,000 mL with DDD water in a volumetric flask.

0.1 M Phosphate buffer solution (pH 7.4)

0.1 M Phosphate buffer solution (pH 7.4) was prepared by adding 100 mL of 1 M phosphate buffer solution (pH 7.4) to 900 mL of DI water. This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH. 5 M Sodium chloride solution 5 M Sodium chloride solution was prepared by adding 292.2 g of sodium chloride (NaCl; Sigma) to 600 mL of DDD water. This solution was then adjusted volume to 1000 mL with DDD water in volumetric flask.

5 M Sodium chloride solution

5 M Sodium chloride solution was prepared by adding 292.2 g of sodium chloride (NaCl; Sigma-Aldrich) to 600 mL of DDD water. This solution was then adjusted volume to 1000 mL with DDD water in volumetric flask.

0.1 M Phosphate buffer saline (PBS) solution (pH 7.4)

0.1 M Phosphate buffered saline solution was prepared by adding 30 mL of 5 M sodium chloride to 970 mL of 0.1 M phosphate buffer (pH 7.4). This solution was

then adjusted volume to 1000 mL with DDD water in volumetric flask. This solution was then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

0.1 M Washing solution (PBS-T) solution

0.1 M washing solution was prepared by adding 3 mL of 0.3% v/v Triton X-100 (Panreac Analytical Reagent and Fine Chemical, Spain) to 95 mL of 0.1 M phosphate buffer saline (pH 7.4). This solution was adjusted volume to 100 mL with 0.1 M phosphate buffer saline (pH 7.4) water in volumetric flask. This solution was then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

3% Hydrogen peroxide solution

3% Hydrogen peroxide solution was prepared by 79 mL of 0.1 M phosphate buffer saline (pH 7.4) adding 1 mL of hydrogen peroxide (H_2O_2; Merck Schuchardt OHG., Hohenbrunn, Germany) and 20 mL of methanol (CH_4O; BDH). This solution was prepared immediately prior to use.

Preincubation buffer

Preincubation buffer was a prepared by adding 5 mL of normal goat serum (Millipore Corperation, USA) to 95 mL of washing solution (0.1 M PBS-T).

Primary antibody

Rabbit polyclonal anti-Fos and anti-ChAT (Calbiochem; USA) were diluted 1:5,000 in preincubation buffer.

Secondary antibody

Secondary antibody was a washing solution containing 1% v/v biotinylated anti-rabbit immunoglobulin and 3% v/v normal goat serum (Vectastain Elite ABC Kit rabbit IgG's, Vector Laboratories, Burlingame, USA).

Avidin-biotinylated horseradish peroxidase complex (ABC)

ABC solution was a washing solution containing 2% v/v Avidin and 2% v/v biotinylated horseradish peroxidase (Vectastain Elite ABC Kit rabbit IgG's). This solution was left to incubate for at least 30 minutes prior to use.

0.1 M Acetate buffer (stop solution)

0.1 M Acetate buffer was prepared by adding 0.82 g of sodium acetate (CH_3COONa; BDH Ltd, UK) to 100 mL of DDD water. This solution was then adjusted to pH 6.0 with acetic acid.

DAB-H_2O_2 Solution

DAB-H_2O_2 solution was prepared by mixing 98 mL of 0.1 M of PBS solution (pH 7.4), 2 mL of diaminobenzidine (DAB) (3,3´-diaminobenzidine tetrahydrochloride hydrate, $C_{12}H_{14}N_4 \cdot 4HCl \cdot H_2O$; Sigma), and 50 µl of hydrogen peroxide (H_2O_2; Merck). This solution was then filtered through No.1 Whatman filter paper (Whatman) prior to use.

Ni-DAB-H_2O_2 Solution

Ni-DAB-H_2O_2 solution was prepared by mixing 98 mL of 0.1 M of PBS solution (pH 7.4), 0.05 g of ammonium nickel (II) sulfate ($NiN_2H_8S_2O_8 \cdot 6H_2O$; Sigma), 2 mL of diaminobenzidine (DAB) (3,3´-Diaminobenzidine tetrahydrochloride hydrate, $C_{12}H_{14}N_4.4HCl \cdot H_2O$; Sigma), and 50 µl of hydrogen peroxide (H_2O_2; Merck). This solution was then filtered through No.1 Whatman filter paper (Whatman) prior to use.

Chrome alum gelatin

Chrome alum gelatin solution was prepared by adding 1.3 g of gelatine pellets (Ajax, Australia) and 0.1 g of chromium (II) potassium sulfate dodecahydrate ($CrKO_8S_2.12H_2O$; Fluka) to 1000 mL of DI water.

9.3.3.2 Procedure

Free-floating sections were double labeling for Fos and ChAT according to the avidin-biotin complex (ABC) methods.

Day 1

Sections were washed three times (×10 min) with 0.1 M phosphate buffer (pH 7.4), fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, washed three times (×10 min) with 0.1 M phosphate buffer (pH 7.4) and then washed six times (×15 min) with washing solution. Endogenous peroxidase was then deactivated with 3% hydrogen peroxide solution for 15 min, washed three times (×10 min) with washing solution and then blocked background staining with preincubation buffer for 30 min. After that, the sections were incubated with anti-Fos primary antibody (1: 5,000 diluted in preincubation buffer) for 48 h in 500 µL labelled plastic bottles (Sterilin®, Sterilin Ltd., Aberbargoed, UK) at 4 °C.

Day 3

Sections were then washed eight times (×5 min) with washing solution, incubated in secondary antibody solution for 1 h at room temperature and then washed three times (×10 min) with washing solution. After that, the sections were incubated in ABC complex solution for 1 h at room temperature and then washed two times (×10 min) with washing solution. The sections were rinsed with 0.1 M acetate buffer for 5 min and then incubated with Ni-DAB-H_2O_2 solution for approximately 10 min. The reaction was stopped by stop solution for 5 min. The sections were then rinsed three times (×5 min) with 0.1 M phosphate buffer (pH 7.4) and then washed three times (×15 min) with washing solution. Endogenous peroxidase was then deactivated with 3% hydrogen peroxide solution for 15 min, washed three times (×10 min) with

washing solution and then blocked background staining with preincubation buffer for 30 min. After that, the sections were incubated with anti-ChAT primary antibody (1: 5,000 diluted in preincubation buffer) for 48 h in 500 µL labeled plastic bottles (Sterilin®, Sterilin Ltd., Aberbargoed, UK) at 4 °C.

Day 5

Sections were then washed eight times (×5 min) with washing solution, incubated in secondary antibody solution for 1 h at room temperature and then washed three times (×10 min) with washing solution. After that, the sections were incubated in ABC complex solution for 1 h at room temperature and then washed two times (×10 min) with washing solution. The sections were rinsed with 0.1 M acetate buffer for 5 min and then incubated with DAB-H_2O_2 solution for approximately 10 min. The reaction was stopped by stop solution for 5 min. The sections were rinsed once for 5 min with 0.1 M phosphate buffer saline (pH 7.4) and then rinsed three times (×5 min) with 0.1 M phosphate buffer (pH 7.4). Sections were mounted onto the chrome alum gelatin subbed slides. Slices were then dried at 45 °C on the slide warmer (Medex Nagel GmbH., Germany). After that, the sections were dehydrated with serial dilution alcohol (70%, 90%, 95%, 100%, and 100%, 5 min each) followed by xylene (2×5 min). Slides were finally coverslipped using DPX mountant (BDH). This method was performed with gentle agitation on an orbital shaker VRN-360 (Gemmy Industrial Corp., Taiwan).

9.3.3.3 Quantitative analysis

Quantitative assessment of Fos-positive, ChAT-positive, and double-stained neurons was achieved by counting the number of Fos+/ChAT+ neurons in the mPFC (Cg1, PrL, and IL) and dHip (CA1, CA3, and DG) areas. These areas were counted

the number of Fos+/ChAT+ neurons in 3 fields/3 sections/rat. Cells with distinct black nuclear Fos staining, brown cytoplasmic ChAT staining, and black nucleus with brown cytoplasm double staining for both Fos and ChAT in the mPFC (Cg1, PrL, and IL) and dHip (CA1, CA3, and DG) areas were manually counted under light microscopy (×10 objective, Nikon ECLIPSE 80i, Nikon Corporation Ltd., Japan) and images were captured and stored by DP72 software (Olympus, Tokyo, Japan). Number of cells for each sampled area was transformed to cells per square millimeter (mm^2).

9.4 Statistical analysis

All determinations were carried out in triplicate and the experimental data were presented as mean ± S.E.M. The significant differences between treatment levels were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test (SigmaStat version 3.5). *P* value less than 0.05 ($P<0.05$) was recognized statistically significant. All graphical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

9.5 Results

The present study demonstrated for the first time that the α-mangostin purified from the fruit rind of *G. mangostana* could activate neuron in the brain areas regulating learning and memory in similar manner to donepezil. Scopolamine could not increase the number of cholinergic neurons and the number of Fos expression of cholinergic neurons in dorsal hippocampus (dHip) (Figure 9.7 and 9.8) and medial prefrontal cortex (mPFC) when compared to the control ($P<0.05$) (Figure 9.13 and 9.14). The α-mangostin (50 $mg \cdot mL^{-1} \cdot kg^{-1}$) and donepezil could induced Fos

expression of cholinergic neurons of dHip in NSS-treated rats, but not significantly different with the α-mangostin (100 $mg \cdot mL^{-1} \cdot kg^{-1}$) when compared to their control (P<0.05). Donepezil could increased the number and ratio of Fos expression in cholinergic neurons of dHip in scopolamine-induced amnesic rats, but there were no significant differences in 50 and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ α-mangostin when compared to their control (P<0.05) as shown in Figure 9.8 and 9.9, respectively. There was no significant difference in the number and ratio of Fos expression in cholinergic neurons of mPFC in NSS-treated and scopolamine-induced amnesic rats treated with the α-mangostin (50 and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$) and donepezil, respectively, when compared to their control (P<0.05) as shown in Figure 9.14 and 9.15, respectively.

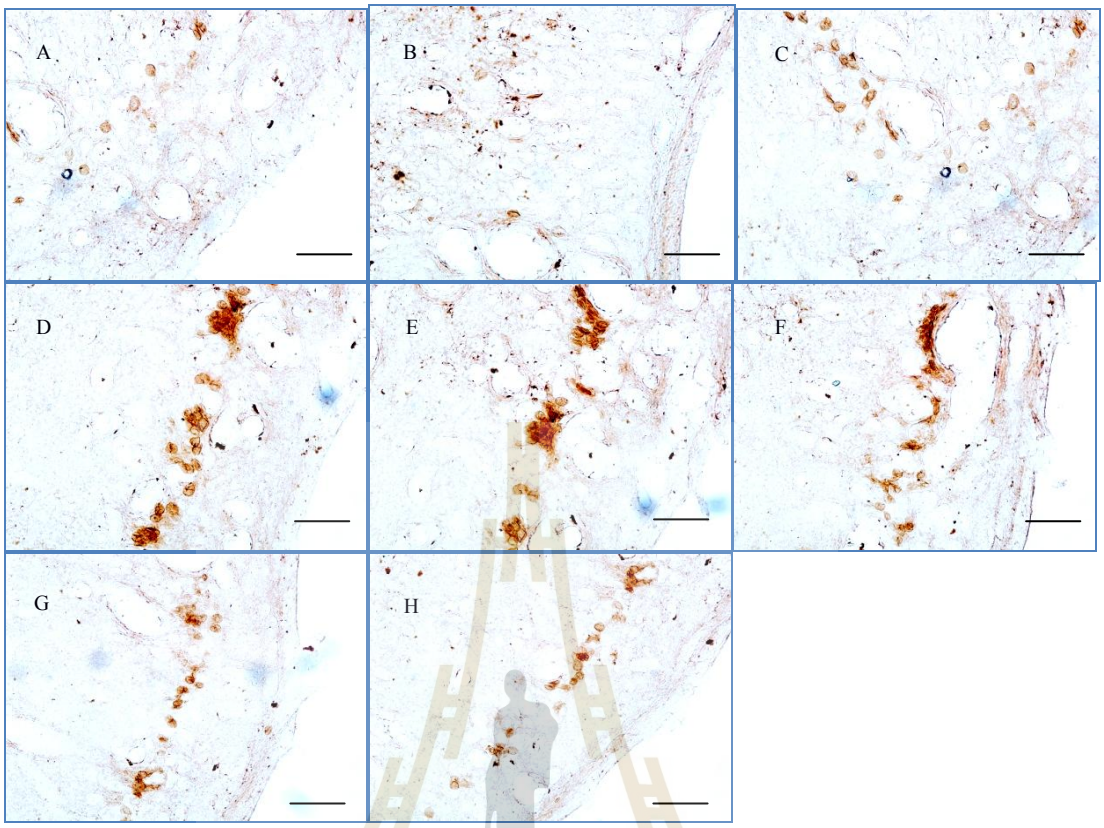

Figure 9.4 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on Fos expression of cholinergic neurons in the CA1 of dHip in NSS-treated rats and SCOP-induced amnesic rats. Photomicrographs of sections dual-immunostained for Fos (black, Ni-DAB) and ChAT (brown, DAB). Fos expression of cholinergic neurons in the dHip after administration of vehicle + NSS (A), DPZ + NSS (B), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (C), 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (D), vehicle + SCOP (E), DPZ + SCOP (F), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (G), and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (H). The results were expressed as mean ± S.E.M. (n = 8). Scale bar = 20 μm. For abbreviations, see Figure 9.1.

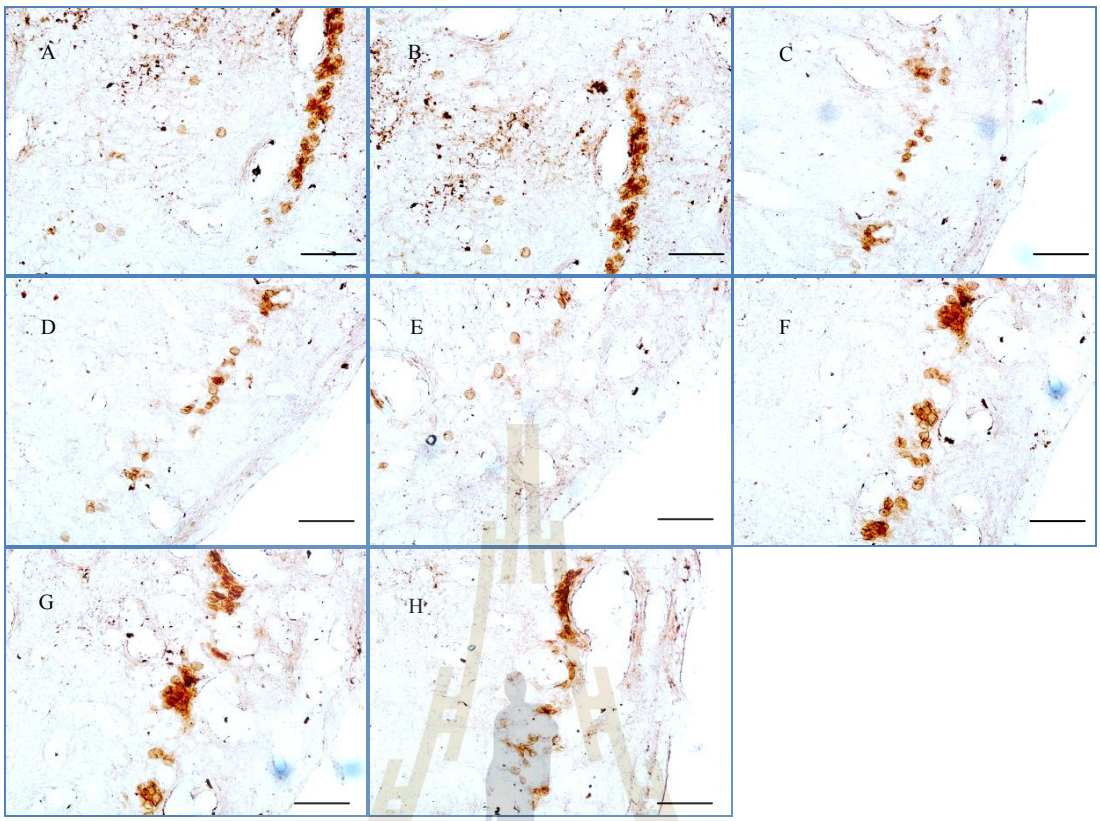

Figure 9.5 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on Fos expression of cholinergic neurons in the CA3 of dHip in NSS-treated rats and SCOP-induced amnesic rats. Photomicrographs of sections dual-immunostained for Fos (black, Ni-DAB) and ChAT (brown, DAB). Fos expression of cholinergic neurons in the dHip after administration of vehicle + NSS (A), DPZ + NSS (B), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (C), 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (D), vehicle + SCOP (E), DPZ + SCOP (F), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (G), and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (H). The results were expressed as mean ± S.E.M. (n = 8). Scale bar = 20 μm. For abbreviations, see Figure 9.1.

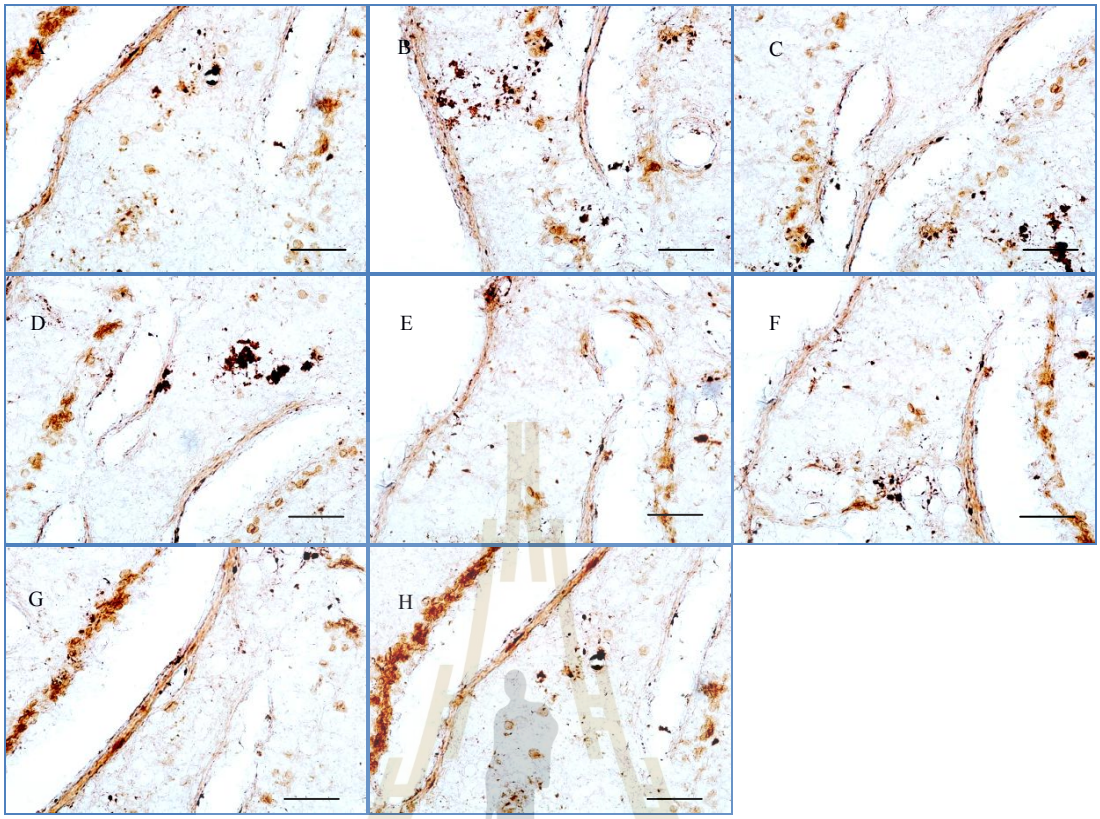

Figure 9.6 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on Fos expression of cholinergic neurons in the DG of dHip in NSS-treated rats and SCOP-induced amnesic rats. Photomicrographs of sections dual-immunostained for Fos (black, Ni-DAB) and ChAT (brown, DAB). Fos expression of cholinergic neurons in the dHip after administration of vehicle + NSS (A), DPZ + NSS (B), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (C), 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (D), vehicle + SCOP (E), DPZ + SCOP (F), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (G), and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (H). The results were expressed as mean ± S.E.M. (n = 8). Scale bar = 20 μm. For abbreviations, see Figure 9.1.

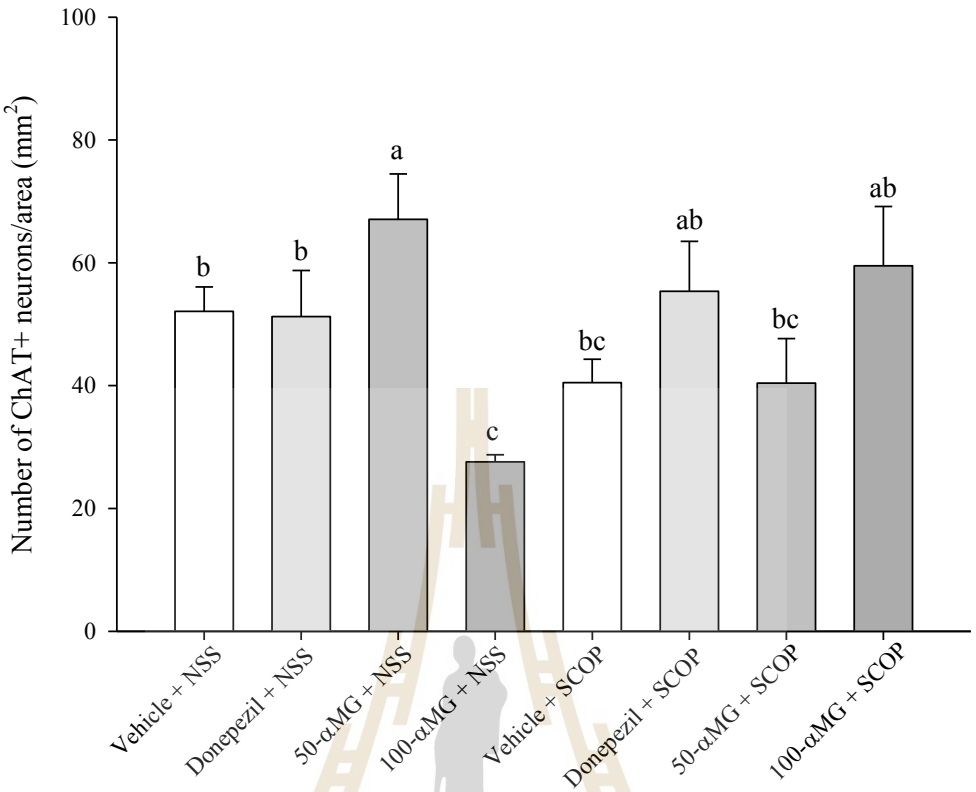

Figure 9.7 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on the number of ChAT+ neurons per area of the dHip ranging from -1.92 to -3.24 mm from bregma in NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as mean ± S.E.M. of number of cells·area^{-1} (mm^2) in the dHip. Different alphabets indicate significant differences, P<0.05. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

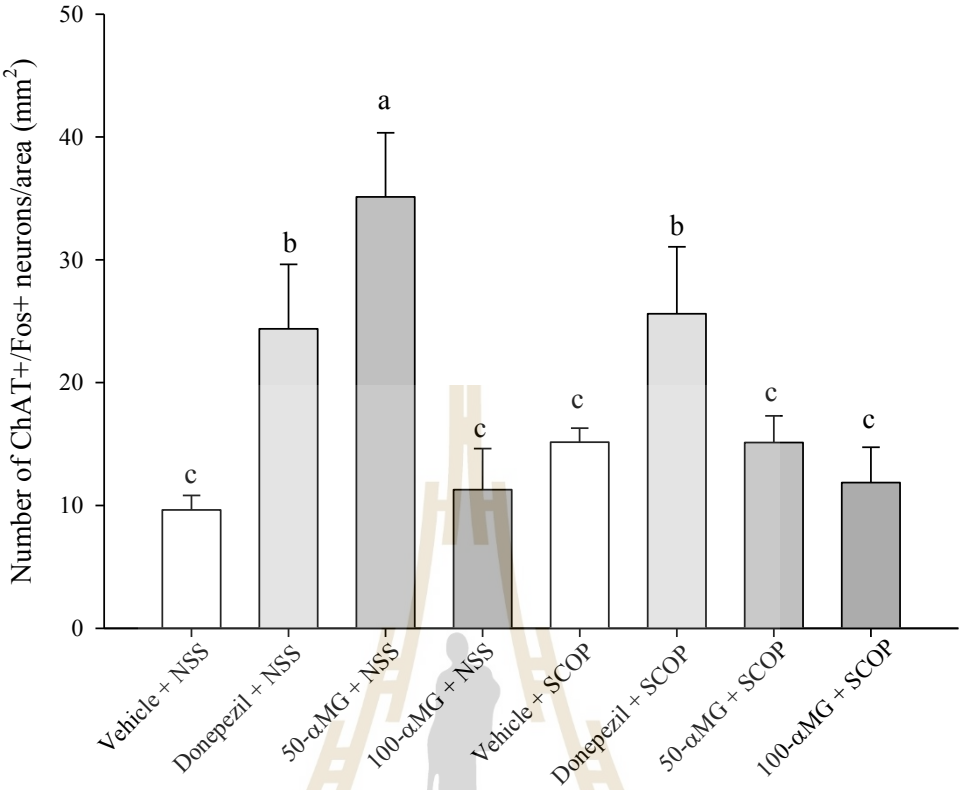

Figure 9.8 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on the number of ChAT+/Fos+ neurons per area of the dHip ranging from -1.92 to -3.24 mm from bregma in NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as mean ± S.E.M. of number of cells·area^{-1} (mm^2) in the dHip. Different alphabets indicate significant differences, P<0.05. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

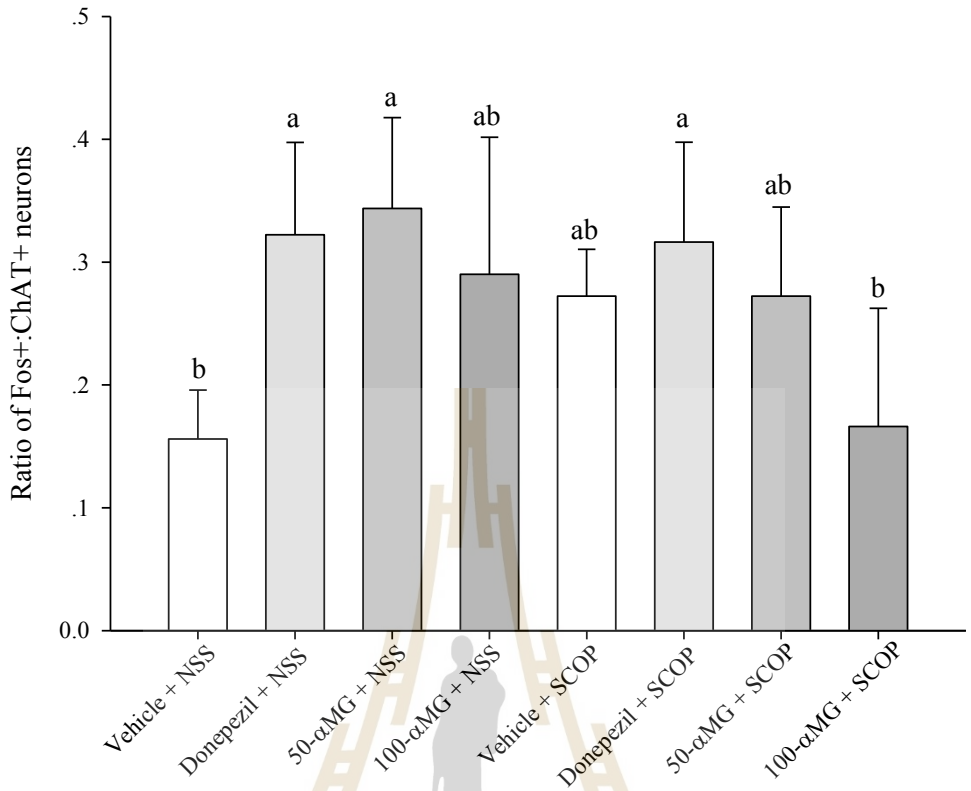

Figure 9.9 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on the ratio of Fos+:ChAT+ neurons of the dHip ranging from -1.92 to -3.24 mm from bregma in NSS-treated rats and SCOP-induced amnesic rats. Values are expressed as mean ± S.E.M. of number of cells·area^{-1} (mm^2) in the dHip. Different alphabets indicate significant differences, P<0.05. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

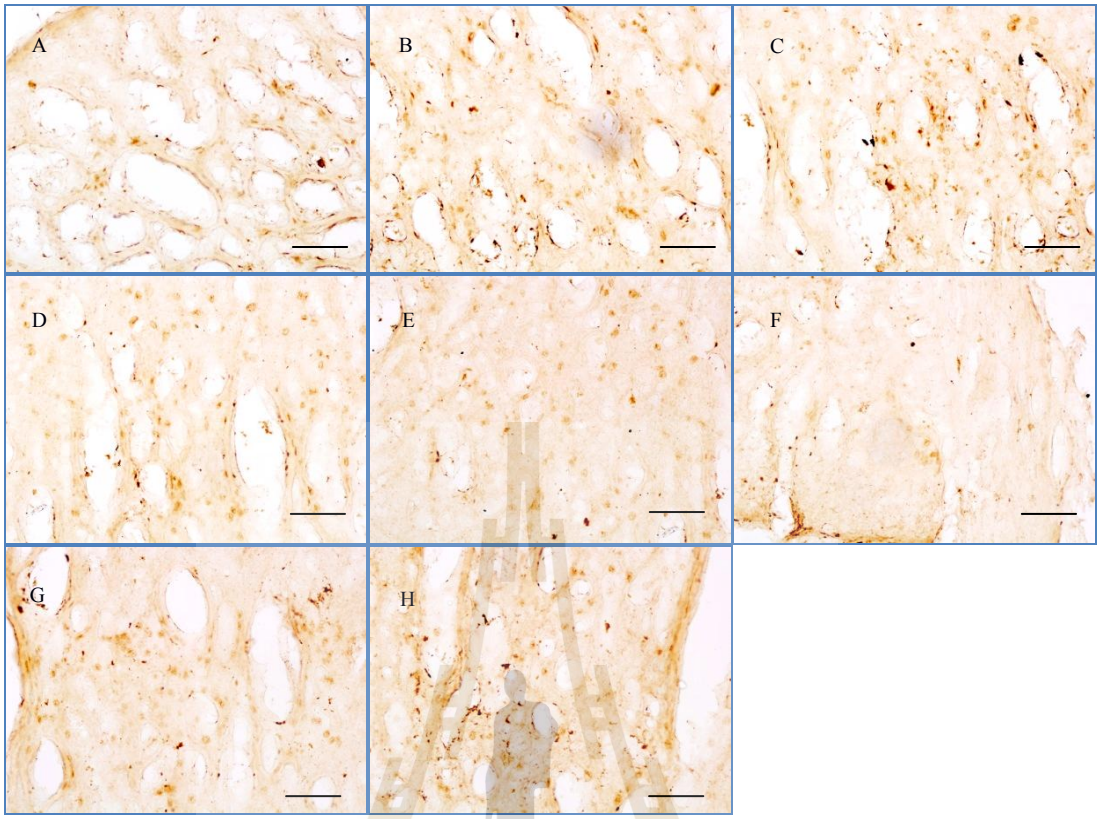

Figure 9.10 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on Fos expression of cholinergic neurons in the Cg1 of mPFC in NSS-treated rats and SCOP-induced amnesic rats. Photomicrographs of sections dual-immunostained for Fos (black, Ni-DAB) and ChAT (brown, DAB). Fos expression of cholinergic neurons in the mPFC after administration of vehicle + NSS (A), DPZ + NSS (B), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (C), 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (D), vehicle + SCOP (E), DPZ + SCOP (F), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (G), and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (H). The results were expressed as mean ± S.E.M. (n = 8). Scale bar = 20 μm. For abbreviations, see Figure 9.1.

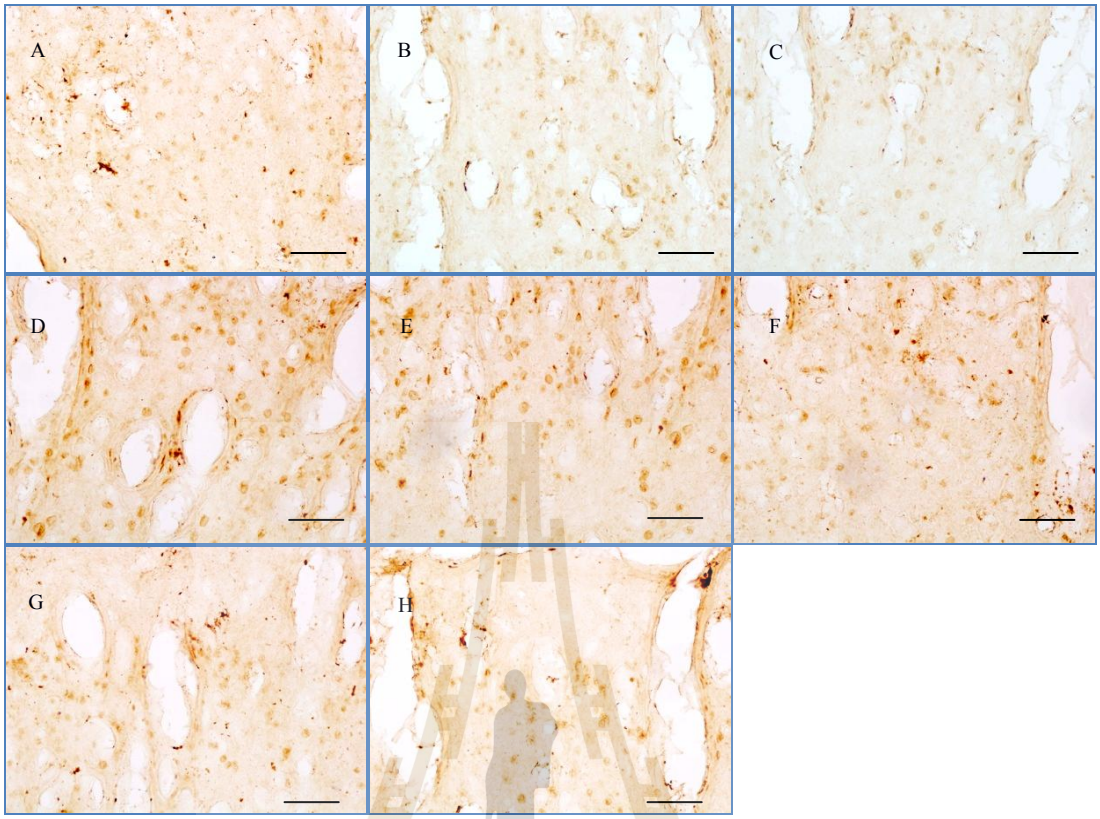

Figure 9.11 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on Fos expression of cholinergic neurons in the PrL of mPFC in NSS-treated rats and SCOP-induced amnesic rats. Photomicrographs of sections dual-immunostained for Fos (black, Ni-DAB) and ChAT (brown, DAB). Fos expression of cholinergic neurons in the mPFC after administration of vehicle + NSS (A), DPZ + NSS (B), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (C), 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (D), vehicle + SCOP (E), DPZ + SCOP (F), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (G), and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (H). The results were expressed as mean ± S.E.M. (n = 8). Scale bar = 20 μm. For abbreviations, see Figure 9.1.

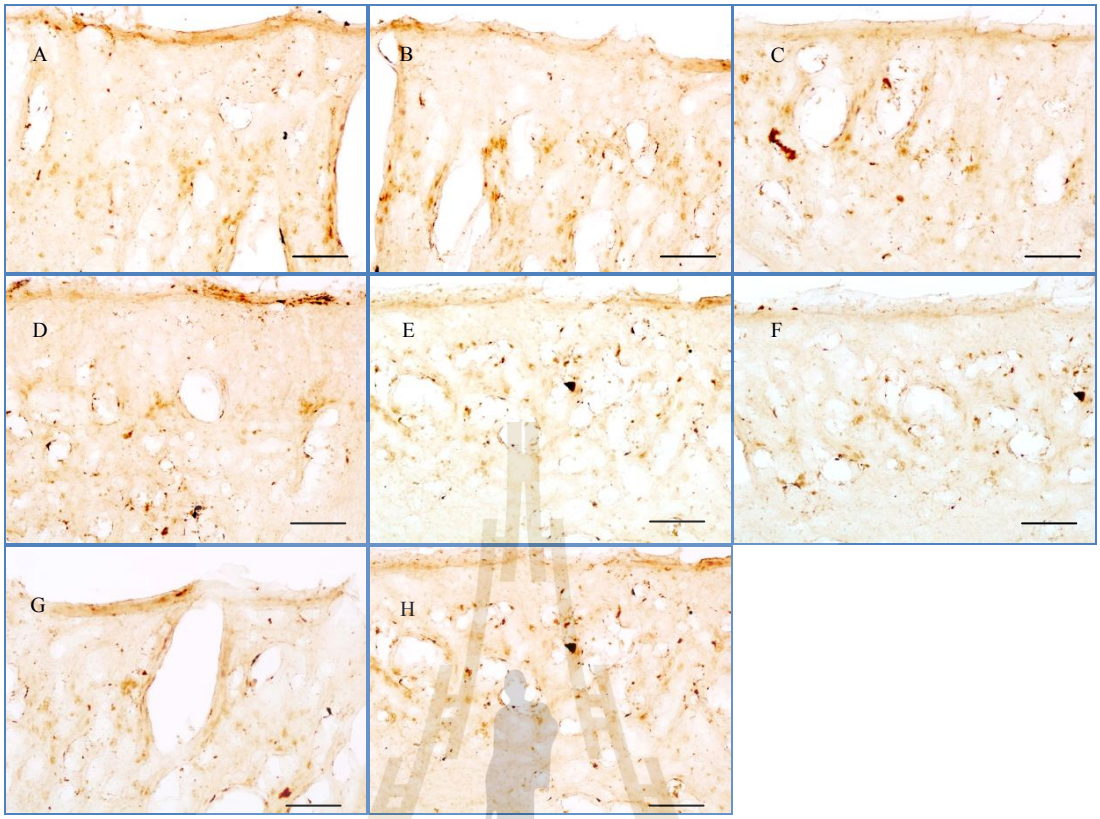

Figure 9.12 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on Fos expression of cholinergic neurons in the IL of mPFC in NSS-treated rats and SCOP-induced amnesic rats. Photomicrographs of sections dual-immunostained for Fos (black, Ni-DAB) and ChAT (brown, DAB). Fos expression of cholinergic neurons in the mPFC after administration of vehicle + NSS (A), DPZ + NSS (B), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (C), 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + NSS (D), vehicle + SCOP (E), DPZ + SCOP (F), 50 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (G), and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$ of α-MG + SCOP (H). The results were expressed as mean ± S.E.M. (n = 8). Scale bar = 20 μm. For abbreviations, see Figure 9.1.

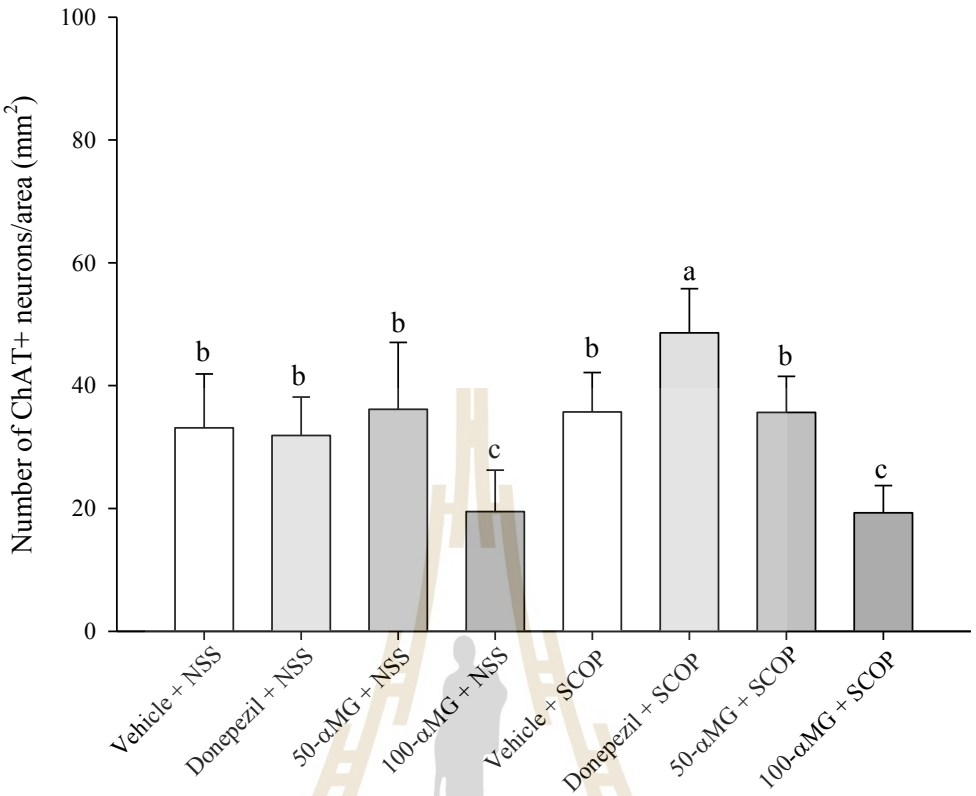

Figure 9.13 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on the number of ChAT+ neurons per area of the mPFC ranging from 4.20 to 2.52 mm from bregma in NSS-treated and SCOP-induced amnesic rats. Values are expressed as mean ± S.E.M. of number of cells·area^{-1} (mm^2) in the mPFC. Different alphabets indicate significant differences, P<0.05. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

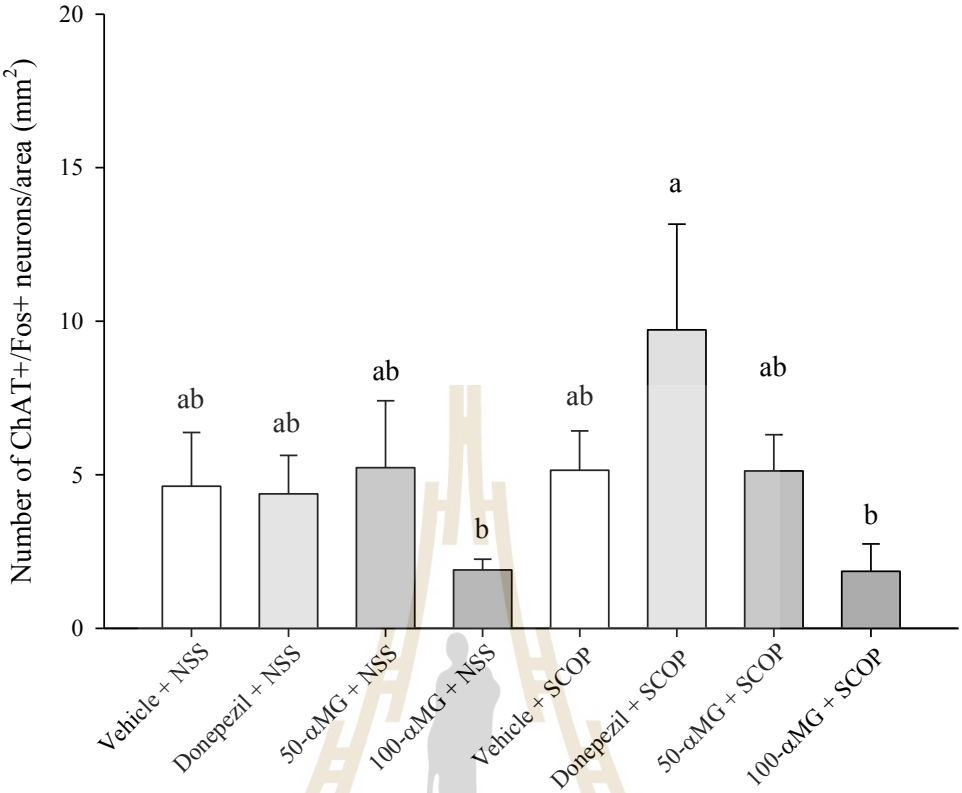

Figure 9.14 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on the number of ChAT+/Fos+ neurons per area of the mPFC ranging from 4.20 to 2.52 mm from bregma in NSS-treated and SCOP-induced amnesic rats. Values are expressed as mean ± S.E.M. of number of cells·area^{-1} (mm^2) in the mPFC. Different alphabets indicate significant differences, $P<0.05$. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

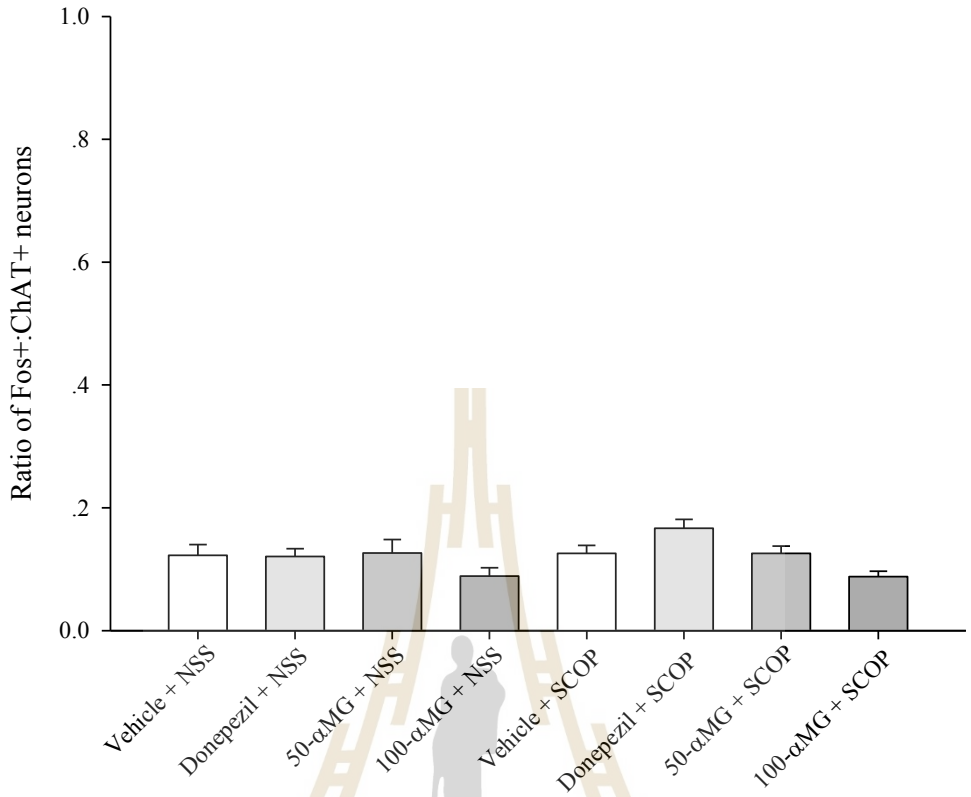

Figure 9.15 Effects of α-MG purified from the fruit rind of *G. mangostana* and DPZ on the ratio of Fos+:ChAT+ neurons of the mPFC ranging from 4.20 to 2.52 mm from bregma in NSS-treated and SCOP-induced amnesic rats. Values are expressed as mean ± S.E.M. of number of cells·area^{-1} (mm^2) in the mPFC. There was no significant difference among NSS and SCOP groups. DPZ, donepezil; α-MG, α-mangostin; NSS, normal saline solution; SCOP, scopolamine.

9.6 Discussion and conclusion

Recent work indicates that the dHip-mPFC network plays a critical role in regulating context-dependent fear memory retrieval after extinction (Jin and Maren, 2015). Neuronal populations within several regions of the dHip integrate signals of learning and memory process. A subpopulation of neurons in the medial prefrontal cortex and dorsal hippocampus expresses the acetylcholine (ACh) that is produced in the cholinergic neurons. Several studies have demonstrated that medicinal plant extracts have a neuronal activity effects in the cerebral cortex and hippocampus that involve the regulation of learning and memory (Chen, Dong, and Li, 2003). Fos expression has been used as an index of neuronal activities in rats (Kim *et al*., 2005). The present study demonstrated that scopolamine did not caused any change in the number of Fos expression of cholinergic neurons in both dHip and mPFC. The present findings revealed the first evidence of the α-mangostin purified from the fruit rind of *G. mangostana* (50 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) and donepezil (2 $mg \cdot mL^{-1} \cdot kg^{-1}$, i.p.) significantly increased Fos expression of cholinergic neurons in the dHip when compared with the vehicle control in NSS-treated group. Donepezil, but not α-mangostin (50 and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$), significantly increased cholinergic neurons with Fos expression in the dHip in scopolamine-induced amnesic rats. The increases in the number of Fos-positive cholinergic neurons of dHip by the α-mangostin in NSS-treated rats were not dose dependent. In the mPFC, donepezil significantly induced the increases of cholinergic neurons with Fos expression in scopolamine-induced amnesic rats, but not NSS-treated rats. α-Mangostin (50 and 100 $mg \cdot mL^{-1} \cdot kg^{-1}$) did not caused any change in the number of cholinergic neurons with Fos expression in the mPFC. In the mPFC, Fos-positive of cholinergic neurons induced by donepezil

(2 mg·mL^{-1}·kg^{-1}) was significantly higher than 100 mg·mL^{-1}·kg^{-1} of the α-mangostin in scopolamine-induce amnesic rats. The Fos expression in the cholinergic neurons activated by the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil were predominantly found in the dHip in NSS-treated rats. Hence, α-mangostin purified from the fruit rind of *G. mangostana* may induce learning and memory by activating cholinergic neurons in the dHip neurons in NSS-treated rats, but not scopolamine-induced amnesic rats.

Activation of cholinergic neurons by donepezil was found predominantly in the dHip of both NSS-treated and scopolamine-induced amnesic rats. The α-mangostin purified from the fruit rind of *G. mangostana* activated cholinergic neurons in NSS-treated rats only of the dHip, but not of the mPFC. The results of the present study suggested that the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil may involved in the regulation of learning and memory by activating neurons of the dHip only in NSS-treated rats, but not in scopolamine-induced amnesic rats. Thus, the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil may enhance learning and memory through the activation of some cholinergic neurons in the CA1, CA3, and DG of the dHip.

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CHAPTER X

DISCUSSION AND CONCLUSION

Dementia is a symptom complex of continuous global weak of intellectual function. It becomes a major medical, economic, and social problem that is deterioration as the increasing number of elderly people. Recently, Youdin and Joseph (2001), Perez-Perez and Rodriguez-Malaver (2005) and Mancuso *et al*. (2007) reported that antioxidants are effective in the amelioration of dementia process, the involvement of oxidative stress component in dementia or Alzheimer's disease (AD). The dementia associated with increased brain oxidative stress during brain aging could be reversed by antioxidants (Socci *et al*., 1995). Moreover, degradation of the central cholinergic system resulting in low levels of acetylcholine is correlated well with severity of dementia. Currently, available drug therapies for AD and other diseases that cause dementia consist primarily of acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine, Huperzine A), an N-methyl-D-aspartate receptor antagonist (memantine) and some neuroprotective agents (Olivares *et al*., 2012). These drugs cause side effects, thus an alternative bioactive compounds from plants and fruits with little or no side effects should be used to replace these drugs. Polyphenolic compounds may be one of candidates because of their antioxidative and anti-aging properties. Polyphenols from many fruits and plants are known to possess cognitive enhancing effects and acetylcholinesterase inhibitory activity (Papandreou *et al*., 2009). *G. mangostana* is a source of bioactive compounds with potential health-

promoting activity. The crude extract from the fruit rind of *G. mangostana* has potent antioxidant profile (Chomnawang *et al*., 2007). Several studies revealed that the fruit rind of *G. mangostana* is the rich source of xanthone (α-mangostin, β-mangostin, γ-mangostin, and gartanin) and high amount of α-mangostin can be found in the crude extract from the fruit rind of *G. mangostana* (Asai *et al*., 1995; Chen *et al*., 2008; Chomnawang *et al*., 2007; Cui *et al*., 2010; Ji *et al*., 2007; Maisuthisakul *et al*., 2007; Yu *et al*., 2007; Zadernowski *et al*., 2009). Thus, the crude extract from the fruit rind of *G. mangostana* may possess biological activities related to polyphenolic compounds and enhance memory probably by inhibiting brain acetylcholinesterase activity in healthy adult and normal aging male Wistar rats (Nontamart *et al*., 2013). The present study demonstrated for the first evidence that the α-mangostin purified from the fruit rind of *G. mangostana* and donepezil could act as antioxidants and centrally acting reversible acetyl cholinesterase inhibitors affecting against learning and memory impairment in amnesic rats by improving memory performance after place learning test by regulating cholinergic enzyme activity, inhibiting apoptotic enzyme activity and lipid peroxidation, and exhibiting neuronal death prevention and neuronal activation in the brain areas involved in the regulation of learning and memory.

The present study found that α-mangostin and donepezil possessed antioxidant and acetylcholinesterase inhibitory activities *in vitro*. The α-mangostin and donepezil enhanced memory in normal saline treated rats by increasing time spent in the target quadrant using Morris water maze test. In scopolamine-induced amnesic rats, α-mangostin and donepezil did not cause any change in time spent in the target quadrant and number of entries into target quadrant. These results were consistent with the results of AChE and ChAT activities in cerebral cortex and hippocampus, but not basal

forebrain. AChE activity in hippocampus was increased by the α-mangostin purified from the fruit rind of *G. mangostana* (50 mg mg·mL^{-1}) in NSS-treated rats, but were decreased in both hippocampus and cerebral cortex of scopolamine-induced amnesic rats. ChAT activity in hippocampus was increased by the α-mangostin purified from the fruit rind of *G. mangostana* (50 mg mg·mL^{-1}) in NSS-treated rats, and were increased in both hippocampus and cerebral cortex of scopolamine-induced amnesic rats. In contrast to those findings, there was no significant difference of AChE and ChAT activities in basal forebrain of both NSS-treated and scopolamine-induced amnesic rats treated with the α-mangostin and donepezil. These findings suggested that the antiamnesic effect of the α-mangostin purified from the fruit rind of *G. mangostana* (50 mg mg·mL^{-1}) could be due to its anti-acetylcholinesterase action in cerebral cortex and hippocampus of scopolamine-induced amnesia, but not of normal saline treatment. Cognitive dysfunction such as learning impairment and delayed amnesia are the most striking age-related changes observed in human being and animals (Foster *et al*., 1994; Gray *et al*., 2008). The cholinergic system is responsible for the storage and retrieval of item in memory and it degradation correlates well with the severity of cognitive and memory impairment. Therefore, it has been suggested that elevation of the acetylcholine level might be helpful in to improve the symptoms of cognitive deficit (Gasparin *et al*., 1998). Loss of cholinergic innervations, demonstrated by reduced choline acetyltransferase (ChAT) and elevated acetylcholinesterase (AChE) activity, is correlated with the degree of dementia (Zubenko *et al*., 1989). Many different theories have been offered regarding the cause of AD; a well-established theory suggests that acetylcholine levels are too low in the brain of AD patients (Davies and Maloney 1976). Therefore, one approach for treating

AD is via the inhibition of AChE. Based on the cholinergic hypothesis, a defect in the cholinergic system is involved in AD (Francis *et al*., 1999). Polyphenols from plants and fruits can enhance memory by inhibiting AChE activity. The polyphenol-rich extract from lotus seed pods was found to inhibit AChE activity (Xu *et al*., 2009). Green tea extract containing polyphenols could inhibit AChE activity in scopolamine-induced amnesia mice (Kim *et al*., 2004). Polyphenol-rich wild blueberry extracts attenuating brain oxidative stress, increasing brain ascorbate and glutathione (GSH) levels, and decreasing AChE activity in mice whole brain homogenates, exhibited a significant improvement in learning and memory tested by the passive avoidance behavioral test (Papandreou *et al*., 2009). Mukhergee *et al*. (2006) and Yang *et al*. (2008) reported that N. *nucifera* rhizome extract inhibited AChE activity and improved learning and memory by enhancing neurogenesis in the dentate gyrus of hippocampus.

The present study also found that the α-mangostin purified from the fruit rind of *G. mangostana* (50 mg mg·mL^{-1}) and donepezil could reduce lipid peroxidation and activity of caspase-3 enzyme in cerebral cortex and hippocampus, but not in basal forebrain. These findings suggested that the antioxidant and anti-apoptotic effects of the α-mangostin purified from the fruit rind of *G. mangostana* (50 mg·mL^{-1}) and donepezil could be due to its inhibitory action on lipid peroxidation in cerebral cortex and hippocampus, but not in basal forebrain of scopolamine-induced amnesia. Moreover, the α-mangostin purified from the fruit rind of *G. mangostana* (50 mg·mL^{-1}) and donepezil could induce Fos expression in cholinergic neurons of dHip in NSS-treated rats, but not in mPFC. Donepezil could induce Fos expression in cholinergic

neurons of both dHip and mPFC in scopolamine-induced amnesic rats, but not the α-mangostin purified from the fruit rind of *G. mangostana*.

These findings suggested that the central modulatory effects of α-mangostin (50 $mg \cdot mL^{-1}$) and donepezil on the neuronal activation of cholinergic neurons indicated by the expression of a marker Fos could be due to its action on increasing Fos-positive neurons in the dHip but not in the mPFC in adult healthy rats. Donepezil also increased Fos-positive neurons in the dHip and mPFC in scopolamine-induced amnesic rats but not for α-mangostin. Thus, α-mangostin and donepezil may involve in the regulation of learning and memory. The present study also demonstrated that α-mangostin and donepezil at any tested doses did not affect the animal behavior and health status and did not produce any plasma biochemical parameters abnormality of adult male Wistar rats during the period of treatment for constitutive 7 days, suggesting that α-mangostin is medically safe for liver and kidneys of rats.

In conclusion, the findings provided the new evidence of the beneficial effects of α-mangostin, an antioxidant and anti-AChE agent, could act against learning and memory impairment in scopolamine-induced amnesic rats by enhancing memory after learning performance in a Morris water swimming maze which may be the result of choline acetyltransferase stimulation and the inhibition of acetylcholine esterase, caspase-3, and lipid peroxidation in the cerebral cortex, hippocampus, and basal forebrain. Moreover, α-mangostin may also prevent neuronal cell death and induced Fos expression in the dorsal hippocampus. The α-mangostin may act as one of the best dietary supplements for the protection of learning and memory impairment.

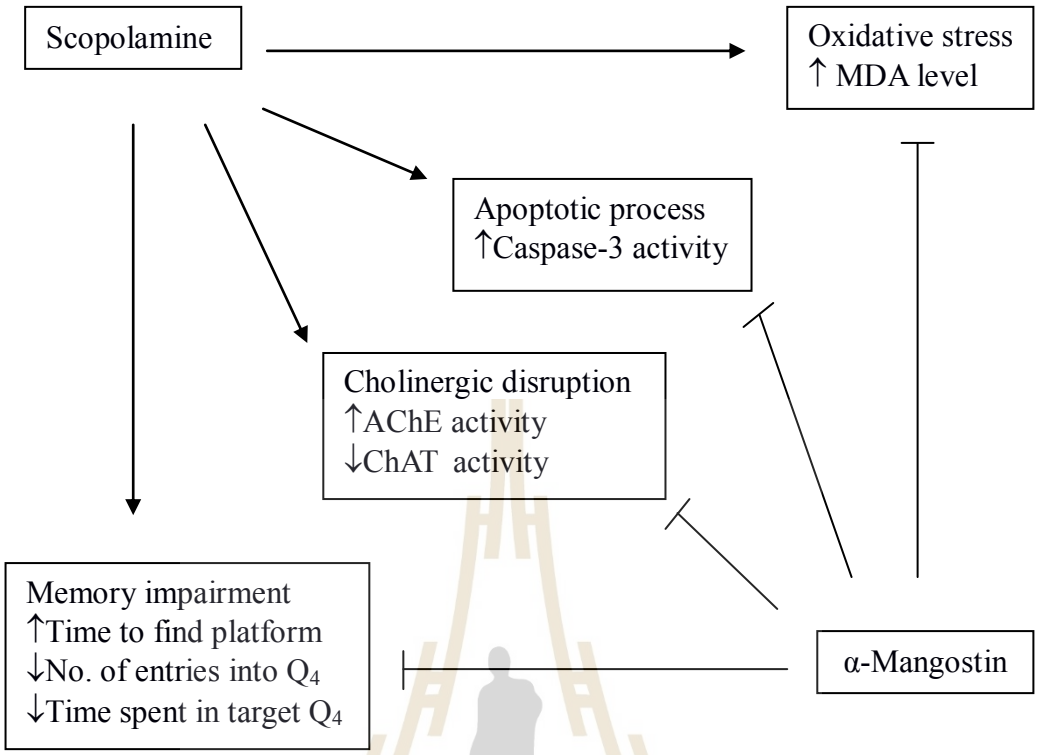

Figure 10.1 The scheme illustrates the possible mechanisms of α-mangostin purified from the fruit rind of *Garcinia mangostana* against oxidative stress, apoptotic process, cholinergic disruption, and memory impairment.

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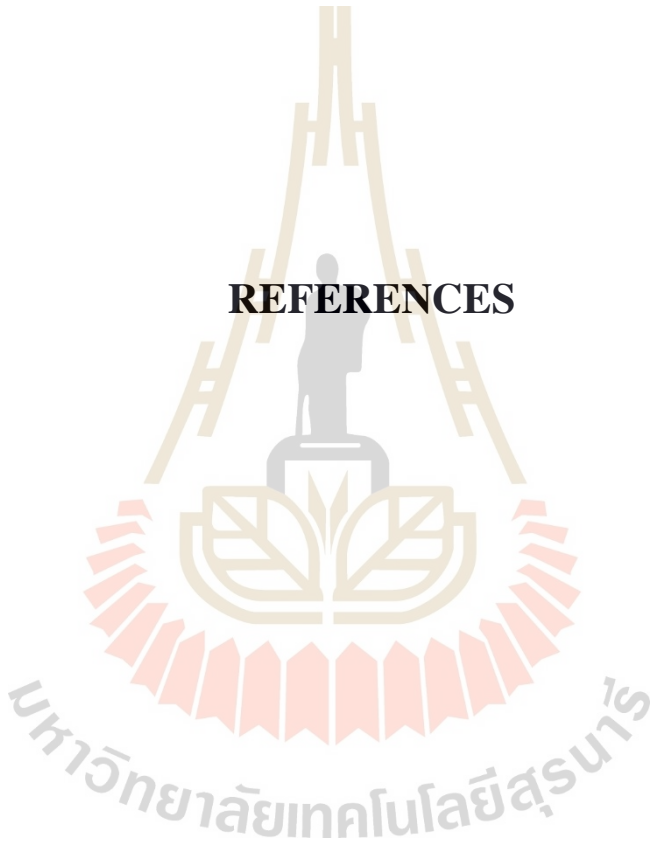

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