ผลของกวาวเครือขาว *Pueraria mirifica* ต่อการออกฤทธิ์ป้องกันและรักษาภาวะเสื่อมของเซลล์ประสาทในสมองส่วนฮิปโปแคมปัสของหนูแรทที่ตัดรังไข่

นางสาวกันยา อนุกูลธนากร

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EFFECTS OF WHITE KWAO KRUA *Pueraria mirifica* ON NEUROPROTECTIVE AND NEUROTHERAPEUTIC ACTIONS IN HIPPOCAMPUS OF OVARIECTOMIZED RATS

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Thesis Title	EFFECTS OF WHITE KWAO KRUA *Pueraria mirifica* ON NEUROPROTECTIVE AND NEUROTHERAPEUTIC ACTIONS IN HIPPOCAMPUS OF OVARIECTOMIZED RATS
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กันยา อนุกูลธนากร : ผลของกวาวเครือขาว *Pueraria mirifica* ต่อการออกฤทธิ์ป้องกันและรักษาภาวะเสื่อมของเซลล์ประสาทในสมองส่วนฮิปโปแคมปัสของหนูแรทที่ตัดรังไข่. (EFFECTS OF WHITE KWAO KRUA *Pueraria mirifica* ON NEUROPROTECTIVE AND NEUROTHERAPEUTIC ACTIONS IN HIPPOCAMPUS OF OVARIECTOMIZED RATS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. สุจินดา มาลัยวิจิตรนนท์ Ph.D., อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. อิชวา เอส พาร์ฮาร์ Ph.D., สุกัญญา เจริญพร Ph.D., 152 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลและกลไกการออกฤทธิ์ของกวาวเครือขาว *Pueraria mirifica* ต่อการป้องกันและรักษาภาวะเสื่อมของเซลล์ประสาทในสมองส่วนฮิปโปแคมปัสในหนูแรทที่ถูกชักนำให้อยู่ในสภาวะพร่องฮอร์โมนเพศโดยการตัดรังไข่ โดยวัดระดับฮอร์โมน E_2, LH และ FSH ในซีรัมด้วยวิธี Radioimmunoassay วัดการแสดงออกของยีนที่เกี่ยวข้องกับการเกิดอะไมลอยด์เบต้า (*App*, *Adam10* และ *Bace1*) และนิวโรไฟบริลลารี แทงเกิล (*Tau3* และ *Tau4*) ในสมองส่วนฮิปโปแคมปัสด้วยวิธีเรียลไทม์พีซีอาร์ และวัดความสามารถในการจดจำของหนูด้วย Morris water maze test ภายหลังจากตัดรังไข่ที่เวลา 0, 1, 2, 3 และ 4 เดือน และภายหลังจากป้อนน้ำกลั่น 1 มล./วัน, ป้อนสารสกัดกวาวเครือขาว (PM) ในขนาด 100 มก./กก. น้ำหนักตัว/วัน, ฉีดพูรารินิ (PU) ในขนาด 7 มก./กก. น้ำหนักตัว/วัน และฉีดเอสโทรเจน (E_2) ในขนาด 80 ไมโครกรัม/กก. น้ำหนักตัว/วัน เข้าทางใต้ผิวหนัง หลังตัดรังไข่เป็นเวลา 2 วัน, 2 เดือน และ 4 เดือน ตามลำดับ เป็นเวลา 4 เดือน พบว่าหลังจากตัดรังไข่นาน 2 สัปดาห์ ระดับ E_2 ในซีรัมลดลงอย่างมีนัยสำคัญทางสถิติ (p<0.005) และระดับ LH และ FSH ในซีรัมเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ (p<0.005) ภายหลังจากตัดรังไข่นาน 1 และ 2 เดือนตามลำดับ การแสดงออกของยีน *Tau3* และ*Tau4* เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติหลังจากตัดรังไข่นาน 1 - 4 เดือน (p<0.05) ในขณะที่การแสดงออกของยีน *App, Adam10* และ *Bace1* เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติหลังจากตัดรังไข่แล้วนาน 4 เดือน (p<0.05) และหนูแรทอยู่ในภาวะความจำเสื่อมหลังจากตัดรังไข่นาน 3 และ 4 เดือน เมื่อให้ PM, PU และ E_2 ในหนูแรทที่ตัดรังไข่แล้ว 2 วัน ที่ระดับ E_2 ในซีรัมลดลง พบว่า PM, PU และ E_2 สามารถป้องกันการเกิดภาวะความจำเสื่อม และลดการแสดงออกของยีน *App*, *Adam10*, *Bace1*, *Tau3* และ *Tau4* ในสมองส่วนฮิปโปแคมปัสได้ และเมื่อให้ PM, PU และ E_2 ในหนูแรทที่ตัดรังไข่นาน 2 เดือน ที่ระดับ E_2 ในซีรัมลดลงและระดับ LH ในซีรัมสูงขึ้นและคงที่ แต่ยังไม่พบภาวะความจำเสื่อม พบว่า PM, PU และ E_2 ให้ผลการทดลองเช่นเดียวกันกับเมื่อตัดรังไข่ไป 2 วัน แต่เมื่อให้ PM, PU และ E_2 ในหนูแรทที่ตัดรังไข่นาน 4 เดือน ที่หนูแสดงอาการความจำเสื่อมแล้ว พบว่า PM, PU และ E_2 สามารถบรรเทาอาการความจำเสื่อมในหนูแรทได้ แต่เฉพาะหนูกลุ่ม PM เท่านั้นที่มีการแสดงออกของยีน *App* ลดลง โดยสรุปการให้กวาวเครือขาวสามารถป้องกันและรักษาภาวะเสื่อมของเซลล์ประสาทในสมองส่วนฮิปโปแคมปัสในหนูแรทที่ตัดรังไข่ได้ แต่ฤทธิ์ในการรักษาจะไม่ดีเท่าฤทธิ์ในการป้องกัน ดังนั้นถ้าต้องให้กวาวเครือขาวในผู้หญิง ควรให้ในระยะก่อนหรือเมื่อเริ่มเข้าสู่วัยหมดประจำเดือนในขณะที่ยังไม่มีอาการภาวะสมองเสื่อมเกิดขึ้น

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KANYA ANUKULTHANAKORN: EFFECTS OF WHITE KWAO KRUA *Pueraria mirifica* ON NEUROPROTECTIVE AND NEUROTHERAPEUTIC ACTIONS IN HIPPOCAMPUS OF OVARIECTOMIZED RATS. ADVISOR: PROF. SUCHINDA MALAIVIJITNOND, Ph.D., CO-ADVISOR: PROF. ISHWAR S. PARHAR, Ph.D., SUKANYA JAROENPORN, Ph.D., 152 pp.

Effects and mechanism of neuroprotective and neurotherapeutic actions of white Kwao Krua *Pueraria mirifica* in the hippocampus of estrogen deficient rats were investigated. Serum E_2, LH and FSH levels were determined by RIA techniques. The expression of the genes associated with amyloid plaques (*App*, *Adam10* and *Bace1*) and neurofibrillary tangles (*Tau3* and *Tau4)* in the hippocampus were examined by real-time PCR. Cognitive performance was examined by Morris water maze test. Rats were ovariectomized and kept for 0, 1, 2, 3 and 4 months, or fed with 1 ml/day of distilled water or 100 mg/kg BW/day of *P. mirifica* (PM) or subcutaneously injected with 7 mg/kg BW/day of puerarin (PU) or 80 mg/kg BW/day of 17beta-estradiol (E_2) after ovariectomy for 2 days, 2 and 4 months, respectively, for 4 months, before those parameters were determined. Serum E_2 levels were significantly decreased ($p<0.005$) after 2 weeks of ovariectomy and serum LH and FSH levels were significantly increased ($p<0.005$) after 1 and 2 months of ovariectomy, respectively. The expression of *Tau3* and *Tau4* was increased as early as 1 month after ovariectomy while the expression of *App*, *Adam10* and *Bace1* was significantly increased after 4 months of ovariectomy. The cognitive impairment was found at 3 and 4 months after ovariectomy. Treatment of PM, PU and E_2 in 2-day ovariectomized rats, while serum E_2 levels were low, could prevent the cognitive impairment and decreased the expression of *App*, *Adam10*, *Bace1*, *Tau3* and *Tau4*. Treatment of PM, PU and E_2 in 2-month ovariectomized rats, while serum estrogen levels were low and serum LH levels were peaked, but the cognitive impairment was yet occurred, the results were similar to those of 2-day ovariectomized rats. However, treatment of PM, PU and E_2 in 4-month ovariectomized rats, while cognitive impairment was appeared, could ablate the cognitive impairment in rats, and only the PM fed rats elicited the decreased *App* mRNA level. Taken together, *P. mirifica* could exhibit either the neuroprotective or neurotherapeutic effect in ovariectomized rats; however, the neuroprotective effect is more promising than the neurotherapeutics. Thus, treatment of *P. mirifica* to women should be started during the time of pre- or peri-menopausal period.

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Student's Signature .....
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LIST OF ABBREVIATIONS

Aβ	=	Amyloid β
ADAM	=	A disintegrin and metalloproteinase
ADME	=	Absorption, distribution, metabolism, and excretion
APP	=	Amyloid precursor protein
BACE	=	Beta-site APP cleaving enzyme
CA	=	Cornu ammonis
cDNA	=	Complementary deoxyribonucleic acid
Co	=	Cornified cell
DG	=	Dentate gyrus
DM1	=	Myotonic dystrophy type 1
EDCs	=	Estrogen disrupting compounds
EGF	=	Epidermal growth factor
EPA	=	Environmental protection agency
ERs	=	Estrogen receptors
ERα	=	Estrogen receptor α
ERβ	=	Estrogen receptor β
E_2	=	17β-estradiol
ERT	=	Estrogen replacement therapy
FTDP-17	=	Frontotemporal dementia and parkinsonism linked to chromosome 17
FSH	=	Follicle stimulating hormone
GnRH	=	Gonadotropin releasing hormone
GPCRs	=	G protein-coupled receptors
HPLC	=	High performance liquid chromatography
HRT	=	Hormone replacement therapy
IGR-1	=	Insulin-like growth factor-1
L	=	Leukocyte cell
LH	=	Luteinizing hormone
LHR	=	Luteinizing hormone receptor
LT	=	Long-term memory
MAP	=	Microtubule-associated protein

MAPK	=	Mitogen-activated protein kinase activation
MFs	=	Moss fibers
mRNA	=	Messenger ribonucleic acid
NFTs	=	Neurofibrillary tangles
NMDA	=	N-methyl-D-aspartate
O	=	Nucleated cell
OECD	=	Organization for economic coopoeration and development
OVX	=	Ovariectomized
pAMPKα	=	Phosphorylated adenosine monophosphate-activated protein kinase-α
PCR	=	Polymerase chain reaction
PKA	=	Protein kinase A
PKC	=	Protein kinase C
RIA	=	Radioimmunoassay
RNA	=	Ribonucleic acid
ROS	=	Reactive oxygen species
SAD	=	Sporadic Alzheimer's disease

CHAPTER I
GENERAL INTRODUCTION

The hippocampus, a bilateral incurved seahorse-shaped structure of the cerebral cortex, belongs to the limbic system which is involved in emotion and memory. The hippocampus is indeed an important part of the brain involved in memory and cognition. Besides, it is also one of the most age-sensitive areas in the brain. During the aging process, the hippocampus greatly diminishes in its plastic capabilities and the decline leads to the age-related impairments which is cognitive output. In neurodegenerative patients, the hippocampus disruption induces cognition/memory loss (Casadesus et al., 2007; Taupin, 2007).

In the present day, the world is now facing the "ageing era" because of the rapid increase in the elderly population which comes with social concerns such as the high incidence of neurodegenerative diseases. Neurodegenerative diseases are progressive neurological disorders which are linked to brain injuries where there is no return. Many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease, occur as a result of neurodegenerative processes. Among the neurodegenerative diseases, Alzheimer's disease is the most common one of which the incidence rate in women is double that of men after the age of 80 years (Zandi et al., 2002). The pathological features of Alzheimer's disease include amyloid β (Aβ) plaque formation and hyperphosphorylated tau proteins (Kim et al., 2012).

The main cause of the high incidence of Alzheimer's disease in aged women is the loss of estrogen during menopause. Following menopause, estrogen deprivation shifts the homeostasis of the hypothalamic-pituitary-ovarian axis which results in a 3 to 4-fold increase in serum luteinizing hormone (LH) concentration and a 4 to 18-fold increase in serum follicle stimulating hormone (FSH) concentration (Chakravarti et al., 1976). The effects of the increased circulating LH and FSH levels due to the loss of negative feedback of estrogen on the aging brain are largely explored. Lei et al. (1993) emphasized that only luteinizing hormone receptor (LHR) had been detected in the brain and they proposed that LH peak at the post-menopausal period might be a critical factor to cause the most brain damage.

The study using ovariectomized (OVX) rodents showed that estrogen deprivation led to impairment of cognitive performance, influenced structural plasticity

of neurons, impaired cholinergic function and reduced neurotropin expression, which are symptoms of Alzheimer's disease (Singh et al., 2008). Estrogen replacement therapy has been widely conducted and assessed afterwards. Estrogen can improve memory function in menopausal women and in OVX rats and mice (Feng et al., 2004; Spencer et al., 2008; Xu et al., 2007). Estrogen can reduce the deposition of Aβ peptide, enhance neurotransmitter release and activity, protect against oxidative damage, and exhibit neurotrophic effects (McEwen, 1999; Xu et al., 1998). However, some clinical trials recently indicated that estrogen has no protective effect on cognitive function in, at least, older women (Shumaker et al., 2004). As such, they questioned whether estrogen treatment could produce beneficial effects in neurodegeneration and cognitive function only if the treatment has been initiated within a critical period of time. This indicates that to ameliorate or ablate the neurodegenerative symptom, estrogen supplement should have been done together with suppression of LH levels.

Thus, the supplement of estrogenic chemicals together with the chemical showing a suppressive effect on LH peak should become a better alternative neuroprotective and therapeutic strategy for neuronal dysfunction in postmenopausal women. However, long-term treatment of estrogen could attribute to the development of some estrogen-dependent cancers, such as breast cancer and prostate cancer (Howes et al., 2003; Usui, 2006). Searching for other chemical agents with fewer side-effects is needed.

Thailand is a tropical country which has large biodiversity, especially for the flora. Using medicinal plant containing estrogen-like substance, namely phytoestrogens, should take a great advantage in this field. *Pueraria mirifica* or white Kwao Krua is an endemic Thai herb which is classified into a Family Leguminosae. Its tuberous root contains phytoestrogens, mainly isoflavones, such as puerarin, daidzin, genistin, daidzein, genistein, and also miroestrol (Chansakaow et al., 2000; Cherdshewasart et al., 2007d). The estrogenic activity of phytoestrogens in *P. mirifica* has been verified by MCF7 proliferation assay (Cherdshewasart & Sriwatcharakul 2008a), vaginal cytology assay and uterotropic assay (Malaivijitnond et al., 2006; Cherdshewasart et al., 2007a). It can also attenuate the increased serum LH levels in OVX rats (Malaivijitnond et al., 2004) and in aged female monkeys (Trisomboon et al., 2006b). In addition, *P. mirifica* can decrease the incidence and growth of mammary tumor in female rats (Cherdshewasart et al., 2007b). Together with these estrogenic

activities, it was recently discovered that *P. mirifica* increased synaptophysin (a presynaptic vesicular protein) immunoreactivity and synaptophysin level in the primary cell culture of hippocampal neurons (Chindewa et al., 2008). Taken together, *P. mirifica* could be a choice of novel chemical treatments for neuronal dysfunction in postmenopausal women. Therefore, the neuroprotective and neurotherpeutic actions of *P. mirifica* in OVX rats were investigated in this study.

OBJECTIVES

1) To investigate the relationship between estrogen deficiency, increased serum LH level and neuronal dysfunction in hippocampus of rats

2) To determine the neuroprotective effects of *P. mirifica* on neuronal dysfunction in hippocampus of estrogen deficient rats

3) To determine the neurotherapeutic effects of *P. mirifica* on neuronal dysfunction in hippocampus of estrogen deficient rats

ANTICIPATED BENEFIT

If *P. mirifica* can prevent and cure neuronal dysfunction in hippocampus of OVX rats, it will be a potential alternative treatment for neurodegenerative disease in postmenopausal women. This will also add value to this medicinal Thai plant.

CHAPTER II
LITERATURE REVIEWS

In the present day, the average life span of humans has been extended by the improved and advance medical technologies which lead to the rapid increase in elderly population. As such, the world is now facing the 'aging era', which comes together with the "aging-associated diseases", for example, neurodegenerative diseases. Neurodegenerative diseases are progressive neurological disorders which are highly linked to brain injuries and have no return. Selective neuronal loss in particular regions of the brain causes different types of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington disease. The most common and fatal neurodegenerative disease with disastrous effects on the senior population is Alzheimer's disease, a neurodegenerative dementia disorder (Kim et al., 2012; Maslow, 2008).

Previous publications indicate that the risk for neurodegenerative diseases is associated with age-related loss of sex steroid hormone in both women and men. However, women show the higher prevalence and incidence of neurodegenerative diseases than men. Many studies have led to the hypothesis that the increase in neurodegenerative diseases in women after menopause is because of the decrease in circulating estrogen (Lovekamp-Swan et al., 2007). Estrogen replacement therapy could be an effective in prevention or treatment of neurodegenerative disease. However, estrogen treatment is not effective in all cases of female patients. Additionally, estrogen causes many undesirable side effects and is expensive, especially for the locals who have no access to the treatment (NIH, 2005). Thus, many researchers are turning to alternative therapies and phytoestrogen supplement should be one of the choices.

This chapter summarizes what we should know about the pathological hallmarks of neurodegenerative disease, hippocampus and its function on memory, estrogen, estrogen deficiency-related neurodegenerative disease, and phytoestrogens. Finally, it focuses on *P. mirifica*, phytoestrogens containing plant, as a novel alternative choice for neuroprotective and neurotherapeutics.

1. Pathological hallmarks of neurodegenerative diseases

The common motifs in neurodegenerative disease are accumulations of insoluble filamentous aggregates in the form of Aβ and neurofibrillary tangles (NFTs) which associated with progressive neuronal cell death in the hippocampus and cortex regions. It results in gradual memory loss, cognitive impairment, behavior dysfunction and finally death (Kim et al., 2012; Skovronsky et al., 2006). The neuropathological features of neurodegenerative diseases are described as follows;

1.1. Amyloid plaques

The accumulation of insoluble amyloid is one of the major pathological lesions of neurodegenerative diseases. Aβ peptide is a major component of senile plaques and generated from amyloid precursor protein (APP) (Selkoe, 2001; Asai et al., 2003). APP is a type-1 integral membrane protein consisting of a large ectodomain, 770 amino acid residue. APP is ubiquitously expressed with highest levels in brain and kidneys, but the precise function is not completely understood (Prox et al., 2012). There are two main secretase activities that are responsible for the processing of APP; α- and β-secretase (Figure 2.1) (Goodenough et al., 2003). The main cleavage is performed by a protease designated as α-secretase that cleaves APP within the Aβ region. Since α-secretase processing of APP prevents the formation of intact Aβ peptide, the pathway is known as "non-amyloidogenic". An alternative route that is particularly present in neurons and brain is known as the "amyloidogenic pathway" which involves cleavage of APP by β-secretase. This cleavage generates Aβ peptide (Prox et al., 2012).

Aβ is generated from APP through sequential cleavages by β-secretase and γ-secretase (Figure 2.1) (Li et al., 2006). Using a variety of techniques, including gene expression, cloning, genomic searches, and protein purification, beta-site APP-cleaving enzyme 1 (BACE1) was identified as the β-secretase (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). Cleavage of APP at two sites by BACE1 produces a secreted form of APP and a 99 or 89-residue membrane-associated C-terminal fragment (C99 or C89, respectively), C99 is subsequently cleaved by γ-secretase at intransmembrane sites to generate Aβ and C-terminal fragment-γ fragments in the amyloidogenic pathway. In a non-amyloidogenic pathway, cleavage of APP first within the Aβ domain by α-secretase, possibly involving A disintegrin and metalloprotease domain 9 (ADAM9), ADAM10, and ADAM17, tissue necrosis factor-

alpha converting enzyme, generates a secreted form APP and C83. C83 is subsequently cleaved by γ-secretase, precluding Aβ generation (Sun et al., 2012). ADAM10 is the major constitutive APP α-secretase in neurons (Prox et al., 2012). Thus, the β-secretase activity in amyloidogenic pathway is represented by BACE1 and the α-secretase activity in non-amyloidogenic pathway is represented by ADAM10.

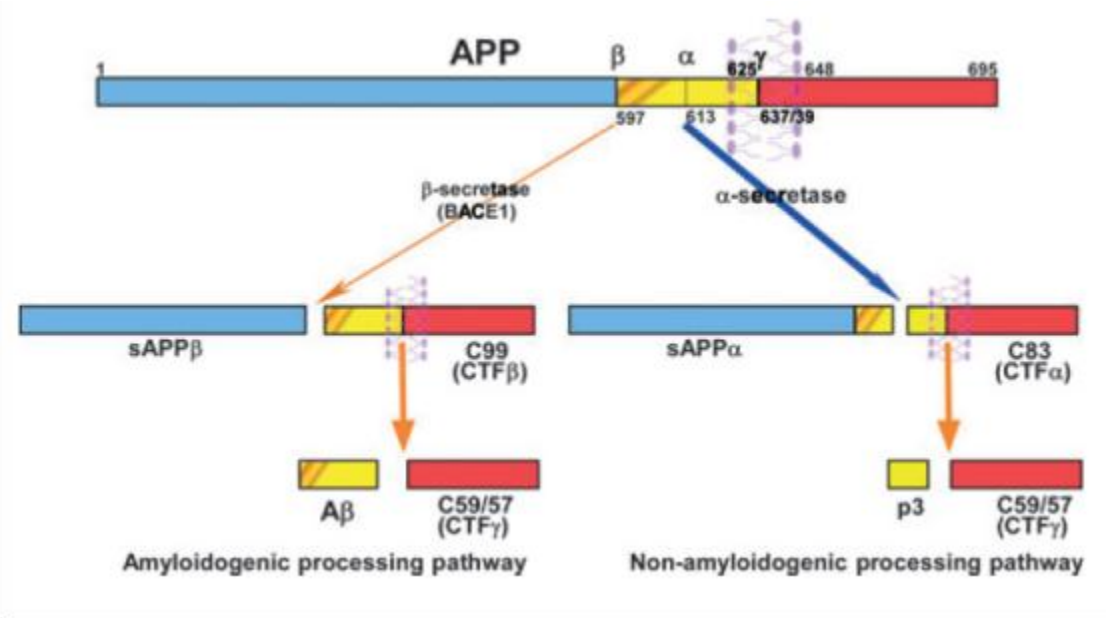

Figure 2.1 Generation of amyloid β (Aβ) from amyloid precursor protein (APP) by cleavage of β and γ-secretase. APP is a type I membrane protein. APP is processed by α, β and γ-secretase. The non-amyloidogenic pathway is the predominant pathway for processing of APP under normal conditions in which APP is cleaved by α and γ-secretase. In the amyloidogenic pathway, APP is cleaved by β-secretase to produce C99, which is subsequently cleaved by γ-secretase to generate Aβ (Sun et al., 2012).

Aβ accumulation is toxic to neuron and may act synergistically with NFTs pathology. The increase in production, aggregation and accumulation of Aβ initiates a cascade of events to neurotoxicity and eventually to clinical symptoms of Alzheimer's disease. It is linked mechanistically to impairment in axonal transport, inhibition of proteosomal activity, defect in DNA transcription, increase in levels of oxidative stress and apoptosis (Skovronsky et al., 2006). The extracellular amyloid plaques throughout the cerebral cortex and the hippocampus manifest as a severe deterioration of cognitive abilities, memory loss and behavioral changes, with a complete loss of autonomy later in the course of the disease.

1.2. Neurofibrillary tangles

NFTs are soluble tau aggregates found within the neurons and glia of people with sporadic of neurodegenerative disease such as Alzheimer's disease, tauopathies, Down syndrome (trisomy 21) and myotonic dystrophy type 1 (DM1). The numbers of NFT correlate with dementia severity. Tau protein is a neuronal microtubule-associated protein (MAP), which localized primarily in the axon. Tau is the most commonly misfolded protein in human neurodegenerative disease and it is a key component of NFTs in Alzheimer's disease. The tau gene is located on chromosome 17 (Robert & Mathuranath, 2007) and its function is to stimulate microtubule assembly and stabilize microtubules. Hyperphosphorylation of tau leads to its aggregation into NFTs, a hallmark structures of many neurodegenerative diseases such as Alzheimer's disease and tauopathies (Goedert & Spillantini, 2011; Shi et al., 2011).

Adult human brain expresses six different tau isoforms from a single gene by alternative splicing of exons 2, 3, and 10 of its pre-mRNA (Figure 2.2). The exon 10 is adult specific and encodes the second (R2) of four repeats that binds microtubule and shows a species-specific difference crucial to neurodegeneration. Alternatively splicing of exon 10 generates tau with three or four microtubule binding repeats (3R-tau or 4R-tau), which under development and cell type-specific regulation. 3R-tau and 4R-tau are expressed in approximately equal amounts (1:1 ratio) in adult human brain. Several mutations in tau gene result in either increase or decrease in 4R-tau expression and cause frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP17), one of the tauopathies. Thus, alteration in the 3R-tau/4R-tau ratio is sufficient to trigger neurodegeneration in frontotemporal dementia and might also play a role in other neurodegenerative disorders such as Pick's disease, progressive nuclear palsy or corticobasal degeneration in which the 3R-tau/4R-tau ratio is markedly altered (Shi et al., 2011).

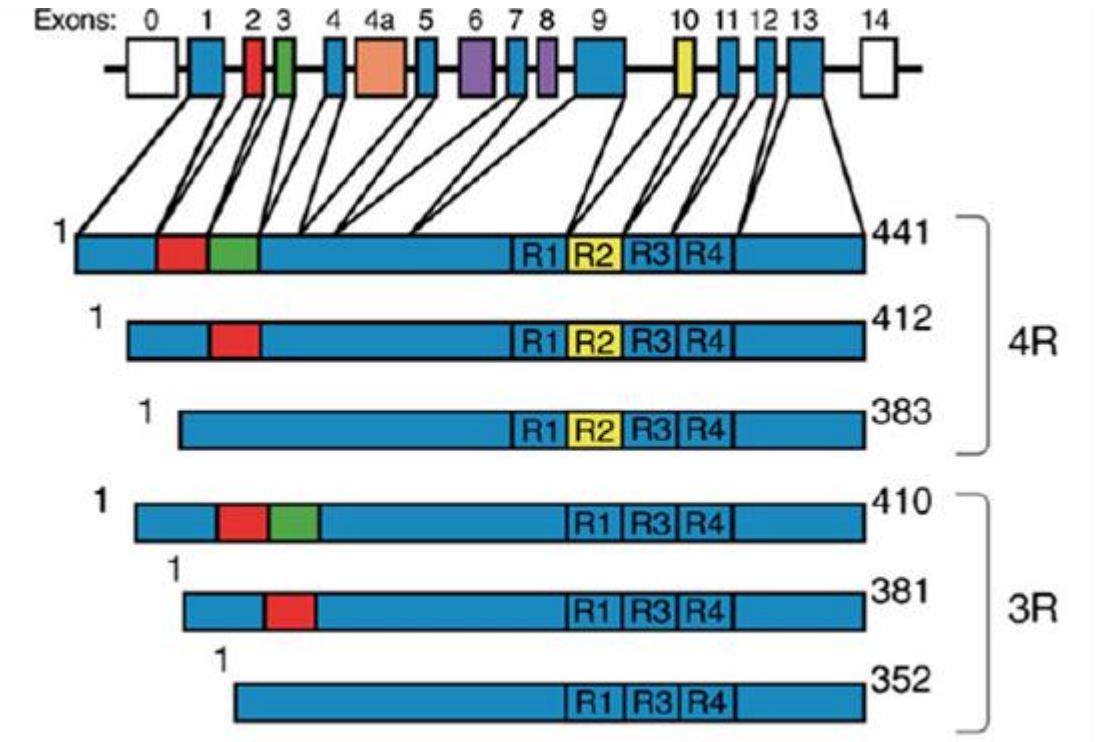

Figure 2.2 MAP tau and the six tau isoforms express in adult human brain. MAPT consists of 16 exons. Alternative mRNA splicing at exon2, exon3 and exon 10 gives rise to the six tau isoforms (352-441 amino acids). Exon0, which is part of the promoter, and exon14 are non-coding regions. Exon6 and 8 are not transcribed in human brain. Exon4a is only expressed in the peripheral nervous system. The repeats of tau (R1-R4) are shown, with three isoforms having four repeats each (4R) and three isoforms having three repeats each (3R). Each repeat is 31 or 32 amino acids in length (Goedert & Spillantini, 2011).

Aβ and tau seem to confer toxicity synergistically, as Aβ exacerbates preexisting tau pathology (Gotz et al., 2001), while Aβ toxicity is tau dependent (Roberson et al., 2007). Therefore, both Aβ and tau hyperphosphorylation are the two degenerative processes that coexist in neurodegenerative disease.

2. Hippocampus

The hippocampus (Gr. hippos = horse, and kampe = curve) is a major component of the human brain and other mammals. It is phylogenetically one of the most ancient structures of the brain. The hippocampus belongs to the limbic system and plays important roles in long-term memory (LT) and spatial navigation. It is a

bilateral structure which locates on each side of the brain beneath the neocortex on the basal medial surface of the temporal lobes (Taupin, 2007). It is surrounded by the entorhinal, parahippocampal and perirhinal cortices (Figure 2.3) (Bird & Burgess, 2008).

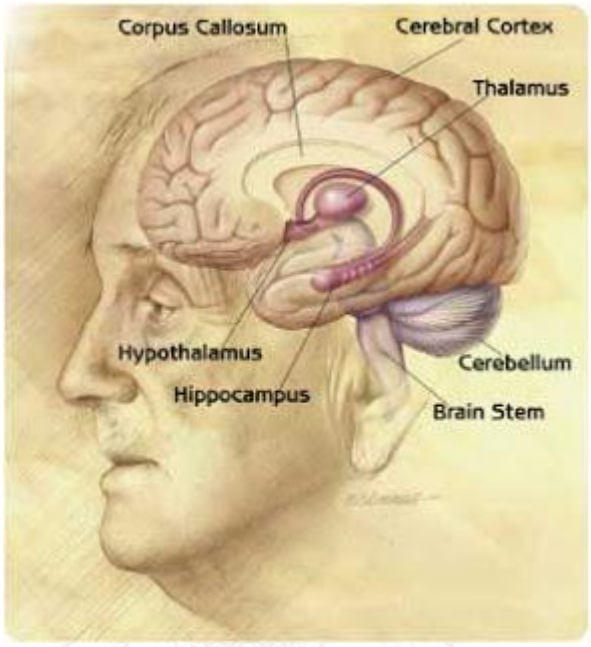

Figure 2.3 Schematic of hippocampus in human brain.
(Available in: http://2.bp.blogspot.com/-fqPGWaJGh6A/UAMFYQvd1QI/AAAAAAAAAf8/3xwP1sXOEao/s320/hippocampus.jpg)

2.1. Cytoarchitecture of hippocampus

The hippocampus is composed of three subregions; the dentate gyrus (DG), the Cornu Ammonis (CA) or the hippocampus proper or Ammon's horn, and the subiculum (Figure 2.4). The DG has a "V" or "U" shape and the CA is a curved structure forming a "U" enchased in the DG. The cells of the principal layers of the DG and CA regions have distinctive morphologies, cell shape, sizes and connections. The CA is divided into 4 subfields; CA1, CA2, CA3 and CA4. The CA1 region is adjacent to the subiculum. The CA3 region is adjacent to the fimbria/fornix region. The CA2 region is a small boundary between CA1 and CA3. However, the identity of CA2 as a subfield of the CA region is the source of debates. The CA4 is located in the hilus of the DG (Amaral & Witter, 1989; Manns & Eichenbaum, 2006; Taupin, 2007; van Strien et al., 2009). The cortex that forms the hippocampal formation has a three-layered appearance. The first layer is a deep layer, comprising a mixture of afferent and efferent fibers and

interneurons. In the DG, this layer is called hilus, whereas in the CA regions it is referred to as the stratum oriens. Superficial to this polymorph layer is the cell layer, which is composed of principal cells and interneurons. In the DG, this layer is called the granule layer, whereas in the CA regions and the subiculum it is referred to as the pyramidal call layer (stratum pyramidale). The most superficial layer is referred to as the molecular layer (the stratum moleculare) in the DG and subiculum. In the CA region the molecular layer is subdivided into a number of sublayers. In CA3, three sublayers are distinguished: the stratum lucidum, which receives input from the DG; the stratum radiatum, comprising the apical dendrites of the neurons located in the stratum pyramidal; and, most superficially, the stratum lacunosum-moleculare, comprising the apical tufts of the apical dendrites. The lamination in CA2 and CA1 is similar, with the exception that the stratum lucidum is missing (van Strien et al., 2009).

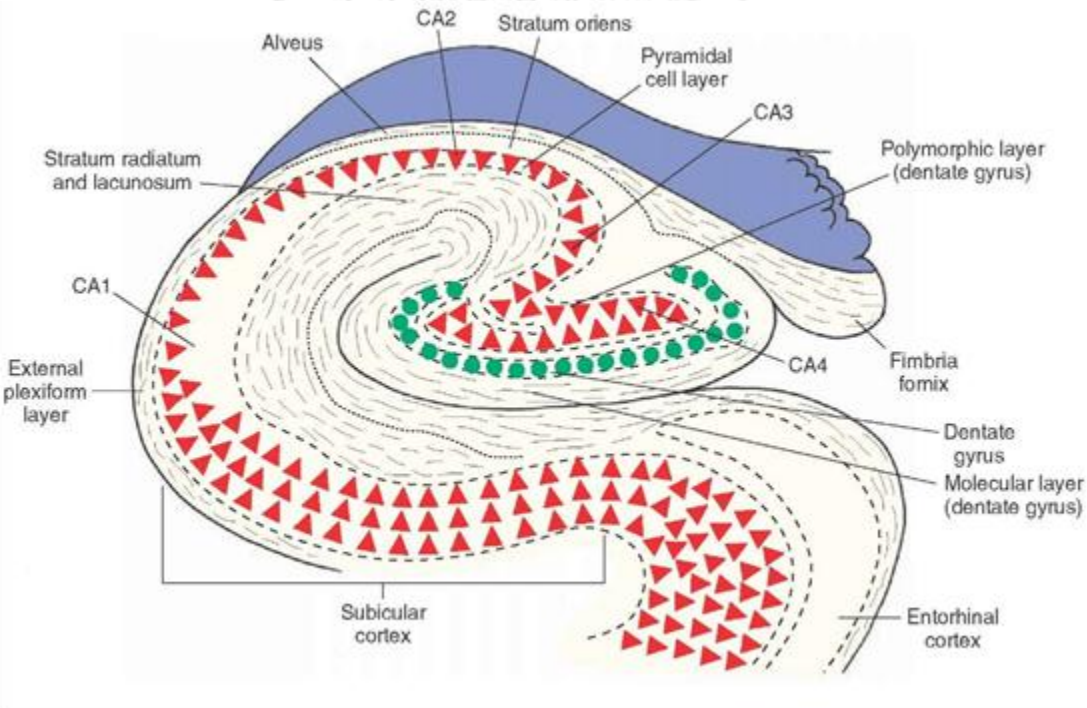

Figure 2.4 Hippocampal anatomy. The histological appearance of the cell layers within the hippocampal loci of the hippocampal field, dentate gyrus and subicular cortex. (Available from: http://what-when-how.com/neuroscience/the-limbic-system-integrative-syatems-part-1/)

2.2. Hippocampal pathway

The hippocampus has a unique pattern of connectivity. The tri-synaptic circuit is the main pathway of the hippocampus (Amaral, 1993; Henze et al., 2000; Taupin, 2007) (Figure 2.5). Major inputs to the hippocampus originate from the entorhinal cortex and synapse on all subfields. The first link of the tri-synaptic circuit responds to the main afferences of the hippocampus, the pyramidal cells layer II of the entorhinal cortex. Their axons, the perforant fibers, project to the dentate granule cells through the hippocampal fissure. The hippocampal fissure is a cell-free natural division that separates the DG from the CA1 region. The granule cells project their axons, the moss fibers (MFs), to the dendrites of the pyramidal cells of CA3 forming the second link of the circuit. The pyramidal cells of CA3 send collaterals, the collateral of Schaeffer, to the pyramidal cells of CA1 forming the third link of the hippocampal tri-synaptic circuit. Thus, the tri-synaptic organization of the main cell layers of the hippocampus implies the information from the enthorhinal cortex flows through the hippocampus primarily unidirectionally. The tri-synaptic circuit is organized near-transverse band or lamella corresponding to a functional unit of the hippocampus. Lateral projections connect each lamella with each other along the septotemporal axis. The fimbria/fornix is the main output of the hippocampus (Allen & Fortin, 2013).

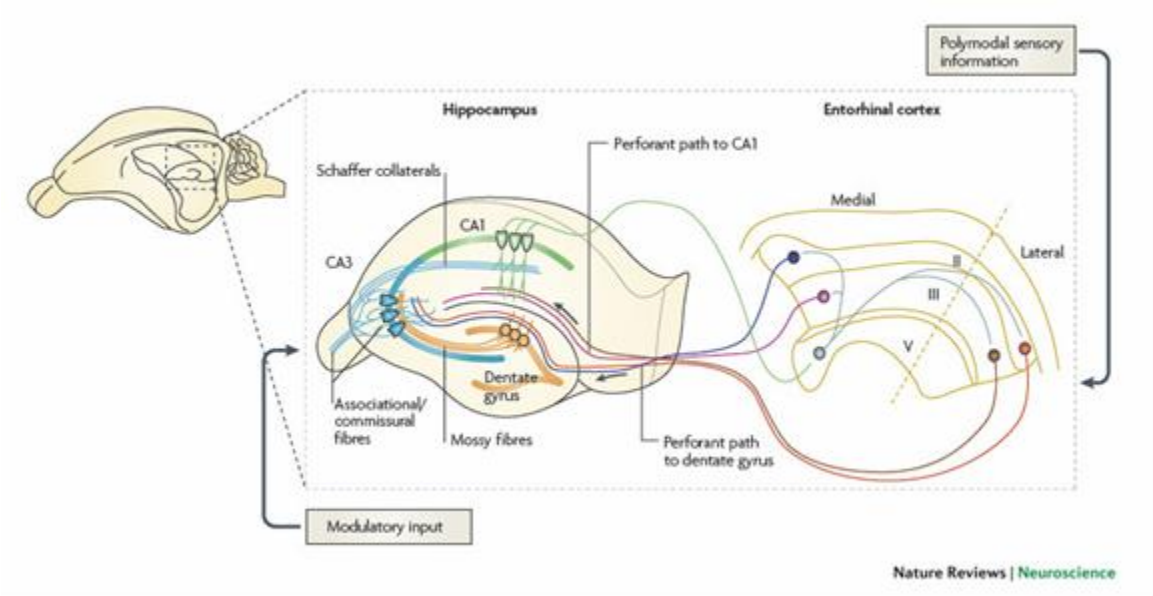

Figure 2.5 The classical image of the tri-synaptic hippocampal circuit (Neves et al., 2008).

2.3. Memory

Memory is essential to behavior, enabling organisms to draw on past experience to guide choices and actions. There are two types of memory; declarative memory and nondeclarative memory. Declarative memory (explicit memory, relational memory) is a brain-system construct, referring to memory that is dependent on the integrity of the hippocampus and anatomically related structures in the medial temporal lobe and the diencephalon. Declarative memory is further divided into episodic and semantic components. Episodic memory refers to autobiographical memory for events that occupy a particular spatial and temporal context, while semantic memory refers to general knowledge about the world. When memory is impaired, the ability to acquire new semantic knowledge through repetition will always exceed the ability to acquire episodic memory because episodic memory is by definition of time and place and cannot be repeated. Nondeclarative (implicit) memory is a heterogeneous collection of separated abilities that can be additionally dissociated form each other, including associative learning, word priming, perceptual learning, stimulus-response habits and motor learning. The procedural memory processes are thought to operate automatically and do not include information about where or when an event or learning experience took place. These memory abilities depend on brain systems outside of the medial temporal lobe and diencephalon (Squire et al., 1993; Good, 2002). Many previous studies indicate that the hippocampus contributes to the encoding and storage of declarative memory, especially episodic memories (Tulving & Markowitsch, 1998; Good, 2002). Then, the Morris water maze has been introduced as an instrument with particular sensitivity to determine the effects of hippocampus lesions in rats.

3. Morris water maze

The Morris water maze is a device to access the rat's ability to learn to navigate to a specific location in a relatively large spatial environment. It was designed by Richard Morris (Morris, 1981; Morris et al., 1982). It is a useful behavioral procedure which is widely used in behavioral neuroscience to study spatial learning and memory. It is used to measure the effect of neurocognitive disorders on spatial learning and the evaluation of possible neurocognitive treatments. The Morris water maze has proven to be a robust and reliable test that is strongly correlated with hippocampal synaptic

plasticity and N-methyl-D-aspartate (NMDA) receptor function. There is extensive evidence of its validity as a measure of hippocampal dependent spatial navigation and reference memory. The Morris water maze learning impairments are independent of locomotor effects because land-based locomotor reductions do not affect swimming speed (Vorhees & Williams, 2006). Lesions in distinct brain regions like the hippocampus, striatum, basal forebrain, cerebellum and cerebral cortex are shown to impair Morris water maze performance (Gallagher et al., 1993; D'Hooge & De Deyn, 2001).

The Morris water maze apparatus consists of a large circular pool with water. The pool is divided into four quadrants: North, South, East and West. The platform is positioned in the middle of one of the quadrants. The platform is usually located half-way between the center and the wall. There are many procedures of Morris water maze test for neurobehavioral laboratory but the most basic Morris water maze procedure is spatial (hidden-platform) acquisition. In principle, the animal must learn to use visual cues to navigate a direct path to the hidden platform when started from different quadrants. Most protocols use four start quadrants: North, South, East and West. Animals are given a series of daily trials using a random or semi-random set of start locations. One trial each day is from each of the four positions (Vorhees & Williams, 2006). In the typical "hidden-platform" version of task, rats are trained to find a hidden (invisible) escape platform that is positioned beneath the water surface. Rats are placed into a large circular pool of water from which they can escape onto a hidden platform. Rats can escape from the water by swimming randomly or in unsystematic search paths throughout the pool; but in practice, normal rats learn very quickly to swim directly towards the platform from any starting position at the circumference of the pool. Swimming activity (latency, distance and strategy of search) of each trial is monitored via video camera. Four strategies of swimming pattern have been described by Feng et al. (2004); line, taxis, circle and random (Figure 2.6).

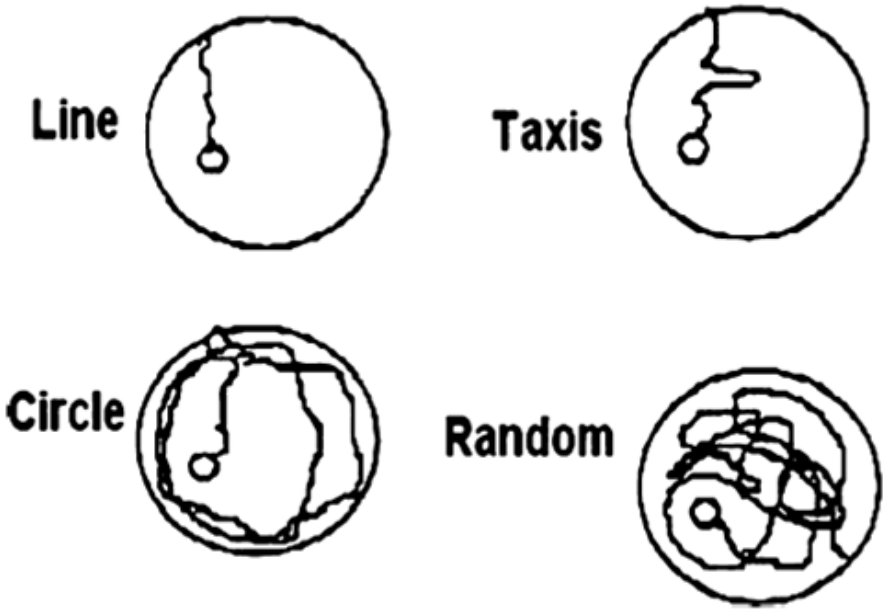

Figure 2.6 Four typical strategies (line, taxis, circle, and random) employed by the rats to find the hidden platform in the Morris water maze (Feng et al., 2004).

4. Hypothalamic-pituitary-ovarian axis and estrogen

Estrogen is an important hormone that regulates multiple tissues and functions in the body. It influences growth, differentiation, maturation, and function of many different tissues including cells of the central and peripheral nervous system. Classically, estrogen is considered as a "reproductive hormone", due to its well-known role in feedback signaling in the hypothalamic-pituitary-ovarian axis (Figure 2.7). The hypothalamus is the site of production of the peptide hormone gonadotropin releasing hormone (GnRH) which is transported to the anterior pituitary gland by a short portal venous system where it stimulates the synthesis and release of gonadotropic hormone, LH and FSH. LH and FSH are glycopeptides consisting of two peptide chains (α and β). The pituitary gonadotropin LH and FSH act on the ovaries to promote gametogenesis and sex steroid production. Steroid feedback can be positive, as demonstrated by the estrogen and LH surge at ovulation, or negative, as demonstrated by the rise in LH and FSH levels after gonadectomy or reductions in steroid levels (Shupnik, 1996).

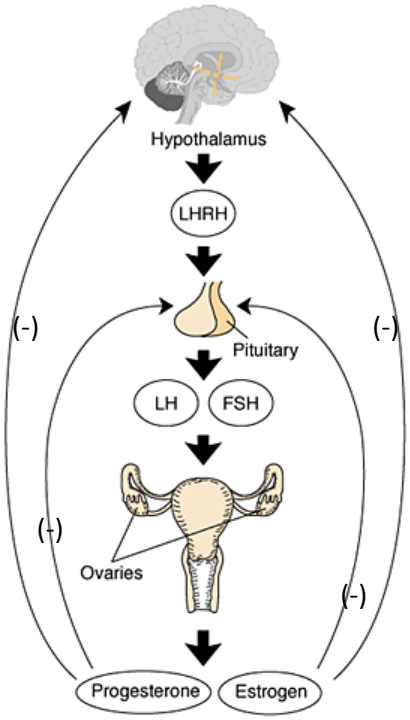

Figure 2.7 The hypothalamic-pituitary-ovarian axis and the negative feedback by estrogen and progesterone (Emanuele et al., 2002).

4.1. Estrogen receptors and their mechanism of actions

Estrogen receptors (ERs) are member of a family of nuclear transcription factors or nuclear receptor. There are 2 subtypes of ERs found in target tissue: ERα and ERβ (Hewitt & Korach, 2002). ERα is the most abundant in the breast, uterus, cervix and vagina, and small quantities are detected in the ovaries, mammary gland, bones and hypothalamus. ERβ exhibits limited expression and is the highest in ovarian granulose cells and gastrointestinal tract, whereas it is moderate to low in mammary gland, hypothalamus and pituitary gland (Hall et al., 2001; Kim & Park, 2012). Recently, Hawkins et al. (2000) show the third estrogen receptor, named ERγ, which is found in teleost fish (Atlantic croaker *Micropogonias undulates*).

There are two molecular mechanisms of estrogen signaling: genomic estrogen mechanism, including ligand-dependent, ligand-independent and DNA binding-independent, and non-genomic mechanism or cell-surface signaling (Hall et al., 2001). **The ligand-dependent mechanism** is the classical mechanism of estrogen action. The binding of ligand induces an activating conformational change within ER and promotes homodimerization and high affinity binding to specific DNA response

elements (EREs), which are cis-acting enhancers located within the regulatory regions of target gene. The DNA-bound receptors contact the general transcription apparatus either directly or indirectly via cofactor protein. Then, the DNA-bound receptor exerts either a positive or negative effect on the expression of the downstream target gene, depending on the cell and promoter context. **The ligand-independent mechanism** of ER is the cross-talk with peptide growth factors. This mechanism can be modulated by extracellular signals in the absence of estrogen. The polypeptide growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1), can activate ER and increase the expression of ER targeted genes. However, the molecular mechanism and the biological role of these processes are not entirely clear. These two pathways are the regulation of genes in which a functional ERE-like sequence can be documented within the promoter. In controversy, the DNA-binding independent mechanism is an agonist-bound ER which led to gene regulation in the absence of direct DNA binding. **Non-genomic mechanism** is rapid biological effects of estrogen which is associated with cell membrane receptors such as G protein-coupled receptors (GPCRs), ion channels or enzyme-linked receptors. This mechanism activates the intracellular signal transduction protein by linking mitogen-activated protein kinase activation (MAPK) to adenylate cyclase and protein kinase C (PKC). The non-genomic mechanism of estrogen is found to play a prominent role in the modulation of steroid non-target tissues such as cardiovascular system, bone and central nervous system (Hall et al., 2001; Acconcia & Marino, 2003; Simoncini & Genazzani, 2003).

4.2. Estrogens and non-reproductive functions

In the past decades, many publications have indicated a new role of estrogen on the “non-reproductive” effects in the brain, especially the neuroprotective and neurotrophic/synaptic plasticity actions (Brann et al., 2007). In the female, the consequence of aging is the reduction of estrogen and entering into the menopause phase. Menopause is a gradual process of changes in cessation of menses, profound reductions in ovarian hormone levels, and irreversible ovarian failure. Estrogen loss, either from natural or surgical menopause, has been associated with a decline in cognitive function. Therefore, postmenopausal women have a risk for neurodegenerative diseases, especially Alzheimer’s disease (Simpkins & Singh, 2008).

4.3. Estrogen and neurodegenerative diseases

Estrogen is an important hormone that regulates multiple tissues and functions in the body. Classically, estrogen is considered as a "reproductive hormone", due to its well-known role in feedback signaling in the hypothalamic-pituitary-ovarian axis. In the last decade, the "non-reproductive" effects of estrogen in the brain, specifically on the neuroprotective and neurotrophic/synaptic plasticity actions have been shown (Brann et al., 2007). Many publications show that the ERs are located in the brain. ERα and ERβ can be found throughout the cerebral cortex, hippocampus, basal forebrain, and amygdala of the mouse, rats, non-human primates and humans (Milner et al., 2005; Milner et al., 2001; Osterlund et al., 2000; Shughrue et al., 1997a; Shughrue et al., 1997b). Besides, estrogen synthesis in the brain was concurrently reported. The first publication of the neurosteroidogenesis in the rat brain was reported by Kimoto et al. (2001). They reported that the hippocampal neurons synthesize neurosteroids under the stimulation and regulation of glutamate-mediated synaptic communication. Hojo et al. (2004) demonstrated that hippocampal neurons of male rats could synthesize estradiol from pregnenolone by cytochrome P45017α and P450 aromatase. They suggested that this synthesis may be regulated by a glutamate-mediated synaptic communication that evoked Ca^{2+} signals.

Although the risk of neurodegenerative diseases is associated with age-related loss of sex hormones in both women and men, the epidemiology of the disease found in female is higher than in male. Therefore, the neuromodulatory actions of estrogen have been extensively investigated. Female rats and mice show memory decline in association with the loss of estrous cycling tested by Morris water maze. These were observed after 17β-estradiol (E_2) loss by 12 months in rats and 17 months in mice (Markowska, 1999; Frick et al., 2000). Plasma level of E_2 is lower in women with Alzheimer's disease in comparison to age-matched control (Manly et al., 2000), and long-term estrogen replacement therapy reduced risk of Alzheimer's disease (Zandi et al., 2002). Unfortunately, estrogen replacement therapy is also associated with increased incidence of stroke and breast cancer (Pike et al., 20009).

Observing the mechanisms of actions of estrogens on neuronal cells, the three major circulating estrogens, estrone, E_2, and estriol, dose-dependently reduce Aβ fibrillation in *ex vivo* studies (Morinaga et al., 2007). E_2 reduced Aβ in HEK293 cells (Chang et al., 1997), increased soluble APP in ZR-75-1 human carcinoma cells (Jaffe et

al., 1994), and accelerated APP trafficking in the trans-Golgi network in vesicles derived from neuroblastoma cells or primary neurons. Thereby estrogens prevent neurodegeneration by reducing the production of Aβ (Greenfield et al., 2002).

It is well known that menopause shifts the balance of the hypothalamic-pituitary-ovarian axis feedback loop and this shift results in an increase in the production of gonadotropins (LH and FSH) along with a decrease in gonadal inhibin production. In women, these changes can be attribute to the loss of negative feedback on LH and FSH by estrogen and inhibin, and result in a 3- to 4-fold increase in serum LH level and a 4- to 18-fold increase in serum FSH level (Chakravarti et al., 1976). Therefore, the effects of increased circulating LH and FSH levels due to the loss of negative feedback of estrogen on the aging brain are speculated. However, only LH receptors have been detected in the brain and the highest density is found within the hippocampus, a region devastated in Alzheimer's disease (Lei et al., 1993). Thus, LH may play an important role in the onset of neurodegenerative diseases.

LH also has the mitogenic capacity and could initiate the cell cycle abnormalities which found in Alzheimer's disease-affected neurons. LH increased amyloidogenic processing of APP of neuroblastoma cell, and pharmacologic suppression of LH reduces plaque formation in transgenic mice model of Alzheimer's disease (Casadesus et al., 2005). The experimental ablation of LH by a selective GnRH agonist (leuprolide acetate) improved Y-maze performance and decreased Aβ load in the hippocampus of APP transgenic mice (Tg2576) (Casadesus et al., 2006). Additionally, Tg-LHβ transgenic mice showed a decreased Y-maze performance when compared to aged-matched wild-type animals (Casadesus et al., 2007). They suggested that the increased LH level may be one of the factors associated with the cognitive decline after menopause.

5. Phytoestrogens

It is well known that estrogens affect cognitive behavior in humans and related with neurodegenerative diseases in aged women. Although estrogen has been used to prevent the symptom, long-term use of estrogen is associated with an increased risk of breast cancer (Ravdin et al., 2007). Therefore, many researchers have focused on the development of alternatives that can mimic estrogenic activities and have no undesirable side effects. Phytoestrogens are a diverse group of plant-derived

compounds that occur naturally in plants which have a similar structure with the E_2 and can bind to both ERα and ERβ (Vitale et al., 2013). Besides, phytoestrogen treatment after menopause offers protection from cardiovascular disease, reduces the extent of osteoporosis, improves cognitive function and relives menopausal symptoms associated with acute ovarian estrogen loss (Breckwoldt et al., 1995). Based on the advantage of phytoestrogens, many women turn to use phytoestrogens as an alternative estrogen therapy.

5.1. Dietary sources of phytoestrogens

Hundreds of foods have been shown to contain phytoestrogen compound. The main dietary sources of phytoestrogens are soybean, chickpea, and alfalfa sprouts (Dixon, 2004). Phytoestrogens can be classified as isoflavonoids, flavonoids, stillbenes, and lignans (Dixon, 2004). The most extensively studied phytoestrogens are isoflavones, and the major ones are genistein and daidzein (Figure 2.8) (Kim & Park, 2012). The isoflavonoids are mostly limited to the subfamily Papilionoideae of the Leguminosae (Bedell et al., 2014).

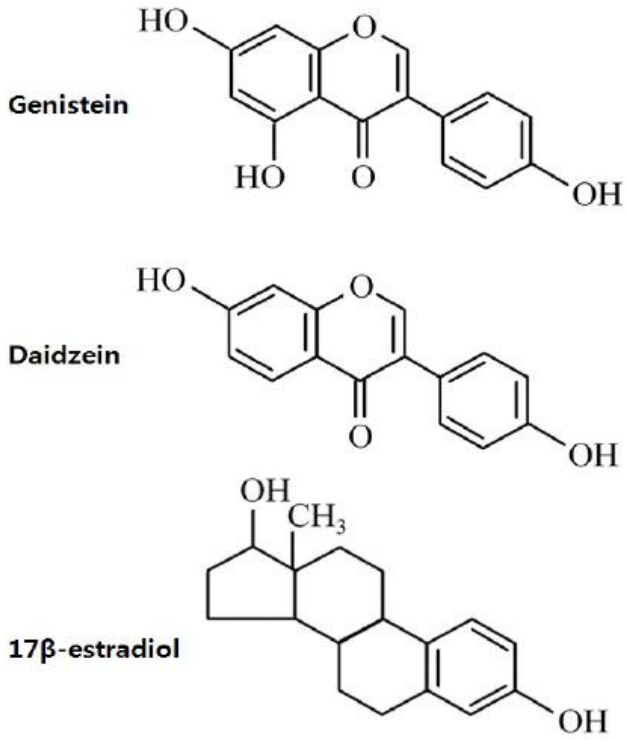

Figure 2.8 The chemical structures of 17β-estradiol, genistein and daidzein isoflavones (Kim & Park, 2012).

After consumption of phytoestrogens, enzymatic metabolic conversion occurs in the gut, and heterocyclic phenols are formed. Isoflavones are metabolized to aglycones, genistein and daidzein, lignans to secoisolariciresinol-digluciside (SDG), and coumestans to coumestol. The breakdown products all contain a phenolic ring that can compete for binding to the "pocket" of ERs (Figure 2.9). These compounds thereby structurally resemble estrogen and have weak estrogenic activity (Bedell et al., 2014).

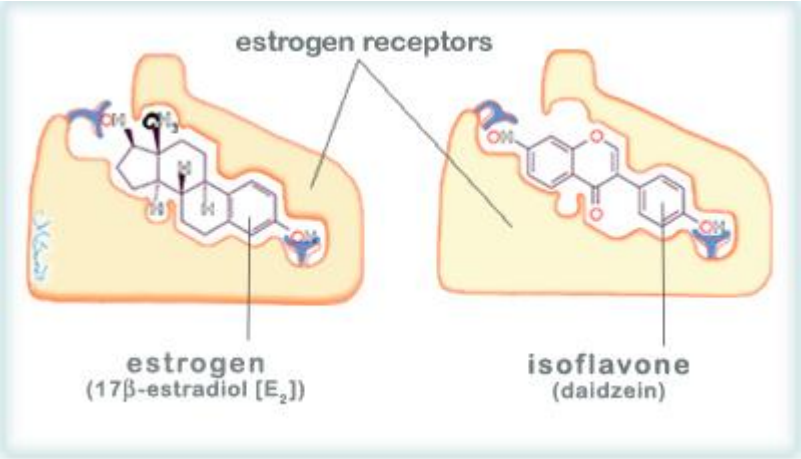

Figure 2.9 Binding of isoflavone and estrogen to estrogen receptors.
(Available in: http://www.womenshealthnetwork.com/menopauseandperimenopause/redcloverformenopausalsymptoms.aspx)

5.2. Mode of phytoestrogen actions

Phytoestrogens have stable structure and low molecular weight therefore they can pass through cell membrane and bind with ERs. Kuiper et al. (1998) indicated that phytoestrogens appear to have a greater affinity for ERβ than for ERα. Genistein has a binding affinity of 87% for ERβ and 4% for ERα, and daidzein is 0.5% for ERβ and 0.1% for ERα. Phytoestrogens can express either agonistic or antagonistic effects depending on its own concentration (Ososki & Kennelly, 2003). At low concentration, genistein appears to act as agonist, stimulates the proliferation of estrogen-dependent breast cancer cells, whereas high concentration inhibits estradiol-stimulated cell growth (Martin et al., 1978).

5.3. Phytoestogens and neurodegenerative diseases

Therapeutic effects of phytoestrogens on neurodegenerations have been widely assessed (Bingham et al., 1998; Howes et al., 2003). All results indicate that phytoestrogens have neuroprotective effects and can improve the memory impairment.

Genistein protects neuronal apoptosis induced by $A\beta_{25\text{-}35}$ in hippocampal neurons (Zeng et al., 2004). Oral administration of 15 and 30 mg/kg of genistein in OVX rats for 6 weeks can attenuate the increased number of neuron apoptosis (Huang & Zhang, 2010). Subcutaneous injection of 5% genistein solution can return the synaptic

connections between hippocampal neurons and improve the ability of learning and memory in OVX rats (Xu et al., 2007). Genistein prevents $A\beta_{(1-40)}$-induced impairment of short-term spatial recognition memory in a Y-maze, and learning and memory in the passive avoidance test in adult male rats (Bagheri et al., 2011). Pretreatment with a single dose of 10 mg/kg of genistein shows protective effect against the development of Parkinson's disease in 6-Hydroxydopamine Hemi-parkinsonian rat (Baluchnejadmojarad et al., 2009).

Puerarin is the main active component which is found only in *Pueraria* plants, belonging to isoflavonoid category (Malaivijitnond, 2012). Previous studies indicate that puerarin has neuroprotective effects on learning and memory dysfunction induced by estrogen deprivation in female mice (Xu et al., 2004) and anti- apoptosis in male rat (Xu et al., 2005). Puerarin associated with changes of synaptic structural modification in the hippocampus of OVX rats leads to memory improvement (Xu & Zhang, 2007). Oral administration of puerarin potentially ameliorated the impairment of spatial memory induced by $A\beta_{(1-42)}$ in rats by attenuating neuronal apoptosis (Li et al., 2010). The attenuation is associated with the activation of Akt (Akt signaling pathway) and phosphorylation of Bad; the Bcl-2 family which is involved in the regulation of apoptosis. Zhang et al. (2011) also found that puerarin decreased Bax/Bcl-2 ratio in neuronal cell hybrid model of sporadic Alzheimer's disease (SAD).

6. *Pueraria mirifica* plant

Pueraria mirifica or white Kwao Krua is a traditional herbal plant found in Thailand. Taxonomically, *P. mirifica* belongs to the family Leguminosae, subfamily Papilionoideae. It is considered as a rejuvenating agent in Thai folklore medicine. *P. mirifica* is a woody perennial climber plant (Figure 2.10a) and mainly grows in deciduous forest. The flowers are bluish purple in color with a long inflorescence (Figure 2.10b). In Thailand, there are two varieties of white Kwao Krua; *P. candollei* var. candollei (syn. *P. candollei*) and *P. candollei* var. *mirifica* (syn. *P. mirifica*). The bioactive compounds in *P. mirifica*, phytoestrogens, are found in the tuberous root. Therefore, its globular or pear-shaped tuberous roots have been used for their rejuvenating properties (Figure 2.10c) (Ingham et al., 2002; Malaivijitnond, 2012).

Figure 2.10 *P. mirifica* (a) cultivated in north-eastern Thailand, and its leaves, flowers (b) and tuberous roots (c) (copy from Malaivijitnond, 2012).

In folklore remedy, the ordinary dose of *P. mirifica* for women and men is about a peppercorn-sized piece, equivalent to about 250 mg/50 kg BW or 5 mg/kg BW, taken once a day at night. It is used as an anti-wrinkle agent for wrinkled skin, to darken white hair and increase hair growth, alleviate cataracts in the eyes, help with memory loss, increase energy and vigor, provide more reflexive bodily movement, increase blood circulation and appetite and alleviate sleep disorder (Suntara, 1931; Malaivijitnond, 2012). Therefore, there are many commercial brands of dietary supplement and cosmetics produced from *P. mirifica*, owing to its rejuvenating and anti-aging effects (Maruyama et al., 2014). In Thailand, the distribution of *P. mirifica* is commonly and abundantly found in the north, west and north-east. Determination of estrogenic activity of wild *P. mirifica* collected from 25 of 76 provinces in Thailand by vaginal cornification assay indicates that the plants of the Central-West region on Thailand showed the highest estrogenic activity (Cherdshewasart et al., 2007a). The plant

genetics and environment could influence the accumulated amount of isoflavonoids in the plant tubers collected from the different sites (Cherdshewasart et al., 2007d).

6.1. Composition of phytoestrogens in *P. mirifica*

Using HPLC technique to determine the phytoestrogen composition in the tuberous root of *P. mirifica***,** three main classes of phytoestrogen compound; isoflavonoids (daidzein, daidzin, genistin, genistein, kwakhurin, kwakhurin hydrate, tuberosin, puerarin, mirificin and puermiricarpene), coumestans (coumestrol, mirificoumestan, mirificoumestan hydrate and mirificoumestan glycol), and chromemes (miroestrol, deoxymiroestrol and isomiroestrol) are isolated (Figure 2.11) (Chansakaow et al., 2000; Cherdshewasart & Sriwatcharakul, 2007c; Cherdshewasart et al., 2007d; Ingham et al., 1986, 1988, 1989; Yusakul et al., 2011) (Figure 2.11).

Chromenes and isoflavonoids are the major active compounds in tuberous root of *P. mirifica*. Deoxymiroestrol is claimed to be as the actual phytoestrogen of *P. mirifica*. However, miroestrol was the first phytoestrogen isolated from *P. mirifica* and showed the highest estrogenic potency (Cain, 1960). The study of Chansakaow et al. (2000), shows that miroestrol and isomiroestrol are artifacts produced by the air oxidation of deoxymiroestrol during isolation work.

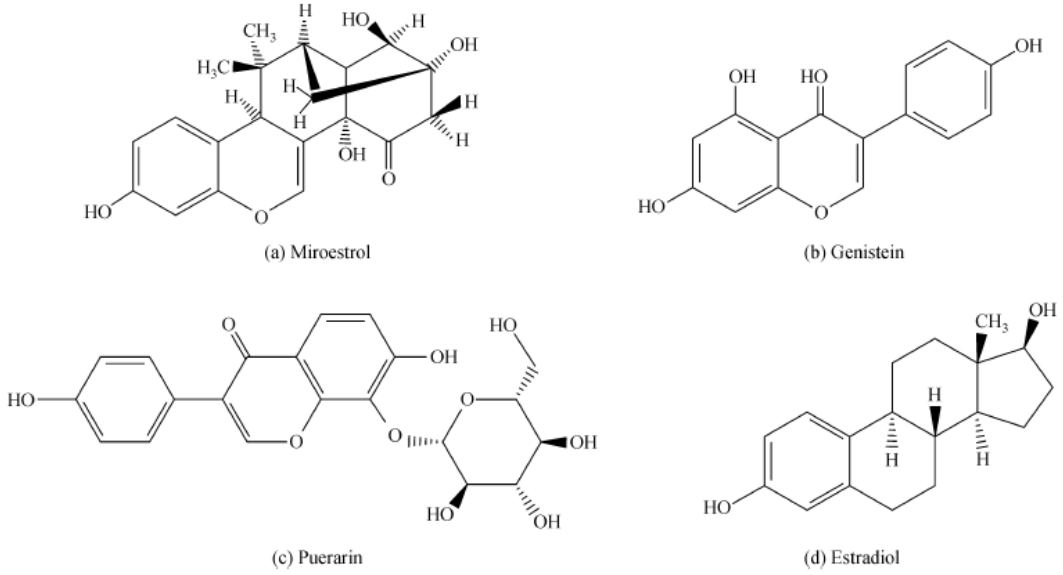

Figure 2.11 Comparison of chemical structure of some phytoestrogens found in *P. mirifica* with estradiol (copy from Malaivijitnond, 2012).

6.2. *P. mirifica*, estrogenic activities and toxicity

The estrogenic activity of *P. mirifica* was investigated in rodents and non-human primates. By oral administration, the OVX rat could exhibit vaginal cornification and increase uterine weight at dosages of 100 and 1000 mg/kg BW of *P. mirifica* treatment, while increase in epididymis weight was found in orchidectomized rat at a dosage of 1000 mg/kg BW. Moreover, the treatment of 1000 mg/kg BW of *P. mirifica* attenuated the gonadotropin (LH and FSH) levels at one week after treatment and it was recovered within 2 weeks in female and 1 week in male (Malaivijitnond et al., 2004; Cherdshewasart et al., 2007a). Moreover, *P. mirifaca* can prevent bone loss in OVX (Urasopon et al., 2007) and orchidectomized rats (Urasopon et al., 2008b) . In non-human primate study, a single high dose of *P. mirifica* (1000 mg) can disturb ovarian function and menstrual cycle in normal cyclic monkey (*Macaca fascicularis*) by increasing the peroid of the follicular phase and menstrual cycle (Trisomboon et al., 2004). Additionally, *P. mirifica* also increased reddish color of sexual skin in aged menopausal cynomolgus monkeys (Trisomboon et al., 2006a).

Based on Pubmed database, two publications of *P. mirifica* and neurons were found recently. One publication indicated the effect of *P. mirifica* increasing the level of synaptophysin immunoreactivity in hippocampal primary cell culture (Chindewa et al., 2008). The other study showed antioxidative activities of *P. mirifica* extract on HT22 neuronal cells (Sucontphunt et al., 2001). Their results indicated the antioxidant and neuroprotecitve activity of *P. mirifica* extract against glutamate-induced toxicity in HT22 cells at the concentrations of 10 and 50 μg/ml. They proposed the possible mechanism that the neuroprotection exerted by *P. mirifica* extract was related to its scavenging activity against hydrogen peroxide (H_2O_2) and related reactive oxygen species. These activities may be the result of the combination effects of various *P. mirifica* extract constituents.

Additionally, the toxicity of *P. mirifica* was investigated in mice and rats by oral administration of its tuber suspension (Chivapat et al., 2000). The LD_{50} value was greater than 16 g/kg and there was no sign or symptom of acute toxicity in mice. Subchronic toxicity was studied in both sexes of Wistar rat at the dosages of 10, 100 and 1000 mg/kg/day of *P. mirifica* for 90 consecutive days. The results indicated that the growth rate and food consumption were significantly lower than the control group at the dosages of 100 and 1000 mg/kg/day of *P. mirifica* treated. Moreover, these dosages did

not cause any abnormalities of hematological or biochemical parameters and any histopathological changes of the visceral organs.

CHAPTER III
A SIMPLE, SENSITIVE AND RELIABLE *IN VIVO* ASSAY TO EVALUATE THE ESTROGENIC ACTIVITY OF ENDOCRINE DISRUPTORS

Introduction

Estrogen disrupting compounds (EDCs) encompass a variety of chemical classes, including drugs, pesticides, industrial by-products, pollutants, and naturally produced botanical chemicals. Since these compounds are fat soluble, it is likely they accumulate from the environment in the fatty tissue of animals or humans exposed to them, and consequently generate outbursts on health effects. Generally, EDCs occur in the very low dose in the environment and are difficult to detect (Propper, 2005; Gore, 2007).

There are two models in determining the estrogenic activity, *in vitro* and *in vivo* assays. In *in vitro* assays, various kinds of estrogen responsive cancer derived cell lines, e.g. MCF-7 human breast cancer cells (Soule et al., 1973; Cherdshewasart et al., 2008c), HeLa cervical cancer cells (Cherdshewasart et al., 2004), HepG2 hepatocarcinoma cells (Lee et al., 2002), CV-1 monkey kidney cells (Okamura et al., 2008) or yeast cells (Boonchird et al., 2010) have been widely used. This assay is based on the ERs binding assay, such as the relative binding affinity assays, and an ER transcriptional activation assay, including cell proliferation assays. Although the *in vitro* assay for estrogenic activity of EDCs is rapid and convenient, and quite applicable for commercial scale assays, it has some disadvantages. For example, using the *in vitro* MCF-7 proliferation assay to determine estrogenic activity levels may not represent the estrogenic response in animals including humans due to the lack of bioavailability and biotransformation (absorption, distribution, metabolism, and excretion or ADME) of the chemicals in whole organisms, and the differences in ER types and expression levels between MCF-7 cells and those in the different tissues of the target organism. Furthermore, the evaluation of the estrogenic activity of EDCs using different estrogen responsive cell lines yielded significantly different results for each chemical between the different cell lines (Cherdshewasart et al., 2008c; Boonchird et al., 2010).

To avoid these pitfalls, *in vivo* methods are often used. The most popularly used *in vivo* assays to assess the estrogenic activity of EDCs are uterotropic and vaginal

cornification assay (Gray et al., 2004; Malaivijitnond et al., 2004; Malaivijitnond et al., 2006; Cherdshewasart et al., 2007a; Sookvanichsilp et al., 2008; Urasopon et al., 2008a; Cherdshewasart et al., 2008b; Siangcham et al., 2010). This is based on the principle that the growth phase of the uterus and vagina in the natural estrous cycle is under the control of estrogens. Uterine growth during the natural estrous cycle is rapid and easily measureable within two days. When the endogenous estrogens are not available, either because the animal is immature or because it has been OVX, then the growth of the uterus becomes sensitive to external sources of estrogen. When the animals are exposed to those chemicals, the uterus of immature or OVX animals can increase in size and weight due to the imbibitions of fluid and cell proliferation stimulated by estrogen. Therefore, the end point of this uterotropic assay is uterine weight, dry or wet weight. The vaginal cytology assay or Allen-Doisy test is used to track changes in the morphology of desquamated vaginal epithelium cells and provides convenient means of assaying changes in estrogen levels. The vaginal epithelium cell is responsive to sex steroids, particularly estrogens, and rising levels of estrogens cause the vaginal epithelium to become "cornified" cells.

Thus, this study aims to search for the inexpensive, simple, sensitive and reliable *in vivo* assays, e.g. uterotropic and vaginal cytology assays, which can be used to determine the estrogenic activity of EDCs. Moreover, recent publications indicate that body weight of OVX rats significantly change after EDCs treatment (Malaivijitnond et al., 2010; Tsai et al., 2010; Yepuru et al., 2010, Zhang et al., 2010), thus this study also assessed if changes of the body weights can be used as one of the *in vivo* assays of estrogenic activity. The E_2 was used as a standard chemical. The *P. mirifica* plant was selected as a representative EDC for this study, because the estrogenic activity of this plant has been widely tested (Chansakaow et al., 2000; Cherdshewasart et al., 2004; Cherdshewasart et al., 2008c; Malaivijitnond, 2012; Sookvanichsilp et al., 2008).

Materials and Methods

Animals

Adult female Wistar rats, 7 weeks old, were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed in stainless steel cages with sawdust bedding with five animals/cage in a room with controlled lighting (light on 06:00-18:00 h) and temperature (25 ± 1 °C) at the

Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. The animals were fed with soybean fee-rat diet (Pokaphan Animal Feed Co., Ltd., Thailand) and water *ad libitum*. All animals were acclimatized to the surroundings for one week before the onset of the study. The experiment protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 0823010.

The preparation of E_2

The powder of E_2 (Fluka, China) was weighed and dissolved in absolute ethanol. After the powder was completely dissolved, the distilled water was added and the solution was allowed to stand at room temperature to evaporate the ethanol. The final concentrations of 0.01, 0.1, 1, 10 and 20 mg/kg BW/day were prepared and kept in the dark bottle at 4 °C until used.

The preparation of *P. mirifica* suspensions

The tuberous roots of *P. mirifica* were purchased from Dr. Sompoch Tubcharoen, Kasetsart University Kampang Sean Campus, Thailand. It was authenticated as the *P. mirifica* by comparing with the voucher specimen numbers BCU010250 and BCU011045, from Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University. The *P. mirifica* roots used throughout this study were from the same lot. The roots were sliced and dried at 70-80 °C, pulverized in a mortar, filtrated through a 100 μm mesh, and the powders were kept in dark bottles. The *P. mirifica* suspension at concentrations of 100 and 1,000 mg/kg BW/day in 1 ml distilled water was prepared for this study. These concentrations were completely verified for their estrogenic activity on reproductive organs (Malaivijitnond et al., 2004; Trisomboon et al., 2005; Malaivijitnond et al., 2006; Trisomboon et al., 2006b; Cherdshewasart et al., 2007a; Cherdshewasart et al., 2008b) However, their estrogenic activity in comparison to the E_2 has never been estimated.

HPLC analysis of isoflavone contents in *P. mirifica* extract

The *P. mirifica* powder was extracted twice by soxhlet extraction process using 95% ethanol as a solvent (1:3, w:v). The extraction was carried out at 80 °C, 8 hours in each time. The two-time extracted solutions were mixed together and filtered by Whatman no.4 paper using a vacuum pump. The extracted solution was then evaporated by rotary evaporator at 50 °C, 70 rpm with a pressure of 300-100 bar. The crude extraction was evaporated again at 60 °C using a water bath until the extracted weight was not changed. The *P. mirifica* extract was kept in a dark bottle and stored in a refrigerator at 4 °C until analyzed for isoflavone contents by high performance liquid chromatography (HPLC) technique.

The concentrations of five isoflavones, puerarin, daidzin, genistin, daidzein, and genistein, were determined by HPLC (Malaivijitnond et al., 2004). Twenty microlitters of the extract solution was injected through a sensory guard column into a HPLC system equipped with a reverse phase Symmetry C18 column. Mobile phase consisted of 0.1% v/v phosphoric acid and acetronitrile with gradient elution. Ultraviolet detection was performed at a wavelength corresponding to the most intense absorption maximum at 255 nm. The determination was done twice. The retention times and the amounts quantifying from the peak area of the standard curves were calculated.

Experimental design

At the age of 8 weeks, the rats were bilaterally OVX and divided into three treatment groups; control, E_2 and *P. mirifica*. The experiment schedule was divided into two periods; pre-treatment and treatment (14 days for each period). During the treatment period, the rats were gavaged daily with 1.0 ml of distilled water (DW) for the control group, 0.01, 0.1, 1, 10 and 20 mg/kg BW/day of E_2 (E-0.01, E-0.1, E-1, E-10 and E-20, respectively) for the E_2 group, and 100 and 1,000 mg/kg BW/day of *P. mirifica* (PM-100 and PM-1000, respectively) for the *P. mirifica* group. The gavage administration was selected for this study as to mimic the usual route that human intakes EDCs in their daily life. Eight rats were used in each group. The treatment was performed at 09:00-11:00 h. At the end of the 28-day study period, each group of rats was euthanized under ether, and the uterus was dissected and weighed (Malaivijitnond et al., 2010).

Measurement of body weight

The body weights were measured and recorded weekly. The body weight changes during the experiment period were calculated by the following equation.

$$\left[\frac{body\ weight\ at\ D_x - body\ weight\ at\ D_0}{body\ weight\ at\ D_0}\right] x\ 100$$

Uterotropic assay

After weighing, the uterus was then fixed in 10% (w/v) neutral buffered formalin solution and manipulated for histomorphometric analysis (Malaivijitnond et al., 2010). The middle region of the formalin-fixed uterus were selected and embedded in paraffin and sectioned in 5 μm thickness. All sections were stained with hematoxylin and eosin following standard procedure (Humanson, 1972). The uterine, myometrium and endometrium areas were determined by the Image-Pro Express program (Media Cybernetics, Inc., USA). The uterine glands were examined and counted under the Olympus compound light microscope using 40x magnifications. Five sections of uterus in each rat (in total, 40 sections/group) were randomly selected and determined.

Vaginal cytology assay

Vaginal epithelial cells were monitored daily at 07:00-09:00 h. The vaginal cells observed under the Olympus compound light microscope were classified into three types; leukocyte cell (L), nucleated cell (O) and cornified cell (Co). A total of 100 cells were counted for calculating the percentage of cornified cells (%Co) as described previously (Malaivijitnond *et al.* 2010). The appearance of cornified cells was used as an indicator of estrogenic activity.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine the differences of means of all the results. The observed significance was then confirmed by using the least significant difference (LSD) test, with the level of significance was set at $p < 0.05$.

Results

Isoflavone contents in *P. mirifica* extract

Five isoflavones, peurarin, daidzin, genistin, daidzein, and genistein, were determined in *P. mirifica* extract by HPLC technique. The retention times were 13.282, 17.776, 23.951, 33.363 and 42.134 minutes, respectively. The major component is puerarin with the concentration of 18.14 mg/100 g of *P. mirifica* extract. The concentrations of daidzin, genistin, daidzein and genistein were 2.58, 2.39, 4.17 and 2.03 mg/100 g, respectively.

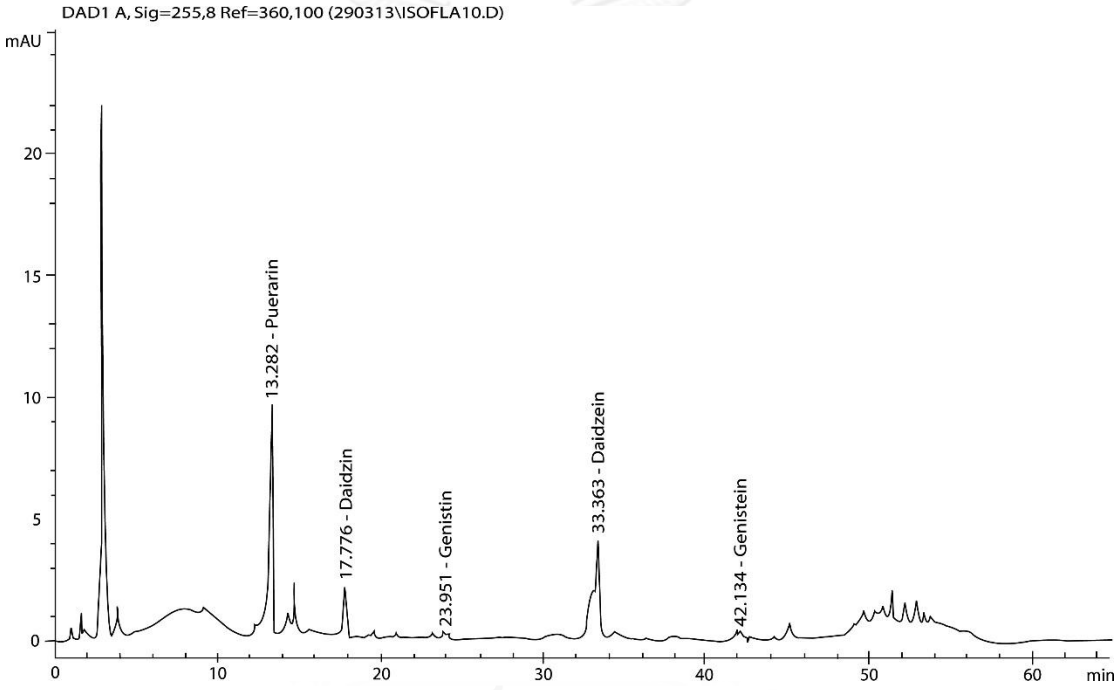

Figure 3.1 A chromatogram of *P. mirifica* extract reveals its isoflavone content as determined by HPLC technique.

Body weight

After ovariectomy, the body weights of rats increased rapidly and was highly significant ($p < 0.01$) within a week and kept a linear pattern of increase for the control (DW) group (Figure 3.2A). However, feeding with high doses of E_2 (E-10 and E-20) for only 1 week (D_{21} of the study period), the body weights were kept constant and tended to be lower than that of the control (DW) group ($p = 0.057$ and 0.123 for E-10 and E-20 groups, respectively). The significant differences of body weights between the control and the E-10 and E-20 groups ($p < 0.05$) could be detected at the second week

of treatment (D_{28} of the study period). On the contrary, the body weights of the lower dose treatment groups of E_2 (E-0.01, E-0.1 and E-1 groups) were not significantly different from the control group throughout the study period.

Feeding of *P. mirifica*, rats showed the broadly similar patterns of changes of body weights to those of the high doses of E_2 treatment (Figure 3.2B). However, the significantly lower body weights from the control group could be detected since the first week of treatment period (D_{21} of the study period).

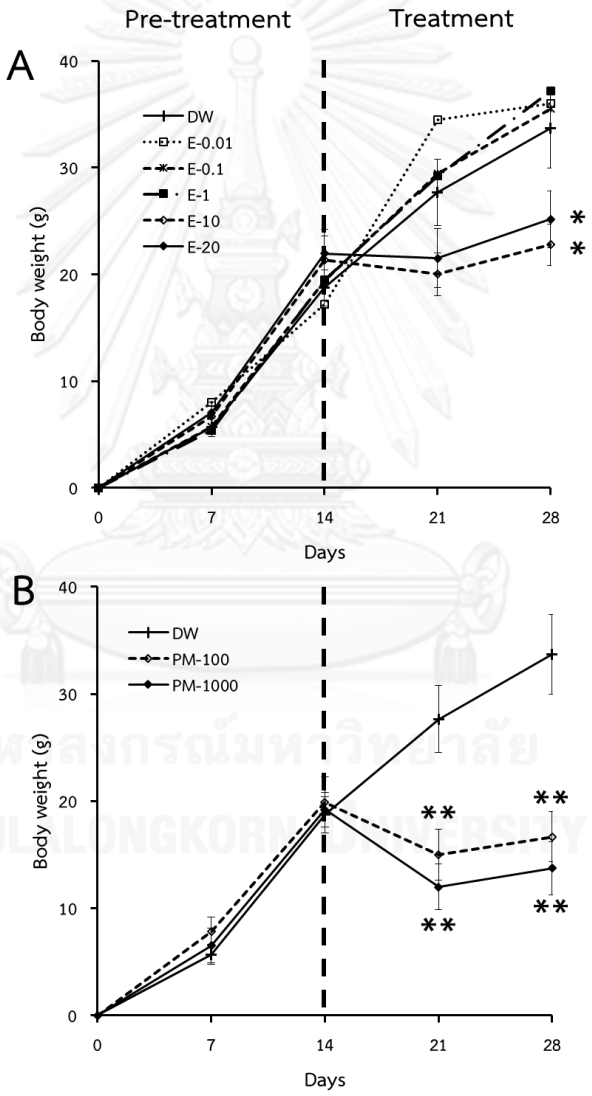

Figure 3.2 Body weights of OVX rats fed with distilled water (DW), E_2 at concentrations of 0.01, 0.1, 1, 10 and 20 mg/kg BW/day (E-0.01, E-0.1, E-1, E-10 and E-20, respectively) (A) and *P. mirifica* at concentrations of 100 and 1,000 mg/kg BW/day (PM-100 and PM-1000) (B) for 14 days. * and ** indicate $p < 0.05$ and 0.001 compared to the DW group.

Uterotropic assay

Patterns of changes in the uterine parameters (uterine wet weight, uterine, myometrium and endometrium areas, and numbers of uterine glands) of the rats fed with E_2 were similar to those of the body weights (Table 3.1), in that the increase was divided into two groups: low and high doses, and no differences between group members. There were no significant differences of the uterine wet weights, uterine, myometrium and endometrium areas, and number of uterine glands of the low doses of E_2 treatment (E-0.01, E-0.1 and E-1 groups) when compared to the control group and also between groups. Those values of the rats treated with the higher doses of E_2 (E-10 and E-20 groups) were significantly higher than those of the control group ($0.001 \leq p \leq 0.05$), and no significant differences between E-10 and E-20 groups.

All of the uterine parameters were significantly increased in rats treated with PM-100 and PM-1000 ($p < 0.005$) when compared to the control group (Table 3.1), except for the number of uterine glands that was a non-significant difference in both PM-100 ($p = 0.867$) and PM-1000 ($p = 0.068$).

The uterus morphology (size, thickness and uterine gland) was similar between the E_2 and *P. mirifica* treatments (Figure 3.3).

Table 3.1 Uterine wet weight, uterine, myometrium, and endometrium areas, and number of uterine glands (mean ± SEM) in OVX rats treated with distilled water (DW group), 0.01, 0.1, 1, 10, and 20 mg/kg BW/day of E_2 (E-0.01, E-0.1, E-1, E-10 and E-20 groups), and 100 and 1,000 mg/kg BW/day of *P. mirifica* (PM-100 and PM-1000) for 14 days

Treatment	Uterine weight (g)	Uterine area (x 10^5 μm^2)	Myometrium area (x 10^5 μm^2)	Endometrium area (x 10^5 μm^2)	Number of uterine gland
DW	0.120 ± 0.004	13.28 ± 0.88	7.68 ± 0.66	5.30 ± 0.31	21.54 ± 1.99
E-0.01	0.150 ± 0.016	16.82 ± 2.21	9.70 ± 1.28	6.67 ± 0.83	21.82 ± 1.91
E-0.1	0.150 ± 0.028	19.02 ± 6.10	10.16 ± 2.90	7.20 ± 1.84	21.91 ± 3.17
E-1	0.141 ± 0.014	16.15 ± 1.39	9.07 ± 0.67	6.71 ± 0.72	17.43 ± 1.87
E-5	0.388 ± 0.016	44.81 ± 3.94	21.22 ± 2.03	20.63 ± 1.33	23.47 ± 1.88
E-10	0.281 ± 0.027[c]	40.48 ± 2.96[c]	22.18 ± 1.35[c]	16.87 ± 1.41c	35.86 ± 4.82[b]
E-20	0.297 ± 0.018[c]	42.41 ± 5.49[c]	23.67 ± 3.27[c]	17.37 ± 2.02[c]	31.22 ± 5.59[a]
PM-100	0.433 ± 0.007[d]	49.35 ± 2.19[c]	27.71 ± 1.35[d]	19.89 ± 0.96[c]	22.26 ± 1.42
PM-1000	0.451 ± 0.020[d]	60.24 ± 1.41[d]	34.16 ± 0.77[d]	22.77 ± 1.34[d]	30.06 ± 2.96

a, b, c and d indicate $p < 0.05$, 0.01, 0.001 and 0.0005 compared to the control group

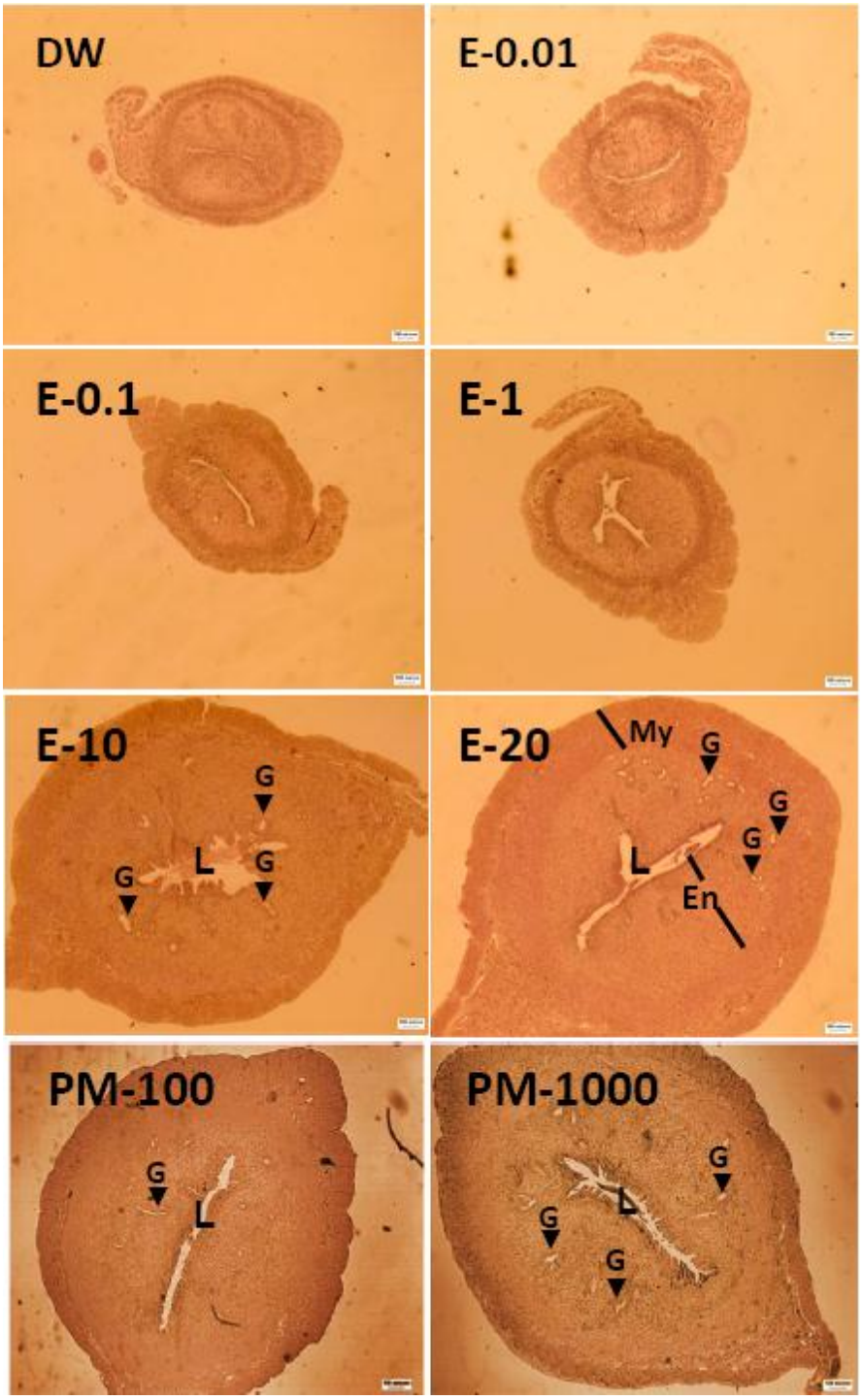

Figure 3.3 Uterine histology of OVX rats fed with distilled water (DW), E_2 at concentrations of 0.01, 0.1, 1, 10 and 20 mg/kg BW/day (E-0.01, E-0.1, E-1, E-10 and E-20, respectively) and *P. mirifica* at concentrations of 100 and 1,000 mg/kg BW/day (PM-100 and PM-1000) for 14 day. L lumen, En endometrium, My myometrium, G uterine gland, respectively

Vaginal cytology assay

After ovariectomy or during the pre-treatment period, the majority of vaginal epithelium cells were L-type. The %Co ranged from 14.6 – 51.7% (or 29.15% ± 0.72, mean ± SEM) during the pre-treatment period in the control, five E_2 and two *P. mirifica* treatment groups (Figure 3.4A, B). It remained lower than 30% during the treatment period for the control group. After 2 days of all E_2 treatments (or D_{16} of the study period), the %Co increased dose-dependently (r^2 = 0.880) (Figure 3.4A, dotted box). This is different from the decrease in body weight and increase in uterine parameters in that there was no changes in the lower dose treatment. Generally, responses of vaginal epithelium cells to the E_2 treatment could be divided into two groups; lesser and greater. The greater response was found in the higher doses of E_2 treatments (E-10 and E-20 groups) when the significant increase in cornified cells, compared to the control group, could be observed as early as the second day of the treatments (D_{16} of the study period) ($p < 0.01$) and ranged from 67.7 - 97.0% from D_{16} onwards (Figure 3.4A). The lesser response was found in the lower doses of E_2 treatment (E-0.01, E-0.1 and E-1 groups) when the significant increase in cornified cells could be observed after 7 days of treatment (or D_{21} of the study period) ($p < 0.05$). Thus, the %Co of all E_2 treatment groups was significantly higher than the control group on D_{21} - D_{28} of the study period (or during 7 - 14 days of E_2 treatment, $p < 0.05$ and 0.01). However, the correlations of the increase of %Co during D_{21} - D_{28} in relation to doses of E_2 were lower than that of the D_{16} (r^2 = 0.530 - 0.828 for $D_{21\text{-}28}$ and o.880 for D_{16}, respectively).

Feeding of PM-100 and PM-1000 showed a significant increase in %Co as early as the second day of treatment (or D_{16} of the study period), similar to those of the higher doses of E_2 treatment (Figure 3.4B), and the %Co of these groups were highly significantly increased throughout the treatment period when compared to the control groups ($p < 0.001$).

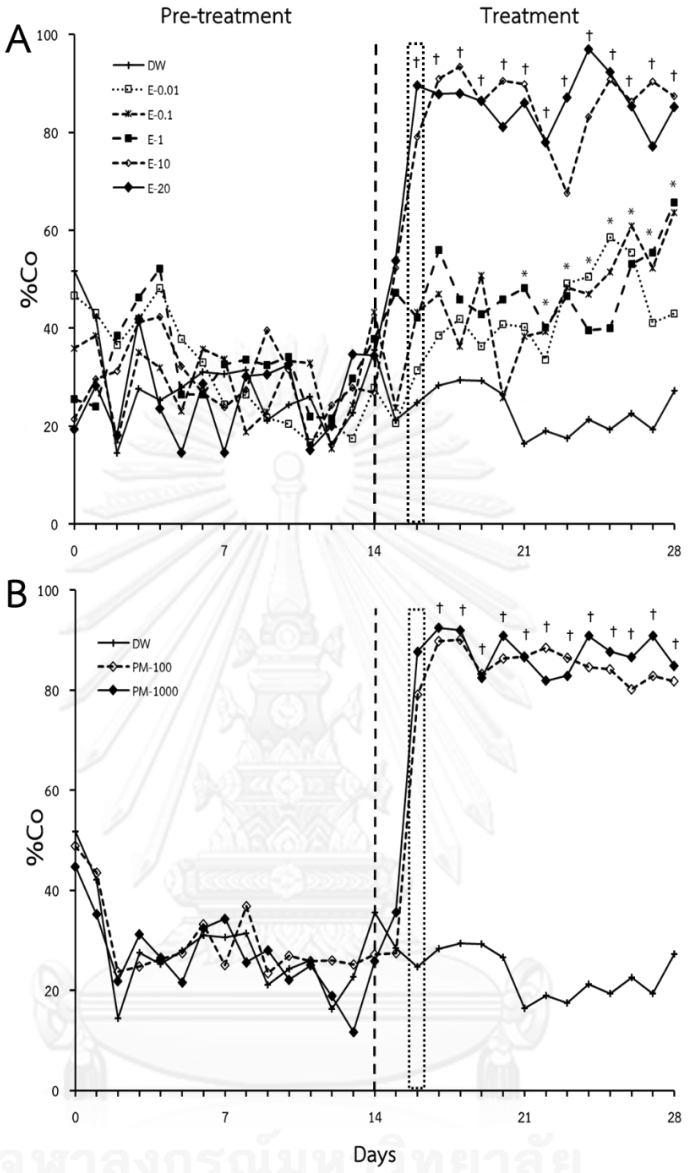

Figure 3.4 Percent of cornified cells (%Co) of OVX rats fed with distilled water (DW), E_2 at concentrations of 0.01, 0.1, 1, 10 and 20 mg/kg BW/day (E-0.01, E-0.1, E-1, E-10 and E-20, respectively) (A) and *P. mirifica* at concentration of 100 and 1,000 mg/kg BW/day (PM-100 and PM-1000) (B) for 14 days. * and † indicate $p < 0.05$ and 0.01 compared to the DW group. As the significant differences between the DW group and all low doses of E_2 (E-0.01, E0.1 and E-1) groups or between the DW group and all high doses of E_2 (E-10 and E-20) groups are similar, the * and † present only in one group. Dotted boxes indicate the second day of treatment when the %Co was first higher than the DW group.

Calculation of estrogenic activity of *P. mirifica* in comparison to E_2

Standard curves of estrogenic activity of E_2 were drawn in terms of changes of body and uterine weights at the end of the study period (D_{28} or 14 days after treatment) (Figure 3.5A, B) and %Co on D_{16} of the study period (or two days after feeding) (Figure 3.5C). The increase in uterine weight was selected as a representative of uterotropic assay, because (1) changes of all uterine parameters are in the same patterns, and (2) increase in uterine weight was clearly seen and easily detected. The standard curve between E_2 concentrations and %Co on D_{16} of the study period was selected because it showed the highest correlation value. Comparing to the E_2, the estrogenic activity of PM-100 and PM-1000 in terms of body weight and uterine weight was > 20 mg/kg BW, whist it was 14.32 and 17.20 mg/kg BW for PM-100 and PM-1000 based on the increase in %Co.

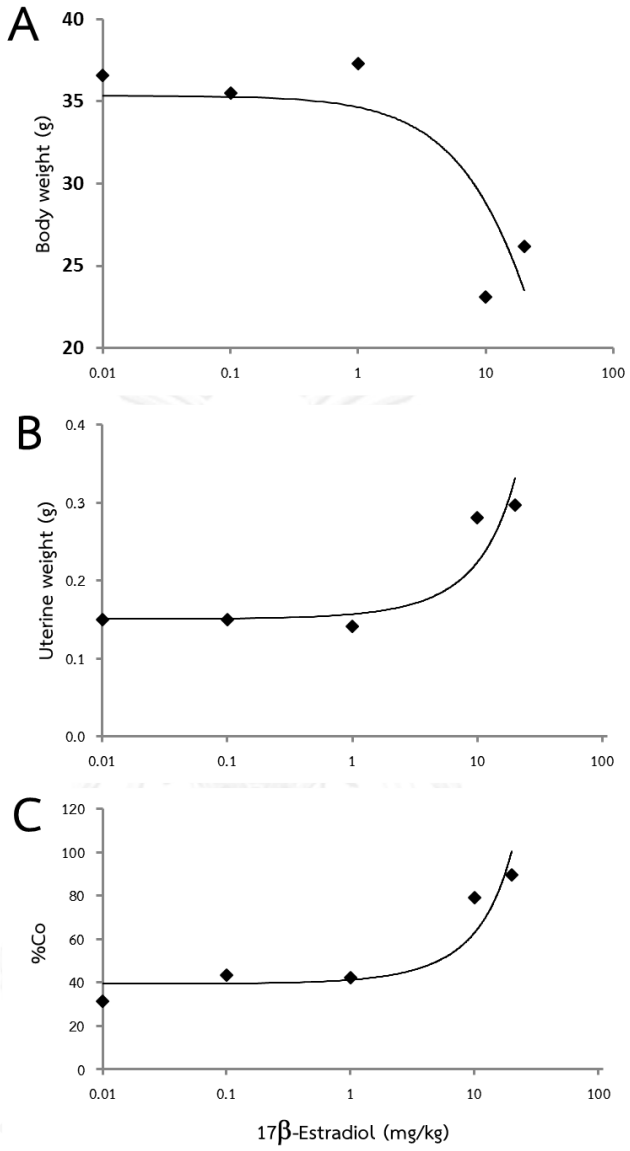

Figure 3.5 Standard curves of estrogenic activity of E_2 in terms of body weight changes (A), uterine weight (B) and %Co (C).

Discussion

After ovariectomy for two weeks, all eight groups of rats dramatically increased in body weight. This might be explained by the effect of estrogen deficiency on the hypothalamus and adipose cells. The hypothalamus, particularly at the paraventricular and arcuate nuclei and lateral hypothalamic area which are involved in control of food intake, and adipose tissue express both ERα and ERβ (Pederson et al., 1996; Anwar et al., 2001; Breton et al., 2011), and estrogens and EDCs can bind to either ERs and exhibit anti-lipogenic (or anti-obesity) effects on the hypothalamus and adipose tissue (Joyner et al., 2001; Naaz et al., 2003; Silva et al., 2010; Tsai et al., 2010). Thus, the ovariectomy induces an increase in food intake and body weight gain in rats (Mehasseb et al., 2011; Silva et al., 2010; Tsai et al., 2010; Zhang et al., 2010). Gene expression analyses indicated that adipose tissue is the center of action for ER-β-selective ligands, and the reduction in body weight after estrogen treatment is likely due to increased energy expenditure via the peroxisome proliferator-activated receptor γ antagonistic actions (Yepuru et al., 2010). Other proposed mechanisms are that ovariectomy led to increase in plasma levels of leptin and adiponectin and expression level of hypothalamic phosphorylated adenosine monophosphate-activated protein kinase-α (pAMPKα) (Silva et al., 2010; Tsai et al., 2010), and decrease in gherin expression in fundus of stomach of rats (Zhang et al., 2010), and, thus, replacement of both E_2 and *P. mirifica* significantly reversed these effects. Although much of the scientific literature has been reported about the effects of estrogen deficiency and estrogen replacement on changes of body weight of rats, to the best of our knowledge, no one uses this simple read-out parameter as one of the *in vivo* assays to evaluate the estrogenic activity of estrogens or EDCs. From this study, it indicated that determination of changes of body weights of rats can be a reliable method to detect the estrogenic activity of chemicals, especially for EDCs. It is also inexpensive, straight forward, simple, and the experimenters do not need any skills on animals weighing.

The uterotropic assay is designed to detect estrogenic activity based on the weight-evidence analysis which is recommended by the US Environmental Protection Agency (EPA) and Organization for Economic Cooperation and Development (OECD) (Gray et al., 2004). It is well known that the uterus is one of the main target organs of estrogen and uterine proliferation requires estrogen stimulation. Therefore, the uterotropic assay was established to determine the estrogenic activity of synthetic

estrogens, xenoestrogens and phytoestrogens (Gray et al., 2004; Malaivijitnond et al., 2004; Malaivijitnond et al., 2006; Sookvanichsilp et al., 2008; Siangcham et al., 2010). Since both types of ERs (ERα and ERβ) can be seen at the uterine glands, and endometrium and myometrium layers of uterus (Mehasseb et al., 2011), the increases in the number of uterine glands, and uterine, myometrium and endometrium areas after E_2 and *P. mirifica* treatments in this study were consequentially in the same line with the increase in uterine weights. Interestingly, the window of the maximal action of E_2 on increasing the uterine parameters seemed to be at 10 mg/kg BW, and the higher dose (E-20) did not show the greater response. On the contrary, the increase in uterine parameters stimulated by *P. mirifica* was higher than those of E-10 and E-20 treatment groups. This can be explained by the fact that the phytoestrogens in *P. mirifica* have a higher binding affinity on ERβ, whilst the estrogen has a higher binding affinity on ERα (Kuiper et al., 1997, 1998), and the ratio of ERβ/ERα was high at the (Mehasseb et al., 2011). As mentioned above, the uterotropic assay is preferable method for the estrogenic activity evaluation on EDCs, because uterine weight is also a simple read-out parameter and weighing the uterus is not difficult. However, compared to the measurement of body weights of rats, the researchers need to kill the animal at the end of the study period for the uterotropic assay, and because of that they could not follow up changes during the time of treatment. Additionally, the researchers need to have experience in uterine histomorphometry. Interestingly, up to two weeks of the low doses of E_2 treatments (E-0.01, E-0.1 and E-1), changes in the body weight of the rats and all uterine parameters could not be detected. This should be due to the fact that an amount of E_2 was likely to be below the threshold for efficacy of the whole body and uterus of rats.

The vaginal cytology assay can be performed in either immature or OVX rodents (Ashby et al., 2000; Diel et al., 2000; Stroheker et al., 2003). In this study, OVX rats were used as an animal model, because OVX can up-regulate the expression of ERs, especially for ERα (Pessina et al., 2006; Traish et al., 2007), and it should improve the sensitivity of the assay. Recent publications indicate that the vaginal cytology assay could detect very weak estrogenic activity of puerarin phytoestrogen (Malaivijitnond et al., 2010). Thus, comparing to the measurement of body weight and the uterotropic assay, the vaginal cytology assay is more sensitive which could detect the estrogenic activity of high doses of E_2 (E-10 and E-20) within 2 days of treatment (D_{16} of the study

period) and also those of the low doses (E-0.01, E-0.1 and E-1) within a week of treatment. Besides the sensitivity, the vaginal cytology assay can follow up changes of the treatment which could not be done by uterotropic assay; and we do not need to kill the animals. However, observation and classification of vaginal cell-types under the compound light microscope every day was a tedious work and we need to practice.

Of the three alternative of the *in vivo* assays proposed, although all are inexpensive, reliable and critical read-out, determination of body weights changes is likely convenient and simple, and vaginal cytology assay appears most promising for sensitivity and shortening the duration of the assay. Thus, the researchers should consider what kind of chemicals they want to study, and how much of the concentration of chemicals, time, complexity, skill and experience is required before they make a decision to use each an *in vivo* assay.

Phytoestrogens, classified as one of EDCs, are plant compounds with estrogen-like biological activity which can bind to ERs and express estrogen-like properties which are similar to endogenous estrogen. Four different families of phenolic compounds produced by plants are considered as phytoestrogens: isoflavonoids, stilbenes, lignans and coumestans (Cornwell et al., 2004). There are more than 300 plant species which contain estrogenic compounds or phytoestrogens, but only few of these are consumed by animals or humans. From HPLC results, puerarin was identified as the major component of the *P. mirifica* extract. Thus, it is possible that the estrogenic activity occurred during treatment period is due to the puerarin found in the *P. mirifica* extract.

Currently, phytoestrogens are widely studied and used as an estrogen replacement therapy in postmenopausal women for the purposes of reducing cardiovascular disease (cardioprotective) (Adlercreutz, 1990), maintaining bone mineral density (Ho et al., 2001; Chiechi et al., 2002), increasing long term and short-term memory (File et al., 2001) and inducing the low risk of breast, endometrial, ovarian, prostate and colon cancers (Rose et al., 1986). However, the plants exhibited variations in the amount of phytoestrogens according to their genetics, location of crop, time of harvest, crop conditions, infection with fungal diseases, and processing in preparation of the raw material (Al-Azzawi & Wahab, 2010; Malaivijitnond, 2012). Therefore, the estrogenic activities of phytoestrogens in each plant should be evaluated for the synthetic estrogen and standardized between lots of plants before applying for the further purposes of use in animals or humans.

It is well-known that the plants in genus *Pueraria* are largely distributed in Asia, and *P. mirifica* is a Thai endemic herb which is now commercially cultivated and widely used in Thailand, Japan, Korea, China and USA (Malaivijitnond, 2012). Its tuberous roots contain various kinds of phytoestrogens, mainly isoflavonoids (Cherdshewasart & Sriwatcharakul, 2007c). Thus, *P. mirifica* is selected as a representative of EDC of this study. Although the estrogenic activity of phytoestrogens in *P. mirifica* has been tested by the *in vitro* MCF7 proliferation assay (Cherdshewasart et al., 2004), and the *in vivo* vaginal cytology and uterotropic assays (Malaivijitnond et al., 2006; Cherdshewasart et al., 2007a), the comparison of its activity in equivalent to that of the endogenous estrogen (17β-estradiol; E_2) for the safety of use in animals and humans has never been done. Comparing to the estrogenic activity of E_2 in terms of body and uterine weights and %Co, the estrogenic activity of PM-100 and PM-1000 was out of or at the upper range of the standard curve which is 14 to >20 mg/kg BW. Thus, this confirms that *P. mirifica* is a phytoestrogen-rich plant exhibiting a high estrogenic activity (Malaivijitnond, 2012) which should be applicable to ameliorate the menopausal symptoms in postmenopausal women. However, the users need to be aware of the suitable and non-toxic doses before use.

In conclusion, comparing the three *in vivo* assays, determining of changes of body weight, uterotropic and vaginal cytology assays, for the evaluation of estrogenic activity of estrogen disrupting compound, *P. mirifica*, in comparison with 17β-estradiol (E_2), although all are reliable and had critical read-out, measurements of body and uterine weights is likely convenient and simple, but the uterotropic assay needs to kill the animals. Vaginal cytology assay appears most promising for sensitivity and shortening the duration of the assay.

CHAPTER IV
MOLECULAR EVENTS DURING THE INDUCTION OF NEURODEGENERATIVE AND MEMORY LOSS IN ESTROGEN-DEFICIENT RATS

Introduction

Neurodegenerative disease is an age-related disease with increasing incidents over the past decade. The progressive loss of the structure and function of neurons in the brain, including neuronal death, is one of the causes of neurodegenerative diseases. The neuropathological hallmarks of neurodegenerative diseases are extracellular senile plaques and NFTs (Goedert & Spillantini, 2011). The extracellular senile plaques are composed largely of a 40- or 42- amino acid Aβ peptide. The intracellular NFTs are composed of aberrantly phosphorylated microtubule-associated proteins called 'TAU' (Boutajangout et al., 2004). These lesions are invariably found throughout the neocortex and hippocampus of patients with neurodegenerative disease that are linked to memory deficit and progressive loss of cognitive function (Prox et al., 2012).

TAU is a major microtubule-associated protein in brain cells, which is localized primarily in the axons. TAU protein helps neurons to maintain the cell shape and to facilitate axoplasmic transport (Boutajangout et al., 2004; Buee et al., 2000; Shi et al., 2011). Post-translational modification, such as phosphorylation, reduces the ability of TAU to promote microtubule assembly (Trinczek et al., 1995). Abnormally hyperphosphorylated and filamentous TAU is the main component of the NFTs found in the brains of neurodegenerative diseases, especially in Alzheimer's disease (Goedert, 1993; Goedert & Spillantini, 2011). The TAU proteins are encoded by a single gene consisting of 16 exons in humans (Kosik, 1993). In the adult human brain, six different *TAU* mRNA isoforms are expressed by an alternative splicing mechanism of exon 2, 3, and 10 of its pre-mRNA (Andreadis et al., 1992). The exon 2 and 3 of *TAU* encode a 29 amino acid region located in the amino-terminal of the full-length TAU protein. Thus, the *TAU* mRNA isoform containing the exons 2 and 3, only the exon 2, or neither of them produces the long (L), short (S), or none (0) TAU protein isoform, respectively. Generally, the proportion of L, S and 0 isoforms in the adult human brain is about 54%, 37%, and 9%, respectively (Robert & Mathuranath, 2007), and this ratio does not

differ between healthy people and patients with serious cognitive impairment, such as Alzheimer's patients (Ginsberg et al., 2006). On the other hand, the exon 10 encodes the second tubulin-binding repeat in the carboxy-terminal of the TAU protein (Goedert et al., 1989), and thus alternative splicing of the exon 10 generates TAU isoforms with four or three tubulin-binding repeats (TAU4 or TAU3, respectively). The tubulin-binding repeat plays an important role in the interaction between TAU and microtubules. The proportion of TAU4 and TAU3 isoforms in the human brain differs between healthy adults (Robert & Mathuranath, 2007; Shi et al., 2011) and patients with neurodegenerative disorders (Goedert et al. 1998; Spillantini et al., 1998). Thus, examination of the TAU4/TAU3 ratio should be considered to gauge the progress of neurodegenerative diseases.

Aβ, a major component of senile plaque, is generated from APP by sequential proteolytic cleavages. The APP processing has two pathways, non-amyloidogenesis and amyloidogenesis, and is mediated by secretases (α, β, and γ). The non-amyloidogenic APP processing is mediated by α-secretase and results in the secretion of a non-toxic, large extracellular APPα fragment (Webb & Murphy, 2012). In this processing, A disintegrin and metalloproteinase (ADAM) family, including ADAM9, ADAM10 and ADAM17, has been suggested to function as α-secretase (Prox et al., 2012). An increase in the expression of ADAM10 lowers the burden of amyloid load and precludes or abrogates Alzheimer's disease (Donmez et al., 2010; Endres & Fahrenholz, 2012). In contrast, the amyloidgenic pathway mediated by β-secretase and γ-secretase in neurons generates a variety of potentially amyloidogenic Aβ species from APP, which are insoluble and neurotoxic (Forman et al., 2004; Prox et al., 2012; Webb & Murphy, 2012). In this pathway, beta-site APP cleaving enzyme (BACE) functions as β-secretase. In the endocytic pathway, BACE1 and BACE2 cleave APP and generates APPβ and a longer C-terminal fragment, which is further processed by γ-secretase and generates Aβ. BACE1 is apparently the only enzyme with APP β-secretase activity in the brain, whilst BACE2 has limited expression in the normal human brain but is largely found in patients with Down syndrome (Cheon et al., 2008; Webb & Murphy, 2012). In addition, BACE1 is highly expressed in the hippocampal and cortical regions of the brain of Alzheimer's disease (Kandalepas & Vassar, 2012; Sun et al., 2012). Thus, α-secretase ADAM10 and β-secretase BACE1 were selected in this study as representative enzymes for the non-amyloidogenic and amyloidogenic pathways of the APP processing.

Recently, many reports indicated non-reproductive functions of estrogen, and the brain is one of the target organs. In the brain, estrogen plays a role in the control of neuronal functions, including neuronal proliferation, neuronal survival, and neuronal plasticity (Brann et al., 2007). Estrogen also has great impacts over cognitive function, since it shows neurotrophic and neuroprotective actions over certain brain areas such as the hippocampus, cortex, and the striatum (Brann et al., 2007). During the aging process or postmenopausal period in women, the reduction of estrogen level induces progressive neurodegeneration in the brain and results in cognitive impairment (Brann et al., 2007). However, there is no report to indicate how estrogen deficiency induces neurodegenerative diseases. To delineate the relationships between estrogen deficiency, neurodegeneration, and cognitive impairment, we examined the effects of ovariectomy on the serum level of E_2, LH and FSH, the expressions of the genes associated with amyloid plaques and NFTs in the hippocampal brain area, and the cognitive ability in adult female rats for 4 months.

Materials and Methods

Animals

Adult female Sprague-Dawley rats, 2 months old, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. They were reared in stainless steel cages with sawdust bedding at five animals/cage in a room with controlled lighting (light on 0600-1800 h) and temperature (25 ± 1 °C) at the Laboratory Animal Room, Faculty of Science, Chulalongkorn University, Thailand. The animals were fed with rat chow diet (Pokaphan Animal Feed Co., Ltd., Thailand) and water *ad libitum*. The rats were reared until they became 4 months old and used in this study following the study of Feng et al. (2004). The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1123009.

Experimental design

At the age of 4 months, animals were divided into 5 groups; M_0, M_1, M_2, M_3 and M_4 with 9, 8, 7, 6 and 8 rats, respectively. In the M_0 (control) group, the rats of the diestrous phase were selected on the first day of experiment. In the M_1, M_2, M_3 and

M_4 groups, rats were bilaterally OVX on the first day of the experiment under sodium pentobarbital anesthesia (40 mg/kg BW, i.p.), and kept for 1, 2, 3 and 4 months, respectively.

One-milliliter of blood sample was collected once a month in each group of rats at 0800-0900 h. In the last month of the experiment, the blood samples were additionally collected in two-week interval. Immediately after the blood clotted, blood serum was separated by centrifugation at 1,000g for 30 min and then kept at -20 °C until being assays for E_2, FSH and LH. Spatial memory test was performed in each group of rats for five consecutive days before the end of the experiment.

At the end of the experiment, the animals were euthanized and the brains were removed. The hippocampus region was then dissected, homogenized in 300 μl of TRIzol reagent (Invitrogen, USA), and kept at -80 °C until RNA extraction.

Hormone assays

Serum E_2 levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands as described previously (Korenman et al., 1974). The antisera against estradiol (GDN 244) were kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and inter-assay coefficients of variation were 4.1% and 10.7%, respectively.

Serum FSH and LH levels were determined using the National Institute of Diabetes and Digestive and Kidney disease (NIDDK) kit for rat FSH and LH (Baltimore, MD, USA) as describes previously (Jaroenporn et al., 2011). Iodinated preparations used in this study were rat FSH-I-7 and rat LH-I-7, and the antisera were anti-rat FSH-S11 and anti-rat LH-S10, respectively. The results obtained are expressed using the rat FSH-RP-2 and rat LH-RP-2 reference standards. The intra- and inter-assay coefficients of variations were 6.5% and 7.6% for FSH and 6.4% and 8.6% for LH, respectively.

Spatial memory test

Morris water maze test was used to examine spatial memory of the rat (Vorhees & Williams 2006). The test was performed in a circular pool with 180 cm diameter and 70 cm deep, which was made of stainless steel, modified from Feng et al. (2004). The pool was filled with water to a depth of 50 cm and divided into four quadrants. A

platform was submerged 1 cm below the surface of water in the center of north-east quadrant.

Each rat was given four trials per day for five consecutive days to find the hidden platform. The first trial was stated by placing the rat into the water surface facing the pool wall in one of the four quadrants, and rotated the placing position clockwise to cover all four quadrants in the subsequent trials. From the next day onwards, the test was started from a quadrant different from the previous day. For each trial, the rat was allowed to swim in a maximum of 90 sec to find the platform and given a 30 sec rest period on the platform. If the rat could not find the platform within 90 sec, it was guided to the platform manually, and a score of 90 sec was recorded. The latency to reach the platform by swimming and the swimming distance to find the hidden platform were measured using a video tracking system (Smart Junior, Panlab-Havard Apparatus, Barcelona, Spain). Average value of the four trials in each day of each rat was calculated and counted as an individual value. The movement patterns of rats to find the platform were categorized into 4 strategies; line, taxis, random, and circular based on the criteria explained by Feng et al. (2004). The frequency of each strategy accounted by each rat was used for analysis.

RNA extraction and cDNA synthesis

Total RNA of the hippocampal sample was extracted according to the manufacturer's instruction, and dissolved in RNase-free water. The quantity and the purity of the RNA sample were checked by measuring the absorbance at 260 and 280 nm wavelengths. The total RNA sample (5 μg) was reverse transcribed to cDNA in a reaction mixture of 20 μl containing 2 μl of RT buffer, 0.8 μl of dNTP mix, 2 μl of RT random primer, 1 μl of Multi Scribe Reverse Transcriptase, 0.9 μl of RNase Inhibitor using the High Capacity cDNA Reverse Trascription Kit (Applied Biosystems, CA, USA). The samples were incubated for 10 min at 25 °C, 2 h at 37 °C, and finally for 5 min at 85 °C.

Real-time PCR analysis

The expression levels of the genes associated with amyloid plaques (*App*, *Adam10*, and *Bace*1) and NFTs (*Tau4* and *Tau3*) were examined using real-time PCR with the primers shown in Table 1. S28RNA was used as a house-keeping gene in this

study. The primers designed for *Tau4* and *Tau3* specifically amplify the isoform with and without the exon 10, respectively.

Real-time PCR was performed using Step One™ Plus Real-time PCR system (Applied Biosystems, CA, USA) in 10 μl of reaction mixture containing 1x POWER SYBR Green PCR Master Mix, 0.5 μM each of the forward and reverse primer, and 1 μl of the cDNA sample. The reaction was carried out at 95 °C for 10 min, 40 cycles of incubation at 95 °C for 15 sec, 60 °C for 10 min, 40 cycles of incubation at 95 °C for 15 sec, 60 °C for 1 min, and a final dissociation curve. Relative expression levels of the target genes were calculated by $2^{-\Delta\Delta Ct}$ method. The value of the M_0 group was adjusted to 1 and the mRNA levels of the other groups were calculated relative to the M_0 group.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine the differences of means of the hormone and mRNA levels. The observed significance was then confirmed by the least significant difference (LSD) test, with the level of significance was set at $p < 0.05$. For spatial memory test, statistical analysis in both latency and distance for searching the hidden platform was performed using two-way ANOVA with repeated measures ($p < 0.05$), whilst the significant difference of the movement patterns was tested by the chi-square test. SPSS software program (version 17.0) was used for the analysis.

Table 4.1 Nucleotide sequences of the primers used for real-time PCR experiments.

Target gene	Accession No.		Primer sequences (5′-3′)	Product size
App	X07648	Forward	CACACCCACATCGTGATTCCT	105
		Reverse	GTCCATCCGCTCCTGGTGTA	
Adam10	XM_001054737	Forward	GGCCCTCTAGCTAGGCTGAAA	113
		Reverse	ATCAGGGCAATTCCCATAAGC	
Bace1	AF190727	Forward	TTGCCATGTGCACGATGAG	57
		Reverse	GCCGTGACAAACGGACCTT	
Tau4	ENSRNOT00000042984	Forward	GATCTTAGCAACGTCCAGTCCAA	133
		Reverse	TCCCTAAGGAACCACACTTGGAG	
Tau3	ENSRNOT00000045127	Forward	GAGGCGGCAAGGTGCAAATAGTCT	100
		Reverse	CCACCTCCTGGCTTGTGATGGAT	
S28RNA	V01270	Forward	GGCCGAAACGATCTCAACCT	217
		Reverse	GCCACCGTCCTGCTGTCTAT	

Results

Effect of estrogen deficiency on serum hormone levels

Serum E_2 levels during the diestrous phase in the M_0 group were 27 ± 11 pg/ml (ranged between 13 and 85 pg/ml) (Figure 4.1A). After ovariectomy, serum E_2 levels declined below the baseline within 2 weeks in the M_1 group, and remained at low levels throughout the 4-month period. Conversely, serum LH and FSH levels (baseline levels = 0.31 ± 0.10 ng/ml and 4.36 ± 1.02 ng/ml for LH and FSH levels, respectively) were profoundly increased by 2 weeks of estrogen depletion in the M_1 group to approximately 22 and 4 times, respectively (6.99 ± 0.98 ng/ml and 15.06 ± 1.09 ng/ml for LH and FSH levels). The serum LH and FSH levels of the M_2, M_3 and M_4 groups peaked at 1.5 months (16.53 ± 3.14 ng/ml) and 2 months (36.01 ± 3.05 ng/ml) after ovariectomy and reached a plateau phase (Figure 4.1B and C).

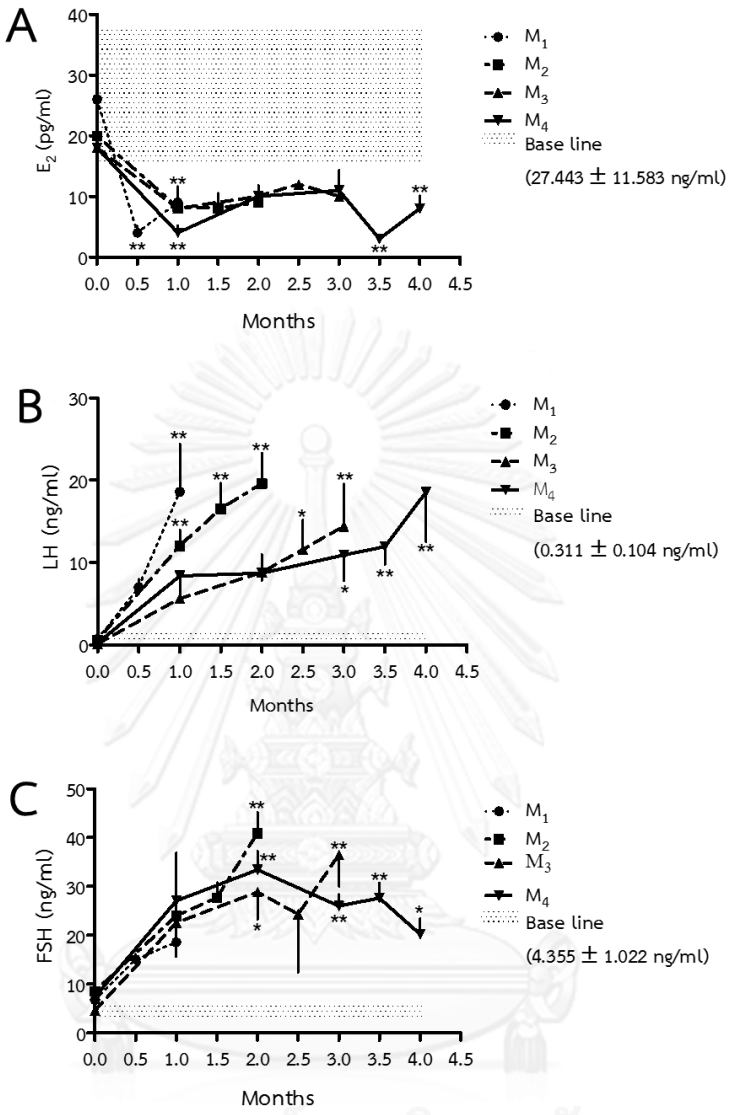

Figure 4.1 Changes in the serum levels of E_2 (A), LH (B), and FSH (C) in female rats after ovariectomy for 0, 1, 2, 3 and 4 months (M_0, M_1, M_2, M_3 and M_3, respectively). Data are expressed as mean ± SEM, and the data points that significantly differ from the control (M_0) group are indicated by * and ** for $p < 0.05$ and 0.005, respectively.

Effect of estrogen deficiency on cognitive performance in OVX rats

The latency of searching for the hidden platform was reduced during five consecutive days of testing in all five groups of rats (Figure 4.2A). There was no significant change in the profiles of the latencies in the M_1 and M_2 groups compared to the control. However, the latencies were significantly longer in the M_3 and M_4 groups ($p < 0.05$).

The pattern of changes in the distance to reach the platform were similar to those of the latency, that is, the distance declined along with the time of five consecutive days of testing (Figure 4.2B). Again only the M_3 and M_4 groups took significantly longer distances to reach the platform compared to the control.

The significant differences in the latencies and distances of the M_3 and M_4 groups were clearly seen on day-5 of the trial. Therefore, the strategies for searching the platform were examined only in the trials on day-5 (Figure 4.3). The strategies used by the control (M_0) rats were mainly line (50%) and taxis (45%). After ovariectomy, random and circular strategies were used more often by the M_3 and M_4 groups (57% and 54% for M_3 and M_4 groups, respectively; $p < 0.001$) compared to the M_0 group (5% with circular strategy only). Although the percentage of the random and circular strategies was higher in the M_1 group compared to the M_0 group, the difference was not large (21% and 5% in M_1 and M_0 groups, respectively; $p < 0.05$).

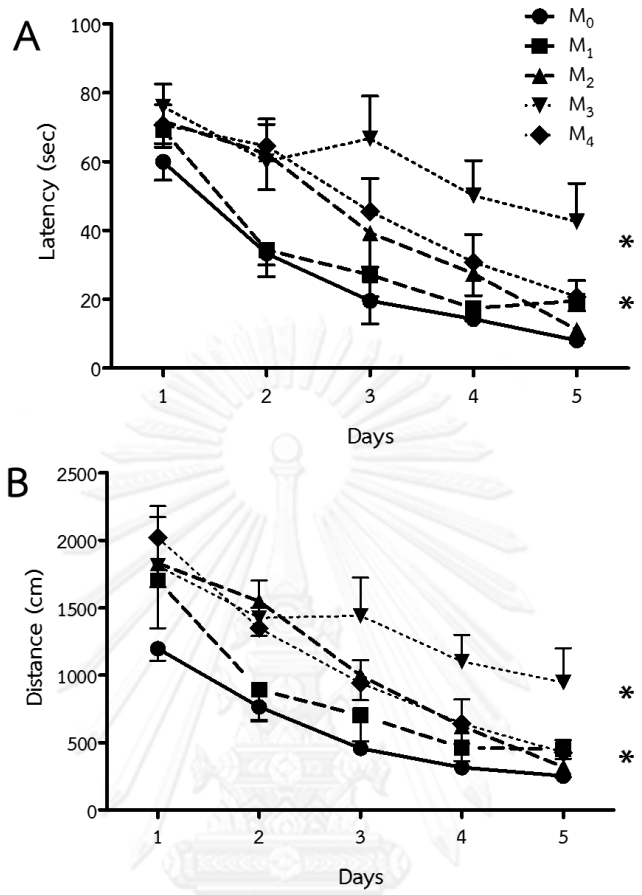

Figure 4.2 Effects of estrogen deficiency on the latency (A) and the distance (B) of searching for a hidden platform in female rats after ovariectomy for 0, 1, 2, 3 and 4 months (M_0, M_1, M_2, M_3 and M_4, respectively). The test was conducted for five consecutive days (day 1-5) before euthanization. Data are expressed as mean ± SEM. The data plots that significantly differ from the control (M_0) group as analyzed by two-way ANOVA with repeated measures are indicated by asterisks; * for $p < 0.05$.

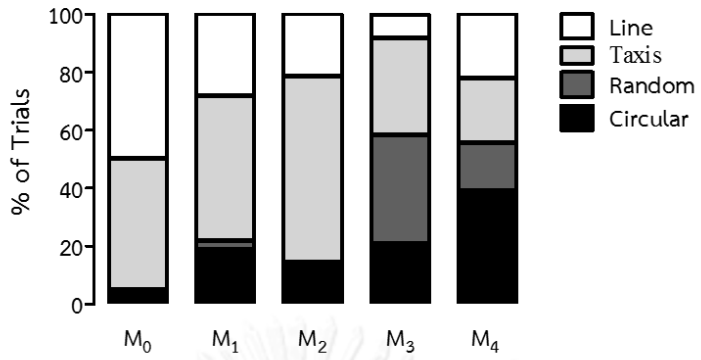

Figure 4.3 Strategies used by female rats after ovariectomy for 0, 1, 2, 3 and 4 months (M_0, M_1, M_2, M_3 and M_4, respectively) on day-5 of the spatial memory test.

Effect of estrogen deficiency on the expression of genes associated with NFTs

The expression levels of *Tau4* mRNA in the hippocampus was significantly higher in the M_1 group compared to the control (2.40 ± 0.23 folds, $p < 0.05$) (Figure 4.4A). After the increase in the first month, the levels of *Tau4* mRNA tended to be further increased gradually (2.14 ± 0.17, 2.85 ± 0.39 and 2.92 ± 0.88 folds for the M_2, M_3 and M_4 groups, respectively) (Figure 4.4A). The *Tau3* mRNA levels were also significantly higher (3.46 ± 0.45 folds) in the M_1 group compared to the control ($p < 0.005$) (Figure 4.4B). The increased *Tau3* mRNA levels remained high throughout the experimental period, but tended to be slightly declined thereafter (2.91 ± 0.25, 2.45 ± 0.52 and 2.54 ± 0.94 folds for the M_2, M_3 and M_4 groups, respectively) (Figure 4.4B). As the increase in the levels of the *Tau3* mRNA was larger than that of the *Tau4* mRNA, *Tau4/Tau3* mRNA ratio tended to be lower compared to the controls in the first two months (0.69 ± 0.13 and 0.61 ± 0.06 for the M_1 and M_2 groups, respectively) and increased during the later third and fourth month (1.21 ± 0.21 and 1.48 ± 0.43 for the M_3 and M_4 groups, respectively), however with no significant differences (Figure 4.4C).

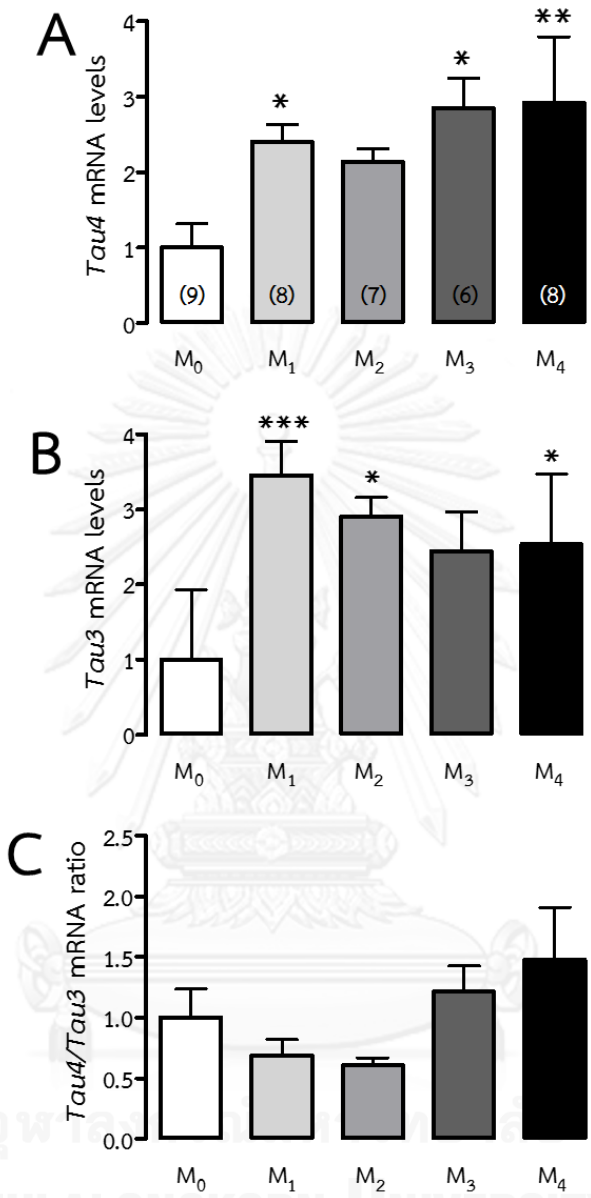

Figure 4.4 The relative mRNA expression levels of the genes associated with NFTs, *Tau4* (A), *Tau3* (B), and the *Tau4/Tau3* mRNA ratio (C) in female rats after ovariectomy for 0, 1, 2, 3 and 4 months (M_0, M_1, M_2, M_3 and M_4, respectively). All data show the fold change in gene expression relative to the M_0 group. Data are expressed as mean ± SEM, and the groups that significantly differ from the control (M_0) group are indicated by asterisks; *'** and *** for $p < 0.05$, 0.01 and 0.005, respectively. The number in each column indicates the number of rats used.

Effects of estrogen deficiency on the expression of genes associated with amyloid plaques

The level of the *App* mRNA tended to increase after ovariectomy and it was significantly higher in the M_4 group compared to the control ($p < 0.05$) (Figure 4.5A). The ratio of *Bace1/Adam10* mRNA levels did not change significantly throughout the experimental period (Figure 4.5B). However, the *Bace1* and *Adam10* mRNA levels showed an increasing trend and were significantly higher in the M_4 group ($p < 0.05$ and $p < 0.005$, respectively; Figure 4.5C and D).

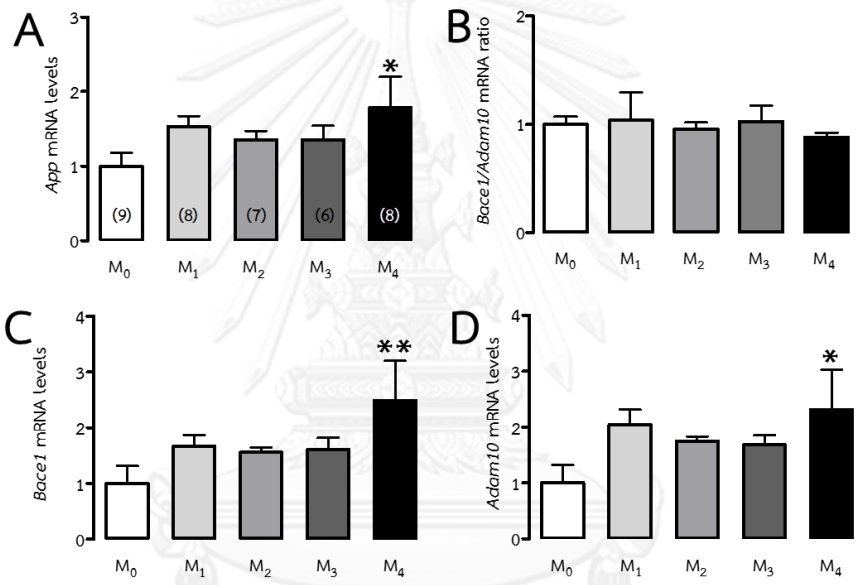

Figure 4.5 The relative mRNA expression levels of the genes associated with amyloid plaques, *App* (A), *Bace1/Adam10* mRNA ratio (B), *Bace1* (C), and *Adam10* (D) in female rats after ovariectomy for 0, 1, 2, 3 and 4 months (M_0, M_1, M_2, M_3 and M_4, respectively). All data show the fold change in gene expression relative to the M_0 group. Data are expressed as mean ± SEM, and the groups that significantly differ from the control (M_0) group are indicated by asterisks; *'*** for $p < 0.05$ and 0.01, respectively. The number in each column indicates the number of rats used.

Discussion

In addition to its role as a reproductive hormone, estrogen has been shown to have neuroprotective and neurotrophic/synaptic plasticity actions in the brain (Brann et al., 2007). Likewise, estrogen deficiency has been implicated in the pathogenesis of neurodegenerative disease, such as Alzheimer's disease, and cognitive decline (Paganini-Hill & Henderson, 1994; Fukuzaki et al, 2008). The hippocampus and the neocortex are highly vulnerable brain areas, which express ERs (ERα and ERβ) (Shughrue et al., 1997a). Although the relationship between estrogen deficiency in elderly, especially in postmenopausal women, and neurodegeneration has been extensively studied, little is known about molecular mechanisms through which neurodegeneration occurs in the brain in conjunction with alterations in reproductive hormones. Thus, the onset of neurodegeneration induced by estrogen deficiency was assessed using a gene-based approach in this study.

OVX rats showed reduction in the serum E_2 levels with accelerated increases in the serum LH and FSH levels as shown previously (Arai et al., 1996; Malaivijitnond et al., 2004; Wu et al., 2008; Yang et al., 2009). These significant alterations in serum hormone concentrations could be observed as early as 2 weeks after ovariectomy. The increased serum FSH and LH levels reached a plateau within approximately 2 months after ovariectomy. This phenomenon reflects disappearance of the negative feedback in the hypothalamus-pituitary-gonadal axis by the removal of ovarian estrogen.

In the current study, the latency and distance to find the hidden platform, and the percentage of the random and circular strategies were significantly increased after 3-4 months of ovariectomy. This corroborates that estrogen deprivation for at least 3 months attributed to the deficiency of spatial learning and memory. The present results are consistent with the previous data obtained from 3- to 4-month OVX rats and 10-month OVX mice by Morris water maze (Feng et al., 2004; Wu et al., 2008; Hou et al,. 2011), indicating that estrogen depletion for 3 months could lead to the spatial learning and memory deficits.

This cognitive defect correlates well with the increased APP protein levels (Wu *et al.* 2008) and the accumulation of Aβ and hyperphosphorylated TAU (Hou et al., 2011) in the hippocampus. However, it was not known when the alteration happens in transcription level. In the present study, ovariectomy significantly increased the

expression levels of the *Tau4* mRNA in the hippocampus. This result is consistent with the increase in *Tau4* mRNA levels reported in Alzheimer's patients (Yasojima et al., 1999; Glatz et al., 2006). Thus, the increase in the *Tau4* mRNA leads to overproduction of TAU4 protein, and induces the formation of filamentous TAU pathology, which is particularly found in frontotemporal dementia (Spillantini et al., 1998; Robert & Mathuranath, 2007). Interestingly, these *Tau4* mRNA increments were observed as early as 1 month after ovariectomy (in the M_1 group).

This early increment in the expression of *Tau4* mRNA suggests that the alteration in the *Tau4* expression starts in early stages of estrogen deficiency prior to the progress of cognitive impairment. Several studies have shown that the estrogen treatment after the initiation of neurodegenerative disease generally fails to ameliorate the decline in cognitive function (Brenner et al., 1994; Henderson et al., 2000; Mulnard et al., 2000). Many current researches thus aim to determine the biomarkers indicative of the progress of neurodegenerative diseases, which can be detectable years before the development of pathology. This study is the first report that shows the increase in *Tau4* mRNA expression in the hippocampus, which can be used as a biomarker implicated in the early-onset of neurodegenerative diseases in estrogen deficit animals.

In the adult human brain, TAU4 and TAU3 exist in approximately equal amounts (Robert & Mathuranath, 2007). An alteration in the ratio of TAU isoforms may be a fundamental mechanism whereby NFTs formation occurs as found in mild cognitive impairment and Alzheimer's disease (Boutajangout et al., 2004; Ginsberg et al., 2006; Shi et al., 2011). In the present study, the *Tau4* and *Tau3* mRNAs showed different expression patterns after ovariectomy. The different expression levels in the *Tau4* and *Tau3* mRNAs appears as an increase in the *Tau4/Tau3* mRNA ratio after 4 months of ovariectomy. Although the increase in the *Tau4/Tau3* mRNA ratio was subtle, it is likely that the imbalance of *Tau4* and *Tau3* expression is involved in hippocampal neurodegeneration under estrogen-deficient conditions as seen in Alzheimer's patients (Ginsberg et al., 2006).

The increase in *Tau4* and *Tau3* mRNA expression also collated well with the increases in the serum FSH and LH levels. In patients with Alzheimer's disease, the serum LH levels are two times higher compared to the age-match controls (Short et al., 2001). Interestingly, the receptors for LH and FSH are expressed in the hippocampus area (Lathe, 2001; Chu et al., 2008). In addition, the LH receptor knock-out mice show

a significant decrease in the number of Aβ plaques and protein content in the hippocampus and the cerebral cortex (Lin et al., 2010). Several lines of evidence suggest that the raised serum LH levels could be a factor to induce Aβ deposition (Casadesus et al., 2006; Wahjoepramono et al., 2011). To understand how LH or FSH is involved in neurodegeneration and cognitive impairment in estrogen deficient animals, further studies are needed.

The *App* mRNA levels increased with advancing time after ovariectomy. This finding is consistent with a previous study that showed the expression of *App* mRNA in the hippocampus of 3-month OVX rats was significantly higher than the sham control animals (Wu et al., 2008). In addition to *App*, expression of the *Bace1* gene was also increased by estrogen deficiency, suggesting estrogen deficiency up-regulates the genes involved in the generation of Aβ. Further, deposition of Aβ plaques correlates with reduced serum estrogen levels during reproductive senescence in aged female mice (Gandy, 2003), and in OVX guinea pigs (Petanceska et al., 2000) and mice (Zheng et al., 2002). Recent studies have shown that BACE activity increases with age and is elevated in Alzheimer's disease brains (Holsinger et al., 2002; Li et al., 2004; Yue et al., 2005). Furthermore, BACE protein expression is down-regulated by estrogen treatment in mixed neuronal/glial cell culture (Nord et al., 2010). Thus, the overexpression of the *App* and *Bace1* mRNAs during estrogen deficiency could directly after Aβ deposition by regulating the expression of the precursor proteins and enzymes involved (Webb & Murphy, 2012).

The *Adam10* mRNA levels were significantly increased in relation to the time after ovariectomy in this study. Previous studies have shown that ovariectomy significantly decreases α-secretase activity together with the serum estrogen levels in rats (Yang et al., 2009). The expression of ADAM10 in cultured human fetal neurons is not altered by estrogen treatment at the gene or the protein level (Nord et al., 2010). To our knowledge, this is the first report that shows an increase in *Adam10* mRNA levels in estrogen deficient rats. Thus, it is possible that the increase in *App* expression after ovariectomy drives the amyloidogenesis (via BACE1) as well as the non-amyloidogenesis (via ADAM10) pathways. Supporting this notion, the *Bace1/Adam10* mRNA ratio in OVX rats did not change throughout the 4-month period.

Although little is known about the relationship between APP and TAU pathology, Aβ and TAU confer toxicity synergistically. Aβ exacerbates pre-existing TAU

pathology (Gotz et al., 2001), while Aβ toxicity is TAU dependent (Roberson et al., 2007). Taken together from these results, we postulate that after ovariectomy the increase in the *Tau4* mRNA expression accumulates TAU4 proteins in early states of estrogen deficiency, while the increases in the *App* and *Bace1* mRNA expressions can synergistically render the pre-existing TAU4 protein. This induces neuronal cell death or cellular dysfunction and subsequently results in the decreases in cognitive ability 3 months later.

The present study shows the sequence of the events linked to neurodegeneration induced by estrogen depletion by examining detailed time course of the change in gene expression, the serum levels of reproductive hormones, and cognitive ability. The present results indicate that estrogen deficiency induces consequent events of the increase in the serum LH and FSH levels, the accumulation of intracellular NFTs through the distortion of the *Tau4/Tau3* expression balance, and the deposition of extracellular amyloid plaques through the increase of Aβ production in the hippocampus. This finally leads to the loss of spatial memory at 3 months after ovariectomy. Translating the knowledge gained in this study from rats whose estrous cycle is 4-5 days to humans whose menstrual cycle is approximately 28 days (Johnson, 2007), suggesting that the estrogen deprivation in aged women can induce neurodegeneration within approximately two years. Using the synthetic estrogen or phytoestrogens to prevent or delay the onset of neurodegeneration in postmenopausal women should be a promising approach.

CHAPTER V
NEUROPROTECTIVE ACTIONS OF *Pueraria mirifica* A THAI HERB IN ESTROGEN-DEFICIENT RATS

Introduction

Estrogen deficiency is a well-known factor that causes Alzheimer's disease (Behl, 2002; Takuma et al., 2007). However, because of the side effects of synthetic estrogen treatment, the use of phytoestrogens should be considered as better and safer substitutes. *P. mirifica*, a plant which is endemic to Thailand, contains high amount of phytoestrogens. Using the HPLC technique, at least 17 phytoestrogens were identified and the puerarin isoflavone is the major constituent (Chansakaow et al., 2000; Cherdshewasart & Sutjit, 2008). Estrogenic effect of *P. mirifica* has been observed in reproductive organs (Malaivijitnond et al., 2004; Trisomboon et al., 2004; Cherdshewasart et al., 2007a) and bones (Urasopon et al., 2007; Urasopon et al., 2008b; Malaivijitnond, 2012). In addition, *P. mirifica* has also shown beneficial effects on the reduction of growth and incidence of breast tumor (Cherdshewasart et al., 2007b). Therefore, development of drugs for the alleviations of postmenopausal symptoms and osteoporosis using *P. mirifica* is of great interest to many pharmaceutical companies.

Extracts of *P. mirifica* exhibit neuroprotective effect in glutamate-induced cell death of HT22 mouse hippocampal cell lines (Sucontphunt et al., 2011) and increase synaptophysin immunoreactivity and synaptophysin levels in rat hippocampal neurons *in vitro* (Chindewa et al., 2008). Therefore, *P. mirifica* may have another potential for use in neurodegenerative diseases such as Alzheimer's disease. However, the use of *P. mirifica* to prevent neurodegeneration and memory loss in laboratory animals has yet been assessed. Here, the effects and mechanism of actions of *P. mirifica* and puerarin on the cognitive performance and expression of the genes associated with amyloid plaques (*App*, *Adam10* and *Bace1*) and NFTs (*Tau3* and *Tau4*) in the hippocampus of early middle-aged estrogen-deficient rats were investigated.

Estrogens exhibit neuroprotective effects via ERs (α and β) (Bohacek & Daniel, 2009). While ERβ is widely expressed in rat hippocampal neurons, there is little or no ERα expression in the hippocampus (Shughrue et al., 1997a). Furthermore, the

expression of *ER* genes in the female rat hippocampus is differentially affected by age (Adams et al., 2002; Mehra et al., 2005; Yamaguchi-Shima & Yuri, 2007), hormone deficiency and hormone replacement (Bohacek & Daniel, 2009). Therefore, it is of great interest to assess changes in *ER*α and *ER*β expression at the transcriptional level in the hippocampus of female rats during aging, after ovariectomy and estrogen/phytoestrogen replacement. This should help to explain the different mechanisms of action between estrogen and phytoestrogens on neuroprotection.

Materials and Methods

Animals

Adult female Sprague-Dawley rats, 2 months old, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Five animals/cage were reared in stainless steel cages with sawdust bedding in a room with controlled lighting (lights on 0600-1800 h) and temperature (25 ± 1 °C) at the Laboratory Animal Unit, Faculty of Science, Chulalongkorn University, Thailand. The animals were fed with a standard rat chow diet (Perfect Companion Group Co., Ltd., Samutprakarn, Thailand) and water *ad libitum*. The rats were reared until they became 4 months old, and were used following the protocols of Anukulthanakorn et al. (2013). The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1123009.

P. mirifica extract

The tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no. 0070317) were purchased from Kasetsart University, Kamphaeng Saen campus, Thailand (Tiyasatkulkovit et al., 2012). The *P. mirifica* extract was prepared as described previously with the %yield of 5.74% (Tiyasatkulkovit et al., 2012). Five major isoflavone contents in the *P. mirifica* extract were analyzed by HPLC and puerarin was a major component with a concentration of 3.53 μg/mg of the extract. The dried *P. mirifica* extract was stored at 4 °C until used. The *P. mirifica* extract at a dose of 100 mg/kg BW/day was freshly prepared from a stock solution of 1,000 mg/ml dissolved in 95% ethanol by diluting with appropriate amounts of distilled water.

Experimental design

The rats were divided into five groups (6-9 rats in each group); sham control, OVX, *P. mirifica*, puerarin and E_2 treated. The sham rats were operated as the OVX rats, but their ovaries were kept intact. In the OVX, *P. mirifica*, puerarin and E_2 groups, rats were bilaterally ovariectomized on the first day of the experiment under sodium pentobarbital anesthesia (40 mg/kg BW, i.p.). After recovery for 2 days, the OVX group was fed daily with 1 ml of distilled water, the *P. mirifica* group was fed with 100 mg/kg BW/day of *P. mirifica* extract (Malaivijitnond et al., 2006; Malaivijitnond et al., 2010), the puerarin group was subcutaneously injected with 7 mg/kg BW/day of puerarin (Malaivijitnond et al., 2010), and the E_2 group was subcutaneously injected with 80 μg/kg BW/day of synthetic E_2 (Feng et al., 2004), respectively, for 4 months.

Spatial memory test was performed using the Morris water maze test for each group of rats for 5 consecutive days before the end of the experiment (Anukulthanakorn et al., 2013). At the end of the experiment, the rats were euthanized, blood sera were collected for the assay of E_2, LH and FSH, uterus and vagina were dissected and weighed, and the hippocampus was collected for mRNA quantification. The mRNA levels of the genes associated with NFTs (*Tau3* and *Tau4*) and amyloid plaques (*App*, Adam10, and *Bace1*) and two-subtypes of estrogen receptor genes (*ERα* and *ERβ*) were analyzed using real-time PCR.

To examine if the hippocampal expression of *ER* genes is differentially regulated by age (Adams et al., 2002; Mehra et al., 2005; Yamaguchi-Shima & Yuri, 2007), the mRNA levels of *ERα* and *ERβ* of rats at the age of 4 months (= early middle-age) and 8 months (= middle-age), the initial and the last age, respectively, of animals in the above mentioned experiment were compared. Nine female Sprague-Dawley rats of 4 months old with at least two consecutive regular 4 or 5 day estrous cycles were selected. They were euthanized during the diestrous stage and the mRNA levels of *ERα* and *ERβ* genes in the hippocampus were analyzed using real-time PCR.

Hormone assays

Serum E_2 levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands (Korenman et al., 1974). The antiserum against E_2 (GDN 244) was kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, USA). Serum FSH and LH levels were determined using the National Institute of

Diabetes and Digestive and Kidney disease (NIDDK) kits for rat FSH and LH (Baltimore, MD, USA) as described previously (Jaroenporn et al., 2011). The results obtained are expressed using the rat FSH-RP-2 and rat LH-RP-2 reference standards. The intra- and inter-assay coefficients of variation were 2.4% and 7.5% for E_2, 4.5% and 5.7% for FSH, and 3.0% and 11.6% for LH, respectively.

Spatial memory test

The Morris water maze test was performed in a circular pool with 180 cm diameter and 70 cm deep (Anukulthanakorn et al., 2013). Each rat was given four trials per day for five consecutive days to find the hidden platform. The first trial was started by placing the rat into the water surface facing the pool wall in one of the four quadrants, and rotated the placing position clockwise to cover all four quadrants in the subsequent trials. From the next day onwards, the test was started from a quadrant different from the previous day. For each trial, the rat was allowed to swim for a maximum of 90 sec to find the platform. When the rat successfully found the platform, it was allowed to have a 30 sec rest on the platform. If the rat could not find the platform within the 90 sec, it was guided to the platform manually and was given a 30 sec rest on the platform, and a score of 90 sec was recorded. The latency to reach the platform and the swimming distance to find the hidden platform were measured using a video tracking system (Smart Junior, Panlab-Harvard Apparatus, Barcelona, Spain). The average value of the four trials in each day of each rat was calculated and counted as an individual value. The movement patterns of rats to find the platform were categorized into 4 strategies; line, taxis, random, and circular based on the criteria by Feng et al. (2004). The frequency of each strategy accounted by each rat was used for analysis.

Hippocampal RNA extraction and cDNA synthesis

Fresh brains were carefully removed from the skull, frozen immediately on a dry-ice chip, and stored at −80 °C for subsequent RNA extraction. The dissection of the brain was performed by transverse section using cryostat with 40 μm of thickness. The transverse section was started from corpus callosum to the third ventricle according to the rat brain Paxinos atlas (Paxinos & Watson, 2007). Total RNA was extracted from the left hippocampus using 300 μl TRIzol reagent (Invitrogen, Carlsbad, CA, USA)

according to the manufacturer's protocols. The quantity and the purity of the RNA sample were checked by measuring the photo absorbance at 260 and 280 nm wavelength. The total RNA sample (5 μg) was reverse transcribed to cDNA in a reaction mixture of 20 μl containing 2 μl of RT Buffer, 0.8 μl of dNTP Mix, 2 μl of RT Random Primer, 1 μl of MultiScribe Reverse Transcriptase, 1 μl of RNase Inhibitor using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). The samples were incubated for 10 min at 25 °C, 2 h at 37 °C, and finally for 5 min at 85 °C.

Real-time PCR analysis

The expression levels of the genes associated with neurofibrillary tangles (*Tau3* and *Tau4*) and amyloid plaques (*App*, *Adam10*, and *Bace1*) were examined using real-time PCR with the primers following Anukulthanakorn et al. (2013). The primers for *ERα* and *ERβ* were as follows: *ERα*, 5'-ACCAATGCACCATCGATAAGAAC-3' (forward) and 5'-TCTTTTCGTATCCCGCCTTTC-3' (reverse) and *ERβ*, 5'-GCGTTTGGTCATGTGAAGGA-3' (forward) and 5'-GCCGGTTCTTGTCTATGGTACAC-3' (reverse). The S28 ribosomal RNA was used as a housekeeping gene (Anukulthanakorn et al., 2013).

Real-time PCR was performed using Step One™ Plus Real-Time PCR System (Applied Biosystems) in a 10 μl reaction mixture containing 1x POWER SYBR Green PCR Master Mix, 0.5 μM each of the forward and reverse primer, and 1 μl of the cDNA sample. The reaction was carried out at 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, followed by a dissociation curve step. Relative expression levels of the target genes were calculated by $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA), with LSD post-hoc test, was used to determine the differences of means of the hormone and mRNA levels, and the uterus and vagina weights. For the spatial memory test, the statistical analysis for the latency and the distance travelled to search the hidden platform was performed using two-way ANOVA with repeated measures. The significant difference of the movement patterns was tested by Kruskal-Wallis and Mann-Whitney test. SPSS software program (version 17.0, SPSS Inc., Chicago, IL) was used for the analysis. Significance levels were set at $p < 0.05$.

Results

Serum hormone levels

Ovariectomy induced an estrogen-deficient stage compared to the sham control (E_2 = 32.05±5.05 pg/ml and 57.77±8.53 pg/ml in the OVX and sham groups, respectively) with significantly higher serum LH and FSH levels (LH = 33.20 ± 5.50 ng/ml and 0.39 ± 0.06 ng/ml, and FSH = 56.11 ± 8.96 ng/ml and 4.50 ± 0.70 ng/ml in the OVX and sham groups, respectively) (Figure 5.1A, B). Treatment with *P. mirifica* and puerarin had no effect on serum LH and FSH levels compared to the OVX group. However, treatment with E_2 significantly attenuated the increased serum LH and FSH levels (~10 fold reduction for LH ($p < 0.001$) and ~2 fold reduction for FSH ($p < 0.05$) in comparison to the OVX group; Figure 5.1A, B). The serum LH and FSH levels in the E_2 group were higher than the sham group and the difference in the FSH levels was statistically significant ($p < 0.05$; Figure 5.1A, B).

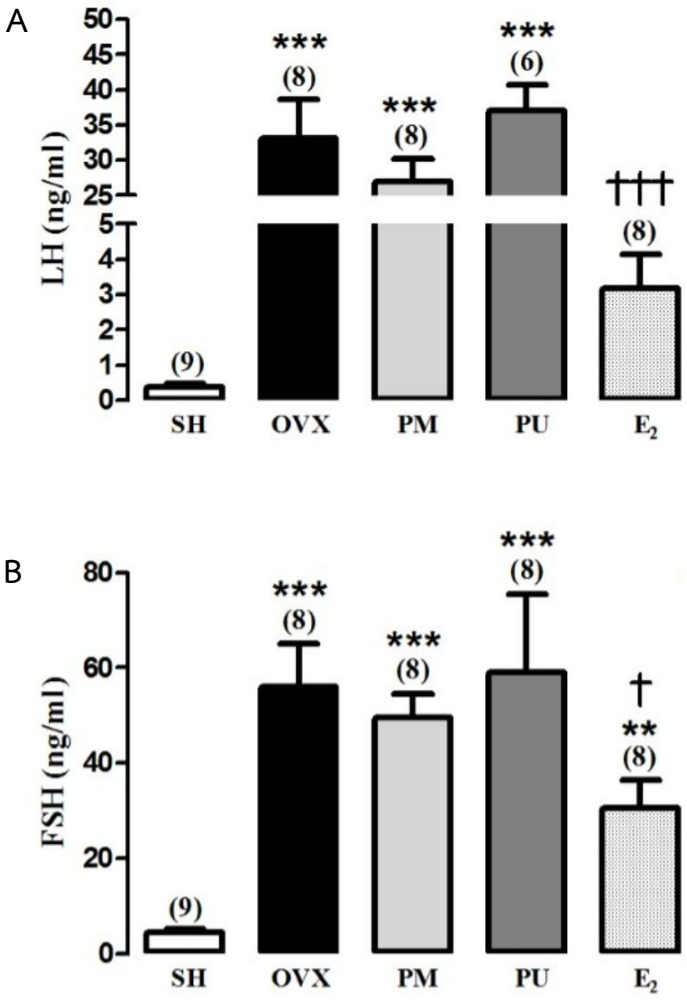

Figure 5.1 Serum levels of LH (A) and FSH (B) in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). **, *** $p < 0.01$ and 0.001 compared to the sham control, respectively. †,††† $p < 0.05$ and 0.001 compared to the OVX group. The number in parentheses indicates the number of rats used in each group.

Relative weights of uterus and vagina

After 4 months of ovariectomy while the rats had very low E_2 levels, the relative weight of uterus and vagina weights were significantly decreased compared to the sham control ($p < 0.001$) (Table 5.1). Although *P. mirifica* could not ablate the increased serum LH and FSH levels induced by ovariectomy, the significant increase in relative weight of uterus and vagina to the sham control levels were detected ($p < 0.001$), but

slightly less than those of the E_2 group. Puerarin had no effects either on the relative weights of uterus or vagina.

Table 5.1 Relative weights of uterus and vagina of the ovary-intact rats (sham; SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2) for 4 months.

Treatment group	Number	Uterus weight (10^3)	Vagina weight (10^3)
SH	9	1.495 ± 0.080	0.689 ± 0.027
OVX	8	0.415 ± 0.026**	0.358 ± 0.035**
PM	8	1.340 ± 0.082†	0.633 ± 0.055†
PU	8	0.444 ± 0.025**	0.327 ± 0.028**
E_2	8	1.735 ± 0.148†	0.824 ± 0.064*†

* $p < 0.001$ compared to the sham control group. † $p < 0.001$ compared to the OVX group

Expression of estrogen receptors

Expression of estrogen receptor subtypes (*ERα* and *ERβ*) was observed in the hippocampus of female rats with significantly lower relative mRNA levels in the middle age group (8 months old) compared to the early-middle age group (4 months old) (~2.5 fold reduction for *ERα* ($p < 0.01$) and ~1 fold reduction for *ERβ* in comparison to the 4 months old rats (Figure 5.2).

In comparison to the sham group, no significant change in relative mRNA levels of *ERα* and *ERβ* was observed in the OVX group (Figure 5.3). The *P. mirifica*, puerarin and the E_2 groups showed no difference in the mRNA levels of *ERα* and *ERβ* when compared to the sham and the OVX groups, except that the *ERα* mRNA level in the E_2 group was significantly higher when compared to the sham group ($p < 0.05$; Figure 5.3A).

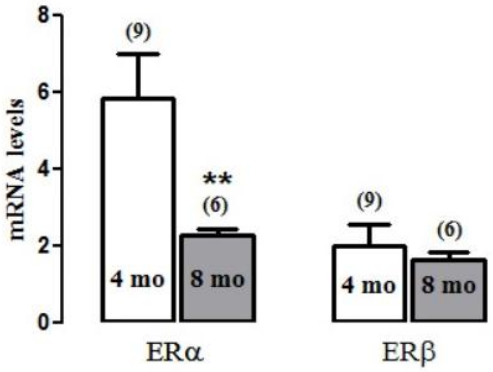

Figure 5.2 The mRNA levels of the two estrogen receptor subtypes (*ERα* and *ERβ*) in the hippocampus of 4 and 8 months old female rats. ** $p < 0.01$ compared to the 4 months old female rats. The number in parentheses indicates the number of rats used in each group.

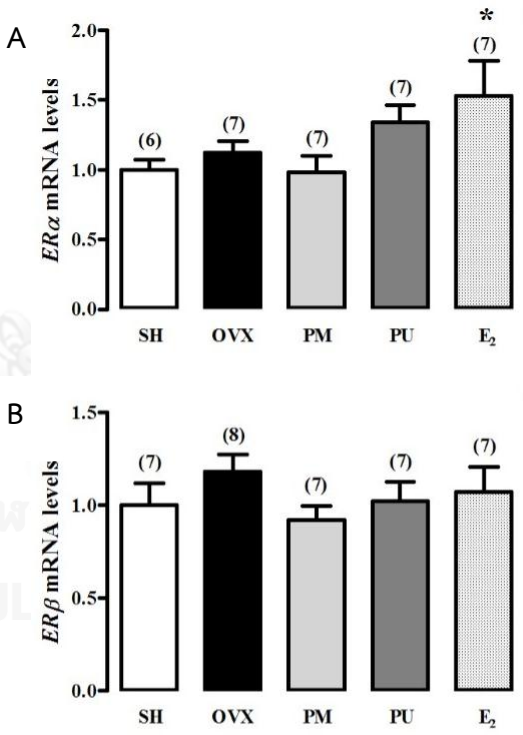

Figure 5.3 The relative mRNA levels of *ERα* (A) and *ERβ* (B) genes in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). * $p < 0.05$ compared to the sham group. The number in parentheses indicates the number of rats used in each group.

Expression of genes associated with NFTs and amyloid plaques

In the OVX group, the relative mRNA levels of *Tau3* and *Tau4* genes were significantly higher ($p < 0.05$) compared to the sham group (Figure 5.4). Similarly, the mRNA levels of amyloid plaques-related genes in the OVX group were higher compared to the sham group, however the changes were marginal ($p = 0.054$, 0.062 and 0.062 for *App*, *Bace1* and *Adam10*, respectively) (Figure 5.5). The treatments with E_2, *P. mirifica*, and puerarin significantly decreased the *Tau* mRNA levels except for the *Tau3* mRNA levels in the *P. mirifica* group when compared to the OVX group (Figure 5.4). The mRNA levels of amyloid plaques-related genes in the E_2, *P. mirifica*, and the puerarin groups were similar to those in the sham group. However, in comparison to the OVX group, the *App* mRNA level in the *P. mirifica* group and the *Bace1* mRNA levels in the *P. mirifica* and E_2 groups were significantly decreased ($p < 0.05$ and 0.01) (Figure 5.5).

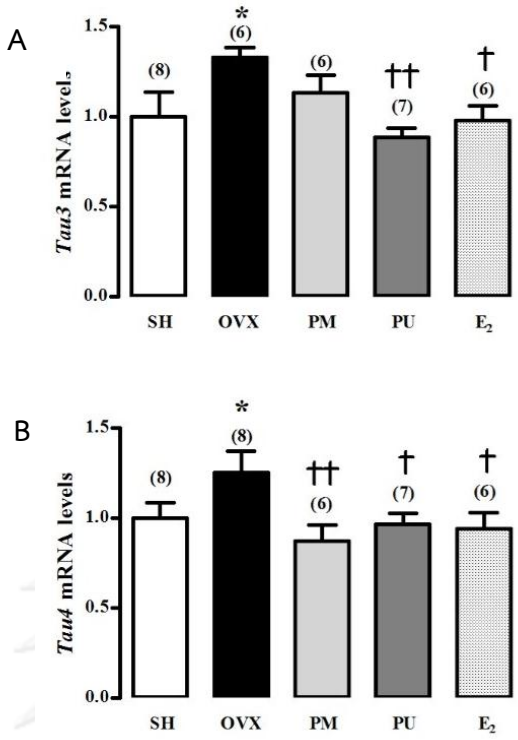

Figure 5.4 The relative mRNA levels of the genes associated to NFTs, *Tau3* (A) and *Tau4* (B), in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). * $p < 0.05$ compared to the SH, †,†† $p < 0.05$ and 0.01 compared to the OVX group, respectively. The number in parentheses indicates the number of rats used in each group.

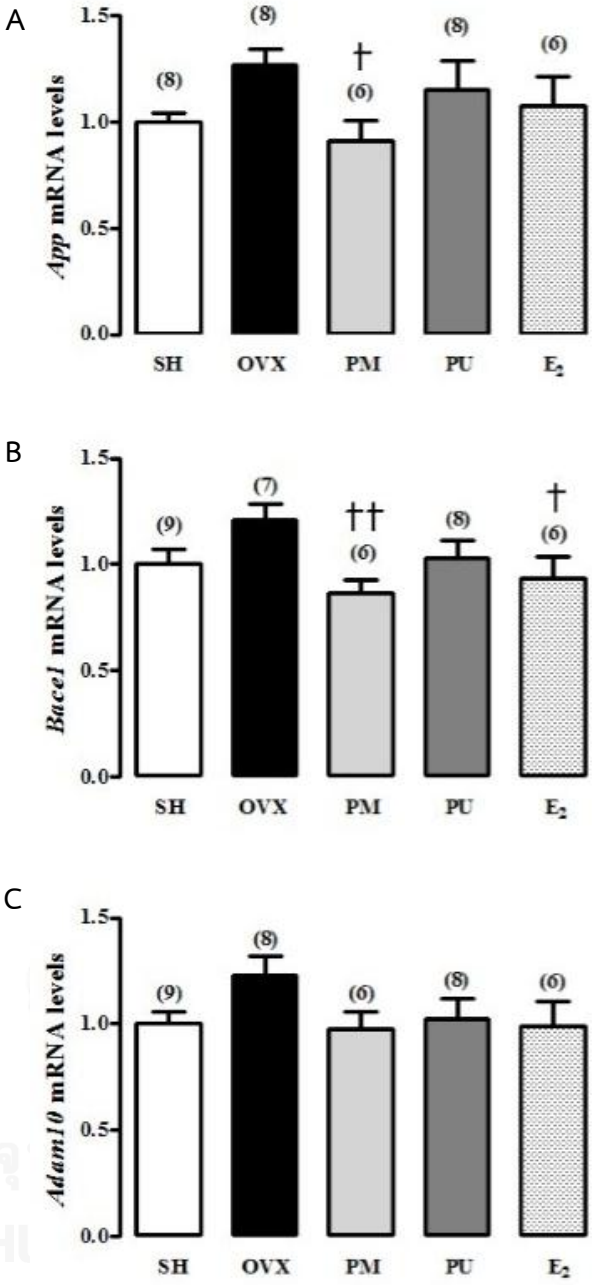

Figure 5.5 The relative mRNA levels of the genes associated to amyloid plaques, *App* (A), *Bace1* (B) and *Adam10* (C), in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). †,†† $p < 0.05$ and 0.01 compared to the OVX group, respectively. The number in parentheses indicates the number of rats used in each group.

Cognitive performance

Estrogen deficiency in the OVX rats induced cognitive impairment, which is indicated by the significantly longer latency ($p < 0.05$) and the longer distance travelled, although marginal ($p = 0.06$), in search of the hidden platform during the 5-day Morris water maze test (Figure 5.6A, B). If only the 5th day of the Morris water maze test was considered, then the latency and the distance travelled were significantly higher ($p < 0.005$ for the latency and $p < 0.05$ for the distance) in the OVX group compared to the sham group (Figure 5.7A, B). The circular strategy for searching the platform was observed in 29% of the OVX rats, but absent in the sham group (Figure 5.7 C).

P. mirifica and E_2 treatments decreased, although not significant, the latency and the distance travelled to the platform compared to the OVX group (Figure 5.6A, B). However, if only the latency and the distance of the 5th day of the Morris water maze test was analyzed, the E_2, *P. mirifica*, and puerarin groups showed significantly lower latency and distance travelled compared to the OVX group (Figure 5.7A, B). In addition, the puerarin and E_2 treated rats exhibited a clear difference in the strategy for searching the platform compared to the OVX rats ($p < 0.01$; Figure 5.7C). Although the strategy for searching the platform by the *P. mirifica* group did not differ from that of the OVX group, the percentage of circular pattern was significantly reduced (Figure 5.7C).

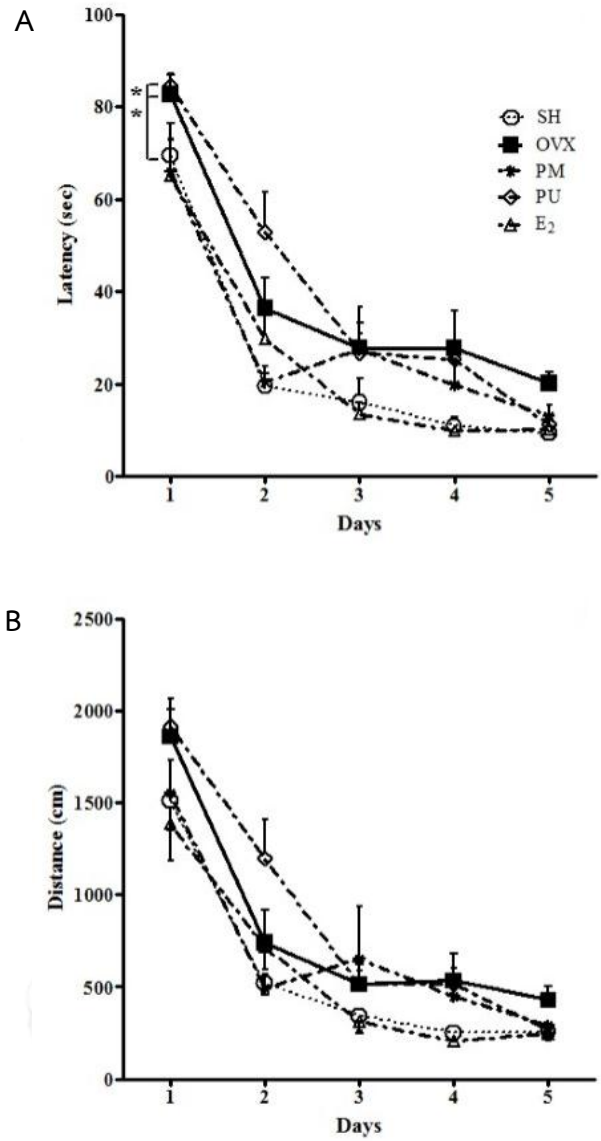

Figure 5.6 The latency period (A) and distance (B) of searching for the hidden platform during the 5-day Morris water maze test in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). * $p < 0.01$ compared to the sham group as analyzed by two-way ANOVA with repeated measures.

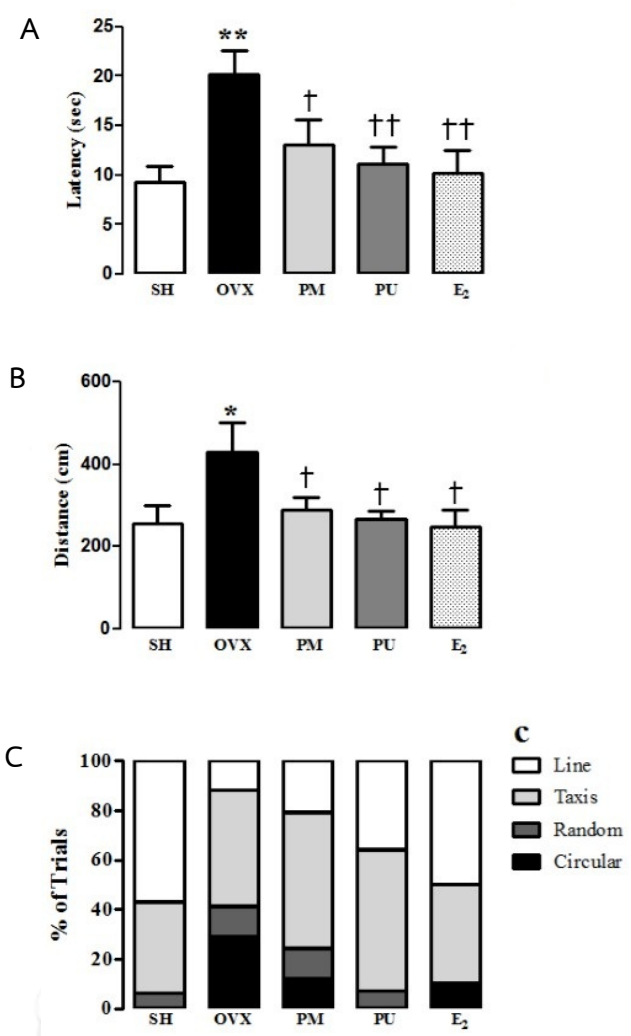

Figure 5.7 The latency period (A), distance (B) and strategy of movement patterns (C) of searching for the hidden platform at the 5th day of the Morris water maze test in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). *, ** $p < 0.05$ and 0.005 compared to the sham control, and †, †† $p < 0.05$ and 0.005 compared to the OVX rats, respectively

Discussion

The significantly lower serum E_2 levels and weights of uterus and vagina, and higher serum LH and FSH levels compared to the sham group confirmed the efficacy of bilateral ovariectomy in all OVX rats. Although no significant effects of *P. mirifica* on serum LH and FSH levels were observed, the recovery of uterus and vagina weights to the sham control levels was detected. This can confirm the estrogenic activity of phytoestrogens in the 100 mg/kg BW/day of *P. mirifica* extract as reported previously (Malaivijitnond et al., 2006; Malaivijitnond et al, 2010). The estrogenic activity of puerarin was unable to be distinguished in this study, regarding either changes of the serum LH and FSH levels or the uterus and vagina weights. However, it was clearly indicated in the previous study that puerarin at a dose of 7.0 mg/kg BW/day had a very weak estrogenic activity on vaginal cornification and uterus proliferation after 140-day subcutaneous injection to rats (Malaivijitnond et al., 2010). Compared to the OVX group, E_2 treatment drastically decreased the serum LH and FSH levels. The lesser extent of the FSH response to E_2, in comparison to the LH response, confirms the involvement of inhibin in the negative feedback on pituitary FSH secretion (Arai *et al.* 1996). The present results accord with previous reports that show the existence of ERs (α and β) in the hypothalamus and pituitary gland (Shughrue et al., 1997a) and the higher affinity of E_2 to both estrogen receptor subtypes compared to phytoestrogens (Kuiper et al., 1998).

It is clear that estrogen deficiency during menopause and after ovariectomy induces neurodegeneration (Dubal & Wise, 2002; Feng et al., 2004). It is also widely accepted that phytoestrogens can mimic several beneficial effects of estrogens in the brain. Indeed, the neurotrophic and neuroprotective properties of phytoestrogens have been documented. For example, phytoestrogens can protect against neurodegeneration after menopause through the preservation of mitochondrial structure and functions (Xu et al., 2008). Phytoestrogens can also increase the mRNA levels of brain-derived neurotrophic factor (Pan et al., 1999a) and choline acetyltransferase (Pan et al., 1999b) in the rat brain. Phytoestrogens isolated from another *Pueraria* species *Pueraria lobata* exhibit neuroprotective effects after Aβ insult in PC12 rat cell line (Choi et al., 2010). Puerarin decreases Bax/Bcl-2 expression ratio in the PC12 cell line (Zhang et al., 2008), and increases phospho-Bad, a member of the Bcl-2 family, and decreases caspase-9 protein levels in *in vivo* rat model (Li et al.,

2010). In addition, neuroprotective effects of genistein, a well-known isoflavone phytoestrogen isolated from soy, are well documented. Genistein inactivates glycogen synthase kinase 3β (GSK3β) and decreases Tau hyperphosphorylation in SH-SY5Y human neuroblastoma cells (Park et al., 2009) and enhances the expression of Aβ degrading enzymes in the rat hippocampus (Zhao et al., 2009). Since *P. mirifica* contains at least 17 phytoestrogens including genistein (Cherdshewasart & Sriwatcharakul, 2008a), the beneficial antagonistic effects of *P. mirifica* on neurodegeneration was expected.

The increase in mRNA levels of *Tau3*, *Tau4*, *App*, *Adam10* and *Bace1* in the hippocampus and cognitive impairment after ovariectomy (Anukulthanakorn et al., 2013) could be reverted by the treatment with phytoestrogens (*P. mirifica* and puerarin) and synthetic estrogen (E_2). This suggests that phytoestrogens and estrogen can mitigate cognitive impairment via the reduction of both neurological hallmarks of neurodegenerative diseases; intracellular NFTs and extracellular senile plaques (Goedert & Spillantini, 2011). As such, this is the first report showing expression of the genes associated with NFTs and amyloid plaques in the rat hippocampus after phytoestrogen treatments. However, it needs to assess further, if these changes of the genes expression after estrogen/phytoestrogen treatments could lead to any pathological evidences in the brain.

P. mirifica could prevent the estrogen deficiency-induced neurodegeneration and memory loss in female rats probably by inhibiting the formation of amyloid plaques (amyloidogenic pathway) as well as NFTs (TAU pathway). Puerarin, the major constituent of phytoestrogens in *P. mirifica*, probably has neuroprotective action mainly through the TAU pathway, because only *Tau 3* and *Tau4* mRNA levels in the puerarin-treated rats showed significant decreases compared to the OVX rats. Although the effect of puerarin on the amyloidogenic pathway through the suppression of Aβ-induced apoptosis has been documented, it was done only in *in vitro* system using PC12 cell (Zhang et al., 2008). Thus, other phytoestrogen substances such as miroestrol and genistein identified in *P. mirifica*, which exhibit very high estrogenic activity (Malaivijitnond, 2012), may help to increase the efficacy of *P. mirifica* on the reduction of the amyloid plaques. Aβ, a major component of amyloid plaque, is generated from the APP, which is cleaved by BACE1 and γ secretase enzymes sequentially (Kandalepas

& Vassar, 2012). *P. mirifica* was more effective than E_2 to reduce *App* and *Bace1* mRNA levels, demonstrating to be a better neuroprotectant.

Using real time-PCR, it was found that the mRNA levels of ER subtypes in the hippocampus were higher in early-middle aged rats (4 months old) compared to middle-aged rats (8 months old). Histological studies showed that the number of ERα-ir and ERβ-ir neurons in the rat hippocampus decrease during aging (Mehra et al., 2005; Yamaguchi-Shima & Yuri, 2007). However, the expression of *ERβ* is higher than *ERα* (Mehra et al., 2005; Foster, 2012). On the contrary, the higher *ERα* mRNA levels in the hippocampus of aged rats were observed. There are three possible explanations for these contradictory results: i) the difference in the age groups between the two studies, ii) the real time-PCR technique used in the present study detected changes at the mRNA level, while the other studies detected protein levels using immunohistochemical (Mehra et al., 2005) or Western blot techniques (Bohacek & Daniel, 2009), and iii) the expression of *ER* mRNA levels which determined, it might also include the cell membrane ERs while those reported by others detected only the intracellular ERs (cytoplasm or nucleus) (Mehra et al., 2005). Indeed, membrane ERs mediate a rapid non-genomic signaling by estrogen and phytoestrogens (Walf & Frye, 2008a; Foster, 2012) and could impact neuroptorective actions via G-proteins in neuroplasma membranes in Alzheimer's brains (Jefremov et al., 2008).

Estrogens and phytoestrogens abrogate neurodegeneration and Alzheimer's disease by binding to ERs (Brann et al., 2007; Bonomo et al., 2009; Foster, 2012). Since E_2 treatment increased only *ERα* mRNA levels, this supports the idea that increased memory could be a result of increased *ERα* expression. Indeed, an increase in hippocampal *ERα* mRNA levels with no changes in *ERβ* mRNA levels by E_2 treatments was also seen in young (2 months old) and middle-aged rats (8-10 months old) (Bohacek & Daniel, 2009; Rodgers et al., 2010).

Contrary to E_2 treatment, phytoestrogens have no effect on *ERα* or *ERβ* mRNA levels *in vivo* (this study) and *in vitro* (Schreihofer, 2005), which might be because of desensitization of ERs by the weak estrogenic effects of phytoestrogens (Bohacek & Daniel, 2009). Thus the neuroprotective actions of phytoestrogens could be through its antioxidant properties (Shi et al., 2006) or through the reduction of reactive oxygen species (ROS), malondialdehyde concentration, cytosolic cytochrome C and caspase-3

in the hippocampus (Liang et al., 2008), and enhanced superoxide dismutase content in the hippocampus (Huang & Zhang, 2010).

It has been proposed that a decrease in E_2 levels or the high levels of LH during estrogen-deficient stage could cause neurodegeneration (Lin et al., 2010; Wahjoepramono et al., 2011). However, the absence of a significant decline in serum LH or FSH levels after *P. mirifica* treatment but enrichment of cognition in the OVX rats to the same degree as E_2 treatment suggests that serum LH level is not the major cause of cognitive impairment. Further comprehensive research is needed to understand the specific neuronal systems influenced by *P. mirifica*.

In conclusion, *P. mirifica* and puerarin, the major constituent of *P. mirifica*, prevent the estrogen deficiency-induced neurodegeneration and memory loss in rats probably by the suppression of expression of genes associated with amyloid plaques and NFTs. This corroborates the high potential of *P. mirifica* as an alternative drug for the prevention of Alzheimer's disease.

CHAPTER VI

NEUROPROTECTIVE EFFECTS OF *Pueraria mirifica* AT THE TIME OF ESTROGEN DEPRIVATION AND LH SURGE, BUT WITHOUT COGNITIVE IMPAIRMENT, IN OVARIECTOMIZED RATS

Introduction

Estrogen is recognized as one of the most important regulators of neuronal function, including neuronal proliferation, survival and plasticity (Unal et al., 2012). Estrogen deficiency is then associated with neurodegeneration in the hippocampus (Unal et al., 2012) and ERT correlates with a reduction and delay in the onset of the diseases (Gandy, 2003). As such, ovariectomy in laboratory animals and menopause in women can result in neuronal changes and impairment of cognitive performance which link to neurodegenerative diseases such as Alzheimer's disease (Singh et al., 2008). The ovariectomized rats or mice have been used as neurodegenerative animal models to assess if estrogens may prevent or attenuate memory deficits.

However, the contradictory reports from the Women's Health Initiative (WHI) depicted that the ERT initiated in elderly post-menopausal women (ages 65 and above) does not improve cognitive performance and may actually increase the risk of developing of Alzheimer's disease (Shumaker et al., 2003). This indicates that estrogen treatment could produce beneficial effects in neurodegeneration and cognitive function only if the treatment has been initiated within a critical period of time. In women, menopause shifts the balance of the hypothalamic-pituitary-ovarian axis feedback loop which attributes to the loss of negative feedback by estrogens and results in a 3 to 4 folds increase in concentration of serum LH and a 4 to 18 folds increase in concentration of serum FSH (Chakravarti et al., 1976). Thus, estrogen deprivation always happens together with increased LH and FSH concentrations. Generally, serum LH and FSH levels significantly increase within 1 week after ovariectomy (Malaivijitnond et al., 2004) and peaked at 6 or 8 weeks before reaching a plateau phase onwards (Anukulthanakorn et al., 2013). The effects of the increased circulating LH and FSH levels due to the loss of negative feedback on the aging brain are largely explored. However, Lei et al. (1993) indicated that only LHR had been detected in the brain, the highest density of which was found within the

hippocampus. There are evidences supporting the relationship between LH and cognitive performance and some reports suggested that LH peak following menopause might also be a critical factor to cause the brain damage. Experimentally abolishing LH synthesis and secretion in the aged APP transgenic mouse model of Alzheimer disease (Tg 2576) using a selective GnRH agonist (leuprolide acetate) improved hippocampally-related cognitive performance and decreased Aβ deposition (Casadesus et al., 2006). Thus, treatment of Alzheimer's disease by chemicals showing both estrogenic activity and suppressive effect on LH synthesis and secretion should become a better alternative neuroprotective strategy for neuronal dysfunction in postmenopausal women.

Recently, researching on natural-based agents with fewer side-effects, cheap and exhibiting estrogenic activity, such as phytoestgrogens containing plants, is challenging. *P. mirifica* is a renowned endemic Thai plant of which its tuberous root was isolated at least 17 phytoestrogens, and puerarin is a major constituent (Cherdshewasart et al., 2007d). The estrogenic activity of phytoestrogens in *P. mirifica* has been tested widely both *in vitro* and *in vivo* (in mice, rats, monkeys and humans) (Cherdshewasart and Sriwatcharakul, 2008a; Cherdshewasart et al., 2007; Malaivijitnonnd et al., 2006; Trisomboon et al., 2006). Moreover, *P. mirifica* can also attenuate the increased serum LH levels in OVX rats (Malaivijitnond et al., 2004) and in aged female monkeys (Trisomboon et al., 2006). Recently, it was reported that treatment of *P. mirifica* for 4 months after 2-day of ovariectomy in rats exhibited neuroprotective actions (Anukulthanakorn et al., 2014, in preparation). Therefore, it was a great of interest to determine if feeding of *P. mirifica* in OVX rats at the time of estrogen depletion and LH peak, but cognitive impairment was yet occurred, could prevent neurodegeneration.

Materials and Methods

Animals

Adult female Sprague-Dawley rats, 2 months old, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were reared in stainless steel cages (five animals/cage) with sterile sawdust bedding in a room with controlled lighting (lights on 0600-1800 h) and temperature (25 ± 1°C) at the Laboratory Animal Unit, Faculty of Science, Chulalongkorn University, Thailand. The animals were

fed with a standard rat chow diet (Perfect Companion Group Co., Ltd., Samutprakarn, Thailand) and water *ad libitum*. The rats were reared until they became 4 months old, and were used following the protocols of Anukulthanakorn et al. (2013). The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1123009.

***P. mirifica* extract**

The tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no. 0070317) were purchased from Kasetsart University, Kamphaeng Saen campus, Thailand (Tiyasatkulkovit et al., 2012). The *P. mirifica* extract was prepared as described previously with the %yield of 5.74% (Tiyasatkulkovit et al. 2012). Five major isoflavone contents, genistin, genistein, daidzin, daidzein and puerarin, in the *P. mirifica* extract were analyzed by HPLC and puerarin concentration was 3.53 μg/mg of the extract. The dried *P. mirifica* extract was stored at 4 °C until used. The *P. mirifica* extract at a dose of 100 mg/kg BW/day was freshly prepared from a stock solution of 1,000 mg/ml dissolved in 95% ethanol by diluting with appropriate amounts of distilled water.

Experimental design

The rats were divided into five groups (3-9 rats in each group); sham control, OVX, *P. mirifica*, puerarin and E_2 treated. The sham rats were operated as the OVX rats, but their ovaries were kept intact. In the OVX, *P. mirifica*, puerarin and E_2 groups, rats were bilaterally ovariectomized on the first day of the experiment under sodium pentobarbital anesthesia (40 mg/kg BW, i.p.). After ovariectomy for 2 months when the serum E_2 level was decline and serum LH levels was elevated (Malaivijitnond et al., 2004; Anukulthanakorn et al., 2013), the OVX group was fed daily with 1 ml of distilled water, the *P. mirifica* group was fed with 100 mg/kg BW/day of *P. mirifica* extract (Malaivijitnond et al., 2006; Malaivijitnond et al., 2010), the puerarin group was subcutaneously injected with 7 mg/kg BW/day of puerarin (Malaivijitnond et al., 2010), and the E_2 group was subcutaneously injected with 80 μg/kg BW/day of synthetic E_2 (Feng et al., 2004), respectively, for 4 months.

Spatial memory test was performed using the Morris water maze test for each group of rats for 5 consecutive days before the end of the experiment. At the end of

the experiment, the rats were euthanized, the hippocampus was collected for mRNA quantification. The mRNA levels of the genes associated with NFTs (*Tau3* and *Tau4*) and amyloid plaques (*App*, Adam10, and *Bace1*) and two-subtypes of estrogen receptors (*ER*α and *ER*β) were analyzed using real-time PCR as described previously (Anukulthanakorn et al., 2013).

Hormone assays

Serum E_2 levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands (Korenman et al., 1974). The antiserum against E_2 (GDN 244) was kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and inter-assay coefficients of variation were 2.4% and 7.5%, respectively.

Serum FSH and LH levels were determined using the National Institute of Diabetes and Digestive and Kidney disease (NIDDK) kits for rat FSH and LH (Baltimore, MD, USA) as described previously (Jaroenporn et al., 2011). The results obtained are expressed using the rat FSH-RP-2 and rat LH-RP-2 reference standards. The intra- and inter-assay coefficients for variations were 4.5% and 5.7% for FSH and 3.0% and 11.6% for LH, respectively.

Spatial memory test

The Morris water maze test was performed in a circular pool with 180 cm diameter and 70 cm deep (Anukulthanakorn et al., 2013). Each rat was given four trials per day for five consecutive days to find the hidden platform. The first trial was started by placing the rat into the water surface facing the pool wall in one of the four quadrants, and rotated the placing position clockwise to cover all four quadrants in the subsequent trials. From the next day onwards, the test was started from a different quadrant from the previous day. For each trial, the rat was allowed to swim for a maximum of 90 sec to find the platform. When the rat successfully found the platform, it was allowed to have a 30 sec rest on the platform. If the rat could not find the platform within the 90 sec, it was guided to the platform manually and was given a 30 sec rest on the platform, and a score of 90 sec was recorded. The latency to reach the platform and the swimming distance to find the hidden platform were measured using a video tracking system (Smart Junior, Panlab-Harvard Apparatus, Barcelona, Spain).

The average value of the four trials in each day of each rat was calculated and counted as an individual value. The movement patterns of rats to find the platform were categorized into 4 strategies; line, taxis, random, and circular based on the criteria by Feng et al. (2004). The frequency of each strategy accounted by each rat was used for analysis.

Hippocampal RNA extraction and cDNA synthesis

Fresh brain of rat was carefully removed from the skull, frozen immediately on a dry-ice chip, and stored at −80 °C for subsequent RNA extraction. The dissection of the brain was performed by transverse section using cryostat with 40 μm of thickness. The transverse section was started from corpus callosum to the third ventricle according to the rat brain Paxinos atlas (Paxinos & Watson 2007). Total RNA was extracted from the left hippocampus using 300 μl TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The quantity and the purity of the RNA sample were checked by measuring the photo absorbance at 260 and 280 nm wavelengths. The total RNA sample (5 μg) was reverse transcribed to cDNA in a reaction mixture of 20 μl containing 2 μl of RT Buffer, 0.8 μl of dNTP Mix, 2 μl of RT Random Primer, 1 μl of MultiScribe Reverse Transcriptase, 1 μl of RNase Inhibitor using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The samples were incubated for 10 min at 25 °C, 2 h at 37 °C, and finally for 5 min at 85 °C.

Real-time PCR analysis

The expression levels of the genes associated with NFTs (*Tau3* and Tau4) and amyloid plaques (*App*, *Adam10*, and *Bace1*) were examined using real-time PCR with the primers following Anukulthanakorn et al. (2013). The primers for *ERα* and *ERβ* were as follows; *ERα*, 5'-ACCAATGCACCATCGATAAGAAC-3' (forward) and 5'-TCTTTTCGTATCCCGCCTTTC-3' (reverse) and *ERβ*, 5'-GCGTTTGGTCATGTGAAGGA-3' (forward) and 5'-GCCGGTTCTTGTCTATGGTACAC-3' (reverse). The S28 ribosomal RNA was used as a housekeeping gene (Anukulthanakorn et al., 2013).

Real-time PCR was performed using Step One™ Plus Real-Time PCR System (Applied Biosystems, CA, USA) in a 10 μl of reaction mixture containing 1x POWER SYBR Green PCR Master Mix, 0.5 μM each of the forward and reverse primer, and 1 μl of the cDNA sample. The reaction was carried out at 95 °C for 10 min, 40 cycles of 95 °C for

15 sec and 60 °C for 1 min, followed by a dissociation curve step. Relative expression levels of the target genes were calculated by $2^{-\Delta\Delta Ct}$ method. The value of the sham control group was adjusted to 1 and the mRNA levels of the other groups were calculated relative to the sham group.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA), with LSD post-hoc test, was used to determine the differences of means of the hormone and mRNA levels. For the spatial memory test, the statistical analysis for the latency and the distance travelled to search the hidden platform was performed using two-way ANOVA with repeated measures. The significant difference of the movement patterns was tested by Kruskal-Wallis and Mann-Whitney test. SPSS software program (version 17.0, SPSS Inc., Chicago, IL, USA) was used for the analysis. Significance levels were set at $p < 0.05$.

Results

Serum hormone levels

Ovariectomy induced an estrogen-deficient stage compared to the sham control (E_2 = 67.54 ± 9.54 pg/ml and 166.61 ± 48.03 pg/ml in the OVX and sham groups, respectively, Figure 6.1A) with significantly higher serum LH and FSH levels (LH = 24.97 ± 3.01 ng/ml and 0.72 ± 0.06 ng/ml, and FSH = 60.62 ± 7.40 ng/ml and 7.40 ± 0.61 ng/ml in the OVX and sham groups, respectively) (Figure 6.1B and C). Treatment with *P. mirifica* significantly decreased serum LH level ($p < 0.05$) while it had no effect on serum FSH level compared to the OVX group. Puerarin had neither effect on serum LH nor FSH levels compared to the OVX group ($p > 0.05$). Serum LH and FSH levels were significantly decreased in the E_2 treatment group ($p < 0.005$), however, only serum LH level returned to the sham control level ($p > 0.05$) (Figure 6.1B and C).

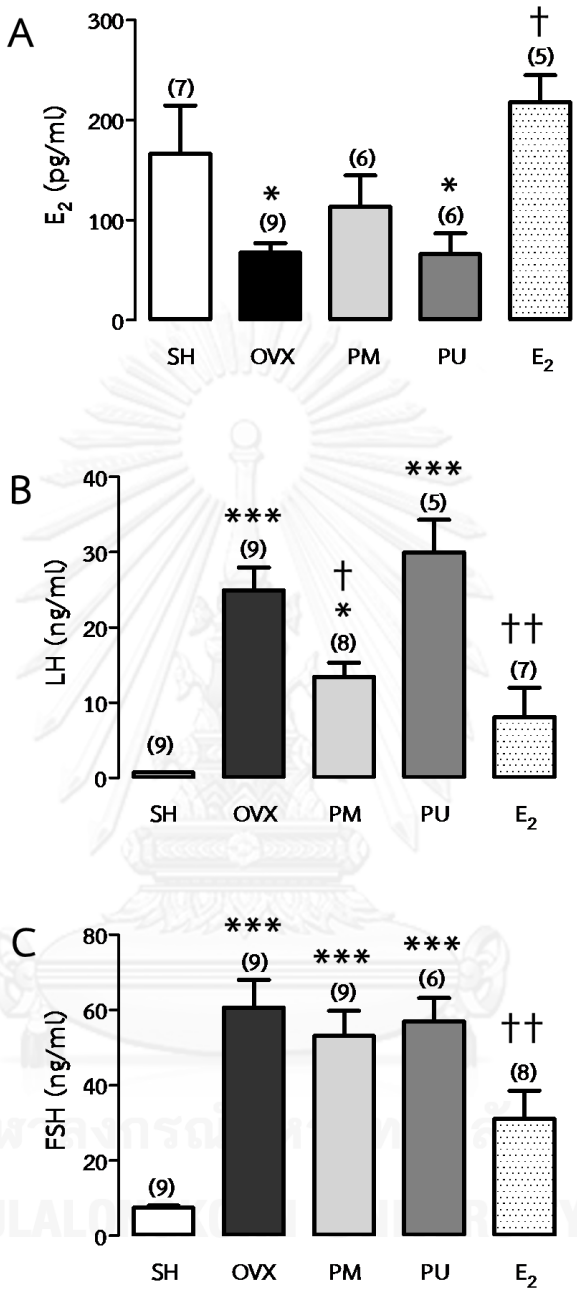

Figure 6.1 Serum levels of 17β-estradiol (E_2, A), luteinizing hormone (LH, B) and follicle stimulating hormone (FSH, C) in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). *, *** $p < 0.05$ and 0.001 compared to the sham control group (SH), respectively. †, †† $p < 0.05$ and 0.005 compared to the OVX group.

Relative weights of uterus and vagina

After 6 months of ovariectomy while the rats had very low serum E_2 level, the relative weights of uterus and vagina were significantly decreased in the OVX group ($p < 0.001$ and 0.05, respectively) (Table 6.1). Treatment with *P. mirifica* significantly ablated the effect of ovariectomy on the relative weights of uterus and vagina. Puerarin had no effect on the relative weight of uterus, however, it was significantly increased the relative weight of vagina to the sham control level. Treatment with E_2 significantly increased both the relative weights of uterus and vagina compared to the OVX group ($p < 0.005$) and returned to the sham control levels.

Table 6.1 Relative weights of uterus and vagina of the ovary-intact rats (sham; SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2) for 4 months.

Treatment group	Number	Uterus weight (10^3)	Vagina weight (10^3)
SH	9	1.627 ± 0.129	0.789 ± 0.039
OVX	9	0.447 ± 0.052**	0.401 ± 0.052*
PM	9	1.243 ± 0.066*††	0.856 ± 0.110††
PU	8	0.594 ± 0.055**	0.709 ± 0.103†
E_2	8	1.425 ± 0.063††	0.850 ± 0.125††

*,** $p < 0.05$ and 0.001 compared to the sham control group. †, †† $p < 0.05$ and 0.005 compared to the OVX group

Expression of estrogen receptors

The mRNA expression of *ERα* and *ERβ* was marginally increased, but non-significant differences ($p > 0.05$), in the OVX group (Figure 6.2A and B). The *P. mirifica* and E_2 treatments showed similar effects on mRNA expression of *ERα* and *ERβ*, viz., decrease in *ERα* mRNA levels ($p < 0.05$) and no changes in *ERβ* mRNA levels ($p > 0.05$), in comparison with the OVX group (Figure 6.2A, B). On contrarily to the *P. mirifica* and E_2 groups, treatment with puerarin significantly decreased *ERβ* mRNA level ($p < 0.05$; Figure 6.2B). Although it was not significant difference from the OVX group, the *ERβ* mRNA levels of the *P. mirifica* and E_2 groups and the *ERα* mRNA level of the puerarin group were apparently comparable to those of the sham control group.

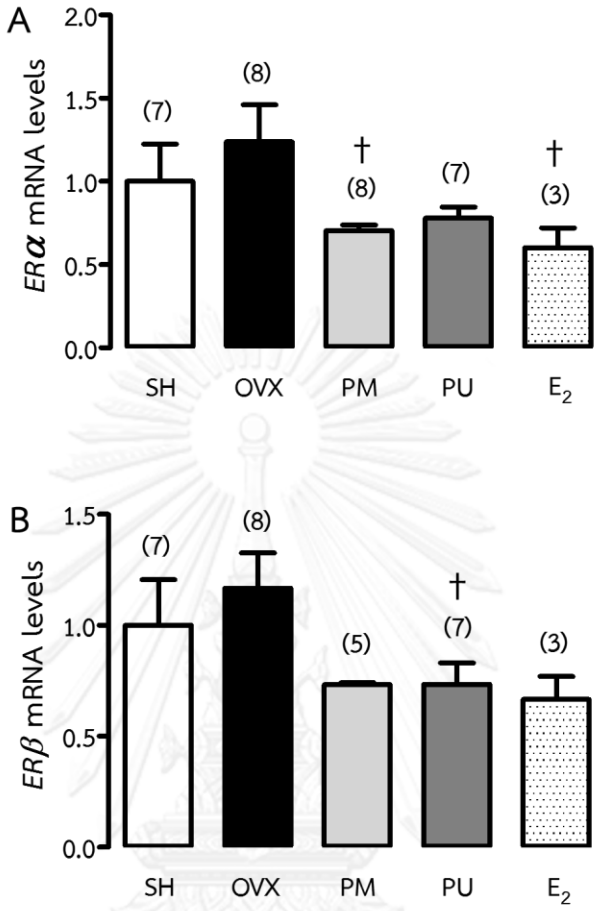

Figure 6.2 The relative mRNA levels of *ERα* (A) and *ERβ* (B) genes in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). † $p < 0.05$ compared to the OVX group. The number in parenthesis indicates the number of rats used in each group.

Expression of genes associated with NFTs and amyloid plaques

The expression of *Tau3* and *Tau4* genes remained unchanged in the OVX group (Figure 6.3A, B). Treatment with *P. mirifica* and E_2 also had no effects on *Tau3* and *Tau4* expression when compared to the OVX and sham control groups. Only the puerarin treatment could decrease the *Tau3* and *Tau4* mRNA levels in the OVX rats ($p < 0.05$) (Figure 6.3A, B).

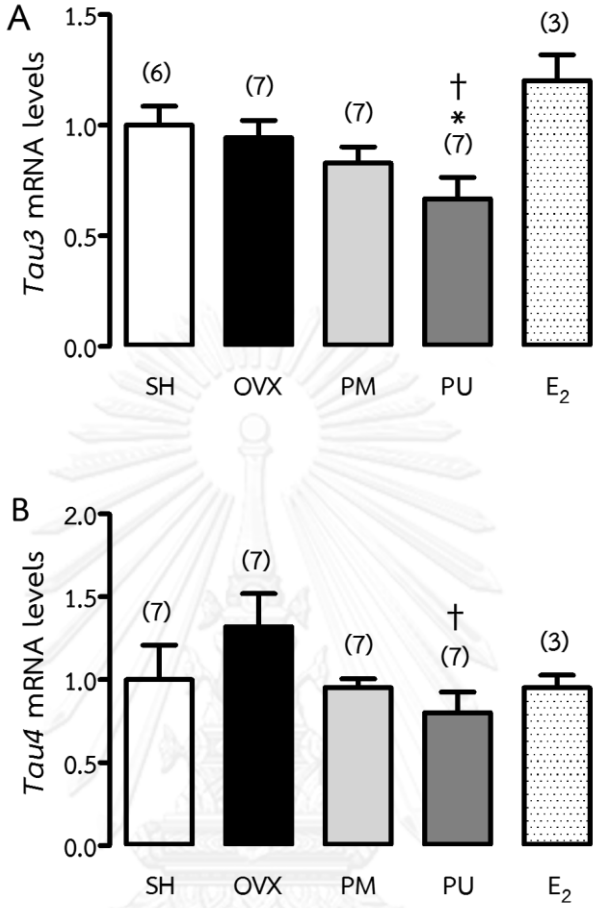

Figure 6.3 The relative mRNA levels of the genes associated with NFTs, *Tau3* (A) and *Tau4* (B), in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). * and † $p < 0.05$ compared to the sham control and OVX groups, respectively. The number in parenthesis indicates the number of rats used in each group.

After ovariectomy for 6 months, only the *Bace1* expression was upregulated ($p < 0.05$) (Figure 6.4B). Consequently, treatments of estrogen (E_2 group) as well as phytoestrogens (PM and PU groups) were significantly decreased the expression of *Bace1* ($p < 0.05$, compared to the OVX group) to the sham control level. In congruent with the decrease in *Tau3* and *Tau4* mRNA levels, only puerarin treatment significantly decreased the mRNA level of *App*. Ovariectomy, treatments with *P. mirifica*, puerarin and E_2 had no effects on expression of *Adam10* (Figure 6.4C).

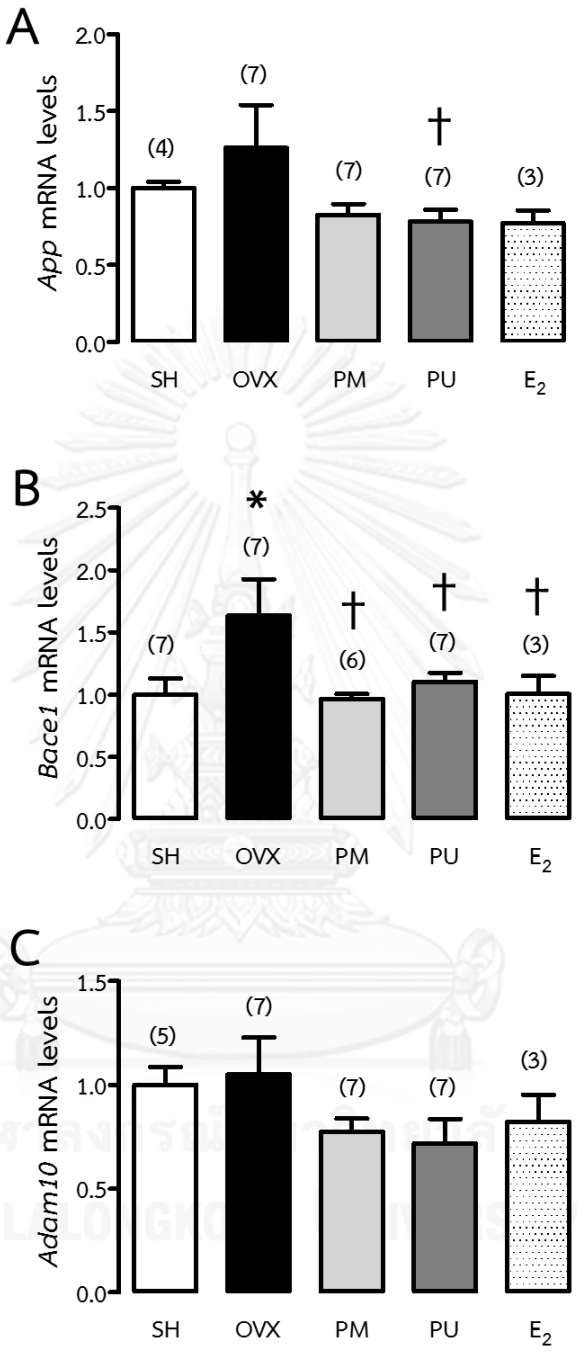

Figure 6.4 The relative mRNA levels of the genes associated with amyloid plaques, *App* (A), *Bace1* (B) and *Adam10* (C), in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). * and † $p < 0.05$ compared to the sham control and OVX groups, respectively. The number in parenthesis indicates the number of rats used in each group.

Cognitive performance

While ovariectomy led to the serum E_2 depletion and serum LH peak in rats, it also induced cognitive impairment indicating by the significant increase in the latency of searching for the hidden platform during five consecutive days of testing ($p < 0.05$, Figure 6.5A), and comparably increase in the latency and distance travelled in the 5th day of Morris water maze test (Figure 6.6A and B). Besides the circular and random strategies of movement were higher in the OVX group (39%) compared to the sham control group (12%) ($p < 0.05$) (Figure 6.6C).

P. mirifica and E_2 treatments were significantly decreased the latency and distance in searching for the hidden platform during five consecutive days of testing ($p < 0.05$ for *P. mirifica* and $p < 0.005$ for E_2, Figure 6.5A and B). In the 5th day of Morris water maze test, the *P. mirifica* and E_2 groups showed significantly lower latency and distance travelled compared to the OVX group ($p < 0.05$), while it was significantly decreased only in the latency in the puerarin group ($p < 0.05$) (Figure 6.6A and B). The percentages of circular and random strategies were also lowered in the OVX rats treated with *P. mirifica*, puerarin and E_2 (12%, 18% and 4%, respectively) ($p < 0.05$) (Figure 6.6C).

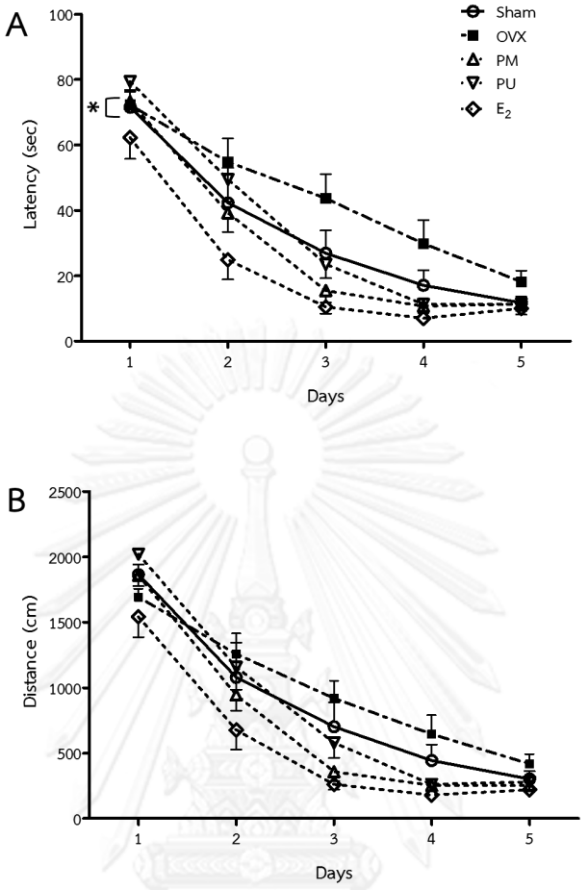

Figure 6.5 The latency period (A) and distance (B) of searching for the hidden platform during the 5-day Morris water maze test in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). * $p < 0.01$ compared to the sham control group as analyzed by two-way ANOVA with repeated measures.

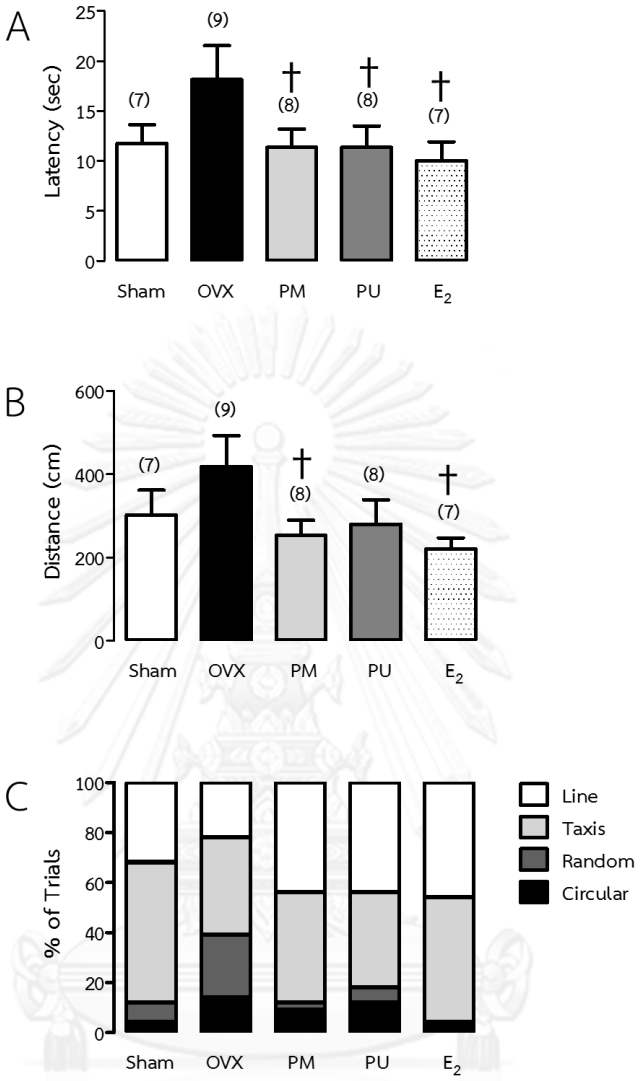

Figure 6.6 The latency period (A), distance (B) and strategy of movement patterns (C) of searching for the hidden platform at the 5th day of the Morris water maze test in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). † $p < 0.05$ compared to the OVX rats.

Discussion

After ovariectomy, the significantly lower relative weights of uterus and vagina and serum E_2 levels, and higher serum LH and FSH levels were found in the OVX group when compared to the sham control group. These results confirmed the estrogen deficient condition in all OVX rats. The *P. mirifica* treatment at a dose of 100 mg/kg BW/day could attenuate the increase in serum LH level but not for the serum FSH level. Additionally, the *P. mirifica* treatment was also significantly increased the relative weights of uterus and vagina. These results confirmed the estrogenic activity of phytoestrogens in *P. mirifica* extract as reported previously (Malaivijitnond et al., 2004). Puerarin is a major component and species-specific phytoestrogen of *Pueraria* genus which has been first recognized in a tuberous root of *P. lobata* (Xu et al., 2005). Puerarin elicited very weak estrogenic activity on reproductive organs (Malaivijitnond et al., 2010). As we reported previously, subcutaneous injection of 7.0 mg/kg BW/day of puerarin for 140 days in OVX rats stimulated vaginal proliferation; however, it had no effect on uterus (Malaivijitnond et al., 2010). In the present study, injection of 7.0 mg/kg BW/day of puerarin for 120 days (or 4 months) significantly increased only the relative weight of vagina, not the relative weight of uterus. However, if the dose is higher and the treatment is long enough, for example, feeding of 3,000 mg/kg BW/day of puerarin for 3 months, could also increase the uterus weight (Rachoń et al., 2007). Even that high dose, 3,000 mg/kg BW/day, weak estrogenicity of puerarin had no effect on pituitary LHβ gene expression and serum LH level (Rachoń et al., 2007). Thus, it is not surprising when puerarin had no effects on serum LH and FSH level in this study as well. On contrarily, subcutaneous injection with 80 μg/kg BW/day of E_2 decreased both serum LH and FSH levels in the OVX rats. The lesser extent of the FSH response to E_2, in comparison to the LH response, confirms the involvement of inhibin in the negative feedback on pituitary FSH secretion (Arai et al., 1996). Feeding of E_2 at doses of 4.3 and 17.3 mg/kg BW/day for 3 months to the OVX rats also suppressed the pituitary LHβ expression and lowered serum LH levels (Rachoń et al., 2007)..

Although it is well known that a lack of estrogen increases the incidence of neurodegeneration, the ERT showed the controversy results in clinical trial. These indicated that other hormonal changes, which are associated with the dysregulation of the hypothalamic-pituitary-ovary axis following menopause, have also been implicated in the pathogenesis of nuerodegeneration (Bowen et al., 2004). The “gonadotropin LH

peak" has attracted attention in the development of Alzheimer's disease (Gregory & Bowen, 2005). LH is known to cross the blood-brain barrier (Lukacs et al., 1995) and LH receptors are expressed in the brain, especially in hippocampus (Lei et al., 1993) . Thus, treatment of *P. mirifica* which can also suppressed serum LH levels (Malaivijitnond et al., 2004, Trisomboon et al., 2006) to the women who are in the early stage of climacteric should better alleviate the cascade of neurodegeneration.

Elevated level of Aβ in the brain is a hallmark of Alzheimer's disease and has been believed to play critical role in the cognitive dysfunction (Li et al., 2010). In APP processing pathway, ADAM10 enzyme is important for preventing the formation of Aβ peptide (non-amyloidogenic pathway) and BACE1 leads to Aβ production (amyloidogenic pathway). Thus, ADAM10 is a key enzyme in the balance of (patho) physiological processes and induction of an increased protease activity of ADAM10 might have beneficial effects of patients with Alzheimer's disease. BACE1 is also a promising therapeutic target but there is no direct physiological role of the processing by BACE1 is known (Prox et al., 2012). Estrogen deficiency was well known to induce Alzheimer's disease by increasing the Aβ deposition (Paganini-Hill & Henderson, 1994; Yue et al., 2005). E_2 diminishes Aβ generation by means of increase in the activity of ADAM10 via the protein kinase C (PKC) (Gandy & Duff, 2000). Estrogen treatment in mixed neuronal/glial cell cultures also downregulated BACE1 protein expression (Nord et al., 2010). Accordingly, ovariectomy for 6 months in 4 months old rats in the present study upregulated *Bace1* mRNA expression and it was decreased by E_2 treatment. In a comparable degree with that of E_2 effect, *P. mirifica* and puerarin treatment decreased the relative mRNA level of *Bace1*. However, changes in *Adam10* mRNA expression could not be observed in all four groups of rats (OVX, PM. PU and E_2 groups). Additionally, the puerarin treatment at a dose of 7 mg/kg BW/day decreased the relative mRNA expression of *App*. Previous study has shown that puerarin treatment ameliorated Aβ (1-42)-induced cognitive impairment and reversed the increase of apoptosis in the hippocampus (Li et al., 2010). Taken together, these results indicated that *P. mirifica* and puerarin are potential candidates to prevent the estrogen deficiency-induced neurodegeneration and memory loss in OVX rats by inhibiting the formation of Aβ in hippocampus brain area.

NFTs consist of intraneuronal bundles of abnormal filaments, composed of a high phosphorylated form of the microtubule-associated protein called TAU. TAU is a

protein which stabilizes neuronal microtubules (Felician & Sandson, 1999). Hyperphosphorylation of TAU leads to its aggregation into NFTs and related to neurodegeneration diseases (Shi et al., 2011). However, the mechanisms by which TAU protein is phosphorylated in the neurodegenerative brain are not fully understood. It is proposed that an imbalanced regulation in protein kinases and protein phosphatases in the affected neurons may be upstream of the abnormal TAU hyperphosphorylation (Pant et al., 1999). There are 2 groups of TAU protein, 3R-TAU (TAU3) and 4R-TAU (TAU4), according to its tandem repeated regions (Robert & Mathuranath 2007; Goedert & Spillantini, 2011; Shi et al., 2011). In this study, only puerarin treatment could decrease the mRNA levels of *Tau3* and *Tau4*, but not in the E_2 and *P. mirifica* group. Aside from the weak estrogenic activity, puerarin also elicited a strong anti-oxidant capacity on neuronal cells (Mahdy et al., 2014). This leads to the suggestion that puerarin prevents a hyperphosphorylation of TAU protein in hippocampus brain area by its anti-oxidative capacity. It was reported that puerarin could improve cognitive impairment in APP/presenilin-1 (APP/PS1) mice by a significant decrease in the levels of lipid peroxidation, but no changes in Aβ deposition (Zhou et al., 2014). Taken together, these results suggest it might be possible that puerarin ablates the Alzheimer's disease primarily by a reduction of NFTs production, but not through the amyloid pathway.

It is well known that there are two subtypes of ERs (α and β) in neural cells (Patrone et al., 2000). Previous study indicated that ERα may play an important role of neuroprotection during the process of Alzheimer's disease (Hu et al., 2003). However, the mRNA expression of *ER*α and *ER*β in the OVX rats were marginally upregulated in the present study, this might indicate that hippocampus cells adjusted for the estrogen deficient condition. Besides, treatment of estrogen (E_2) or phytoestrogens (*P. mirifica* and puerarin) apparently downregulated *ER*α and *ER*β expression compared with the sham control rats. However, it needs to investigate further if changes of mRNA and protein levels of *ER*α and *ER*β link to the Aβ and NTF production and cognitive impairment.

In this study, the 6-month estrogen deficient rats (OVX group) showed significant higher latency and distance to find the platform when compared to the sham control group. This result indicates that the cognitive impairment in the 6-month OVX rats was similar to the 4-month OVX rats as reported previously (Feng et al., 2004;

Anukulthanakorn et al., 2013). Treatment of E_2 (80 μg/kg), *P. mirifica* and puerarin improved the performance as reported previously (Feng et al., 2004). Puerarin also improved cognition in the OVX mice (Xu et al., 2004) and the Aβ-induced cognitive impairment rat (Li et al., 2010). The high percentage of circular and random strategies in the OVX group (39%) and the decline after *P. mirifica* (12%), puerarin (20%) and E_2 (4%) treatments confirmed the improvement of memory deficit

In conclusion, the *P. mirifica* and puerarin could elicit the neuroprotective effect in the OVX rats when the treatment is initiated at the time of estrogen deprivation and LH peak, but without cognitive impairment. The mechanism of action could be associated with the suppression of expression of genes associated with amyloid plaques and TAU hyperphosphorylation.

CHAPTER VII
NEUROTHERAPEUTIC ACTION OF *Pueraria mirifica* IN OVARICETOMIZED RATS

Introduction

Estrogen is an important hormonal signal that regulates multiple tissues and functions in the body. Their actions are not limited to hormonal responses and reproductive behavior. Additionally, the non-reproductive function of estrogen is widely studied especially its effect on the brain. Previous study suggests that estrogens have great impact on cognitive actions in certain brain regions such as the hippocampus, cortex and striatum. The profound reduction of estrogen associated with menopause have been linked to mnemonic decline in both normal aging and dementia (Frick et al., 2002). Dementia is a symptom with multiple causes and Alzheimer's disease is a common dementia which is found in postmenopausal women. Previous study indicated that ERT is associated with a decreased risk of dementia and a decrease in the production of Aβ in female patients with Alzheimer's disease (Schönknecht et al., 2001). However, ERT showed little effect on cognition during midlife but the initiation treatment after surgical menopause seems to improve memory (Henderson, 2008). In non-demented menopausal women, ERT can improve both verbal (Sherwin 2006) and spatial memory (Duff & Hampson, 2000). However, others have found little or no cognitive benefit of estrogen treatment in women (Yaffe et al., 1998; Hogervorst et al., 2000). These results indicate the controversial effects of ERT. One possible explanation for this discrepancy could be the varying time after menopause at which ERT is initiated; neuroprotective or neurotherapeutic action. It has been proposed that a critical period exists during which ERT must be administered to enhance cognitive function (Smith et al., 2010). However, ERT also increases the risk in the incidence of breast cancer and endometrial cancer in women (Mayeauz & Johnson, 1996). Therefore, many researchers are considering to find the alternative and naturally occurring substances that minimize the negative aspect of ERT (Minami et al., 2013).

P. mirifica is an endemic Thai medicinal plant which exhibits estrogenic activity. The estrogenic activity of phytoestrogens in *P. mirifica* has been tested widely both in

cell lines and in mice, rats, monkeys and humans (Cherdshewasart and Sriwatcharakul, 2008a; Cherdshewasart et al., 2007a; Malaivijitnonnd et al., 2006; Trisomboon et al., 2006b). Regarding its estrogenic activity, *P. mirifica* also exhibited neuroprotective actions both *in vitro* (in hippocampal primary cell culture (Chindewa et al., 2008) and glutamate-induced toxicity in HT22 cells (Sucontphunt et al., 2011), and *in vivo* (in estrogen deficient rats) (Anukulthanakorn et al., 2014). Therefore, this study aimed to investigate the neurotherapuetic action of *P. mirifica* treatment for cognitive impairment, in OVX rats. Changes in the expression of genes associated with amyloid plaques (*App*, *Adam10* and *Bace1*) and NFTs (*Tau3* and *Tau4*) together with the cognitive performance were determined in this study.

Materials and Methods

Animals

Adult female Sprague-Dawley rats, 2 months old, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were reared in stainless steel cages (five animals/cage) with sterile sawdust bedding in a room with controlled lighting (lights on 0600-1800 h) and temperature (25 ± 1°C) at the Laboratory Animal Unit, Faculty of Science, Chulalongkorn University, Thailand. The animals were fed with a standard rat chow diet (Perfect Companion Group Co., Ltd., Samutprakarn, Thailand) and water *ad libitum*. The rats were reared until they became 4 months old before used. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1123009.

P. mirifica extract

The extracts of the tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no. 0070317) were prepared as previously described (Tiyasatkulkovit et al., 2012).

Experimental design

Four months old rats were divided into five groups (4-10 rats in each group); sham control, OVX, *P. mirifica*, puerarin and E_2 treated. The sham rats were operated as the OVX rats, but their ovaries were kept intact. In the OVX, *P. mirifica*, puerarin and E_2 groups, rats were bilaterally ovariectomized on the first day of the experiment under

sodium pentobarbital anesthesia (40 mg/kg BW, i.p.). After ovariectomy for 4 months, when the cognitive impairment was appeared, the OVX rats were fed daily with 1 ml of distilled water, the *P. mirifica* group was fed with 100 mg/kg BW/day of *P. mirifica* extract (Malaivijitnond et al., 2006; Malaivijitnond et al., 2010), the puerarin group was subcutaneously injected with 7 mg/kg BW/day of puerarin (Malaivijitnond et al., 2010), and the E_2 group was subcutaneously injected with 80 μg/kg BW/day of synthetic E_2 (Feng et al., 2004), respectively, for 4 months.

Spatial memory test was performed using the Morris water maze test for each group of rats for 5 consecutive days before the end of the experiment as described previously (Anukulthanakorn et al., 2013). At the end of the experiment, the rats were euthanized, the blood sera were collected for E_2, LH and FSH assays, and the hippocampus was collected for mRNA quantification. The mRNA levels of the genes associated with NFTs (*Tau3* and *Tau4*) and amyloid plaques (*App*, *Adam10*, and *Bace1*) and two-subtypes of estrogen receptor genes (*ERα* and *ERβ*) were analyzed using real-time PCR as described previously (Anukulthanakorn et al., 2013).

Hormone assays

Serum E_2 levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands (Korenman et al., 1974). The antiserum against E_2 (GDN 244) was kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and inter-assay coefficients of variation were 2.4% and 7.5%, respectively.

Serum FSH and LH levels were determined using the National Institute of Diabetes and Digestive and Kidney disease (NIDDK) kits for rat FSH and LH (Baltimore, MD, USA) as described previously (Jaroenporn et al., 2011). The results obtained are expressed using the rat FSH-RP-2 and rat LH-RP-2 reference standards. The intra- and inter-assay coefficients of variations were 4.5% and 5.7% for FSH, and 3.0% and 11.6% for LH, respectively.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA), with LSD post-hoc test, was used to determine the differences of means of the hormone and mRNA levels. For the spatial memory test, the statistical

analysis for the latency and the distance travelled to search the hidden platform was performed using two-way ANOVA with repeated measures. The significant difference of the movement patterns was tested by Kruskal-Wallis and Mann-Whitney test. SPSS software program (version 17.0, SPSS Inc., Chicago, IL, USA) was used for the analysis. Significance levels were set at $p < 0.05$.

Results

Serum hormone levels

Both OVX and sham control rats showed low levels of serum E_2 (65.30 ± 10.15 pg/ml for OVX group and 67.32 ± 13.67 pg/ml for sham group, respectively) (Figure 7.1A), while only the OVX group had significantly increased serum LH and FSH levels (35.01 ± 5.55 ng/ml for LH and 29.58 ± 3.01 ng/ml for FSH) (Figure 7.1B and C). The low level of serum E_2 was also found in the puerarin group (48.21 ± 2.51 pg/ml). Although the serum E_2 level was high in the *P. mirifica* group (114.19 ± 19.81 pg/ml), it was not significant difference from that of the sham control and OVX rats. However, treatment with *P. mirifica* was significantly decreased serum LH level, but not the serum FSH level. Puerarin could not ablate the increased serum LH as well as FSH levels induced by ovariectomy. On contrarily, treatment with E_2 increased serum E_2 level significantly (935.10 ± 131.06 pg/ml; $p < 0.001$), and significantly decreased serum LH and FSH levels (0.61 ± 0.14 ng/ml, $p < 0.001$ and 18.67 ± 2.50 ng/ml, $p < 0.005$, respectively) in comparison with the OVX group. The increased serum E_2 level was also significantly higher than the sham control group ($p < 0.001$) (Figure 7.1A, B and C).

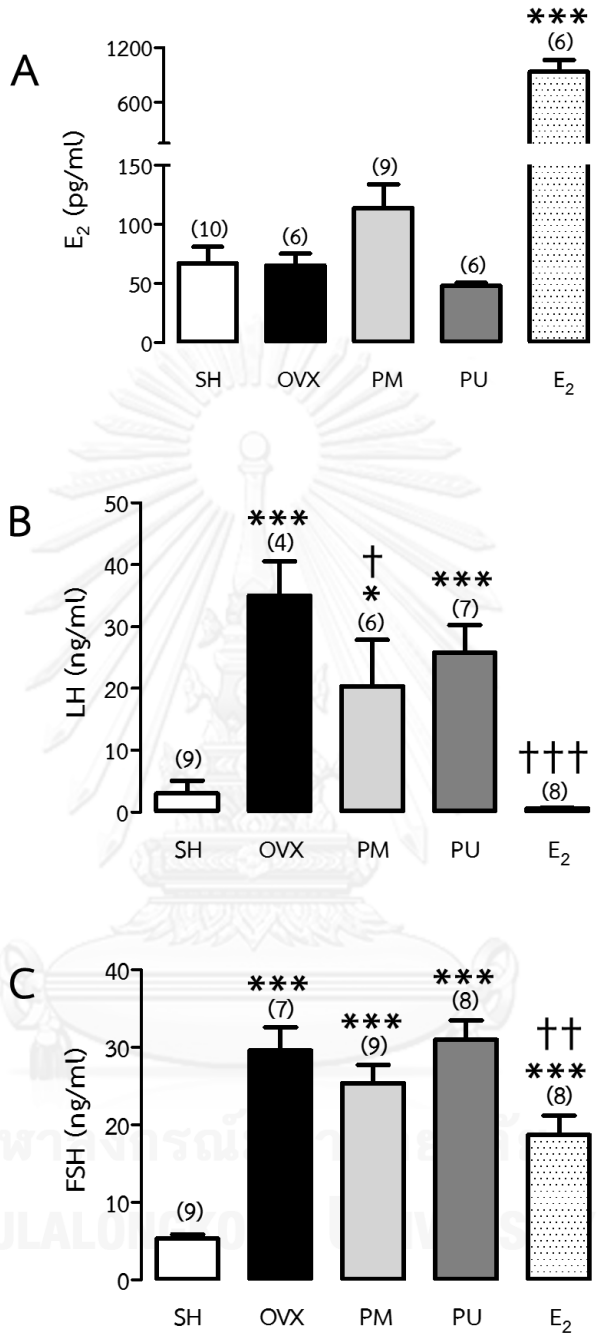

Figure 7.1 Serum levels of 17β-estradiol (E_2, A), luteinizing hormone (LH, B) and follicle stimulating hormone (FSH, C) in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). **, *** $p < 0.005$ and 0.001 compared to the sham control group (SH). †, ††, ††† $p < 0.05$, 0.005 and 0.001

compared to the OVX group. The number in parenthesis indicates the number of rats used in each group.

Relative weights of uterus and vagina

After 8 months of ovariectomy while the rats had very low serum E_2 level, the relative weights of uterus and vagina were significantly decreased in the OVX group ($p < 0.001$ and 0.05, respectively) (Table 7.1). Treatment with *P. mirifica* significantly increased the relative weight of uterus and vagina when compared to the OVX group ($p < 0.001$ and 0.05, respectively). Treatment with puerarin had no effect on the relative weights of uterus and vagina, and the levels were comparable with those of the OVX group. Treatment with E_2 significantly increased both relative weights of uterus and vagina compared to the OVX group ($p < 0.001$ and 0.05, respectively). In congruent with the higher serum E_2 level in the E_2 group, the relative uterus weight was significantly higher than the sham control group ($p < 0.001$).

Table 7.1 Relative weights of uterus and vagina of the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2) for 4 months.

Treatment group	Number	Uterus weight (10^3)	Vagina weight (10^3)
SH	10	1.605 ± 0.113	0.705 ± 0.033
OVX	7	0.570 ± 0.049**	0.362 ± 0.028*
PM	10	1.937 ± 0.228††	0.566 ± 0.070†
PU	8	0.665 ± 0.067**	0.355 ± 0.046*
E_2	8	2.473 ± 0.128**††	0.678 ± 0.109††

*,** $p < 0.005$ and 0.001 compared to the sham control group. †, †† $p < 0.05$ and 0.005 compared to the OVX group

Expression of estrogen receptors

The expression level of *ERα* in the hippocampus was significantly higher in the OVX group compared to the sham group ($p < 0.05$; Figure 7.2 A). Treatment with *P. mirifica* was significantly decreased the expression levels of *ERα* ($p < 0.05$), while

treatments with puerarin and E_2 had no significant effects when compared to the OVX group.

In comparison to the sham group, the expression of *ERβ* in the OVX group was marginally upregulate (p = 0.318). The *P. mirifica*, puerarin and E_2 treated groups showed no significant difference in the *ERβ* mRNA level when compared to the sham and OVX group ($p > 0.05$) (Figure 7.2 B). In congruent with the significant increase in *ERα* mRNA level and marginal increase in *ERβ* mRNA level, the ERα/ERβ ratio was also marginally increased in the OVX group (p = 0.140) (Figure 7.2 C). No changes of ERα/ERβ ratio were observed in the *P. mirifica*, puerarin and E_2 treated groups.

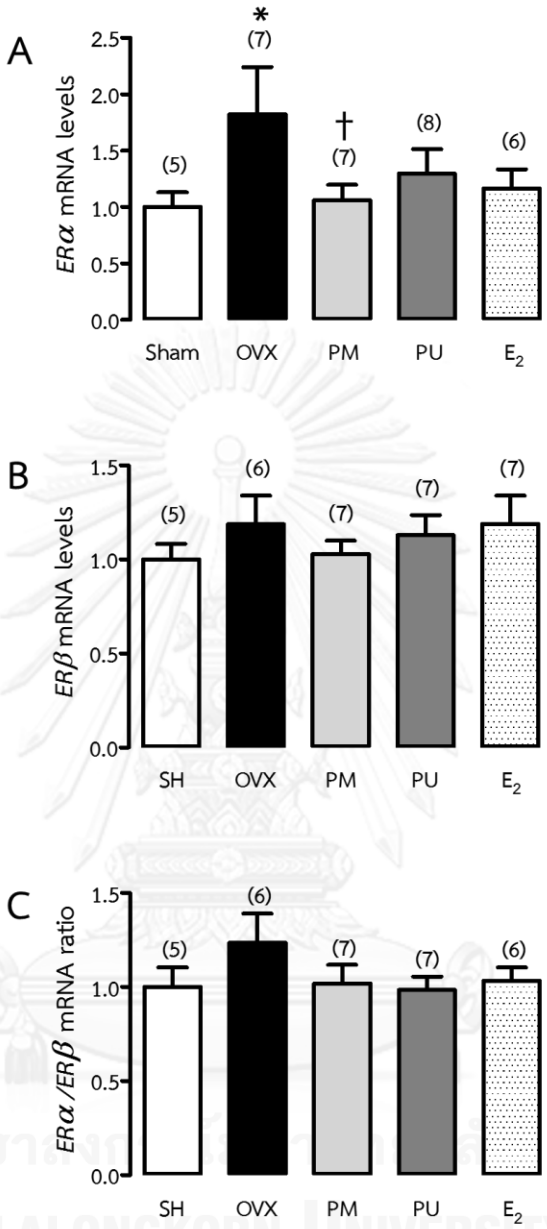

Figure 7.2 The relative mRNA levels of two estrogen receptor subtypes; *ERα* (A) and *ERβ* (B), and *ERα*/*ERβ* mRNA ratio (C) in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). * $p < 0.05$ compared to the sham control group and † $p < 0.05$ compared to the OVX group. The number in parenthesis indicates the number of rats used in each group.

Expression of genes associated with NFTs and amyloid plaques

Although the relative mRNA levels of *Tau3* and *Tau4* genes in the OVX group were increased, it was not significant difference compared to the sham control group (p = 0.411 and 0.269, respectively) (Figure 7.3). Similarly, the *P. mirifica*, puerarin and E_2 groups showed no significant differences in the mRNA levels of *Tau3* and *Tau4* when compared to the sham and OVX group (Figure 7.3)

In comparison to the sham control group, no significant changes in relative mRNA levels of *App*, *Bace1* and *Adam10* were observed in the OVX group, although it tended to increase (Figure 7.4). The *P. mirifica*, puerarin and E_2 treatments had no effects on mRNA levels of *App*, *Bace1* and *Adam10* when compared to the sham and OVX group, except that the *App* mRNA level in the *P. mirifica* group was significantly decreased ($p < 0.05$; Figure 7.3A).

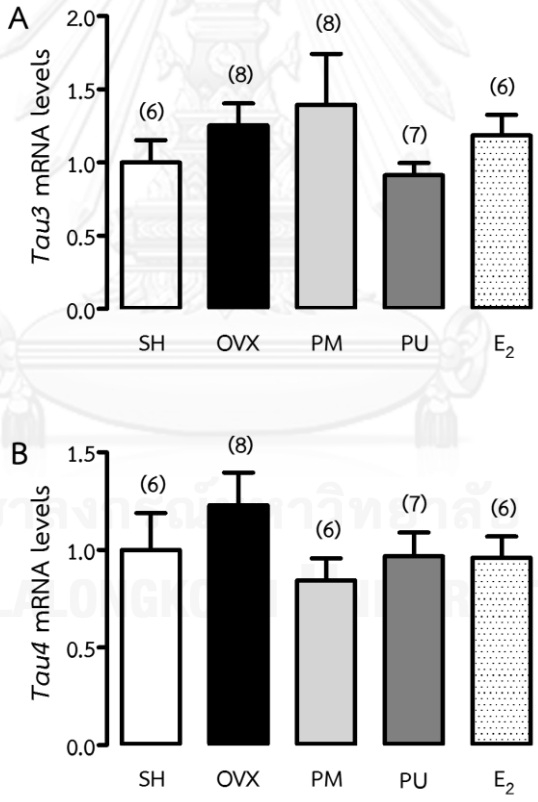

Figure 7.3 The relative mRNA levels of the genes associated to NFTs, *Tau3* (A) and *Tau4* (B), in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradio (E_2). The number in parenthesis indicates the number of rats used in each group.

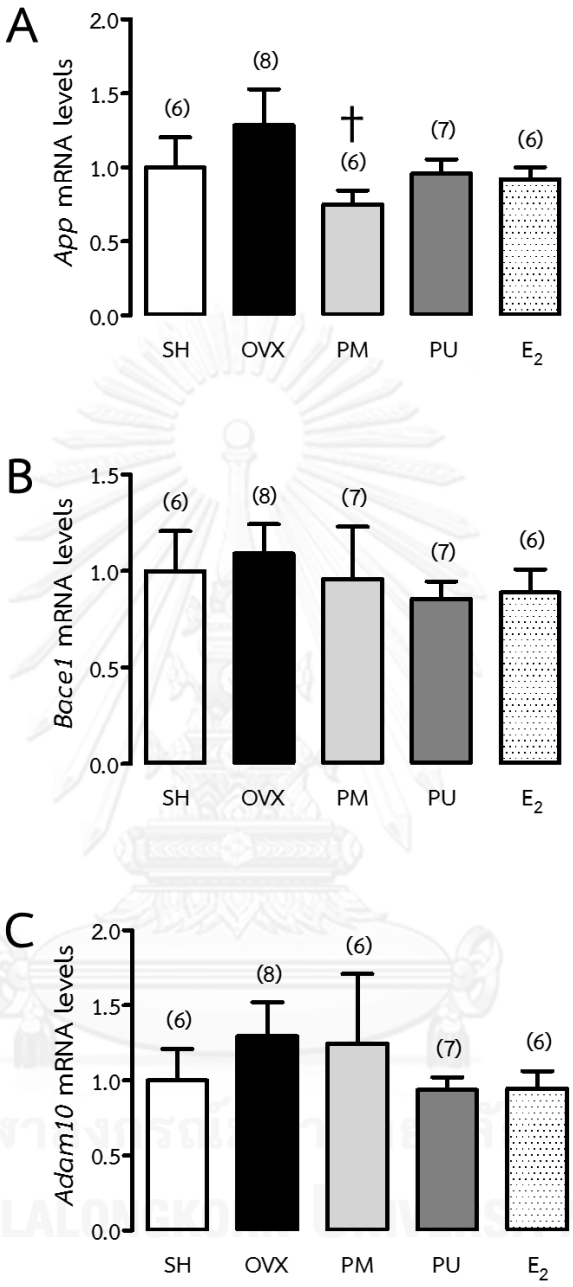

Figure 7.4 The relative mRNA levels of the genes associated to amyloid plaques, *App* (A), *Bace1* (B) and *Adam10* (C), in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). † $p < 0.05$ compared to the OVX group. The number in parenthesis indicates the number of rats used in each group.

Cognitive performance

The latency and distance of searching for the hidden platform were significantly increased during five consecutive days of testing in the OVX group compared to the sham control group ($p < 0.05$; Figure 7.5A, B). These results showed that 8 months of the estrogen deficiency induced cognitive impairment in the OVX rat. In the 5th day of Morris water maze test, the latency and distance travelled were also significantly higher in the OVX group compared to the sham group ($p < 0.05$; Figure 7.6A, B). The percentage of circular and random strategies was observed for 53% of the OVX group while only 7% was observed in sham group ($p < 0.05$) (Figure 7.6C).

P. mirifica, puerarin and E_2 treatments were significantly decreased the latency compared to the OVX group ($p < 0.05$ for *P. mirifica* and puerarin, and $p < 0.001$ for E_2, Figure 7.5A). Treatment with E_2 showed a significant decrease in distance of searching compared to the OVX group ($p < 0.005$; Figure 7.5B). In the 5th day of Morris water maze test, the *P. mirifica* and E_2 groups showed significantly lower latency and distance travelled compared to the OVX group ($p < 0.05$) (Figure 7.6A, B), while it was marginally decreased in the puerarin group ($p = 0.055$). Besides, the percentage of circular and random strategies was found only 25% for *P. mirifica* group, 28% for puerarin group, and 8% for E_2 group (Figure 7.6C).

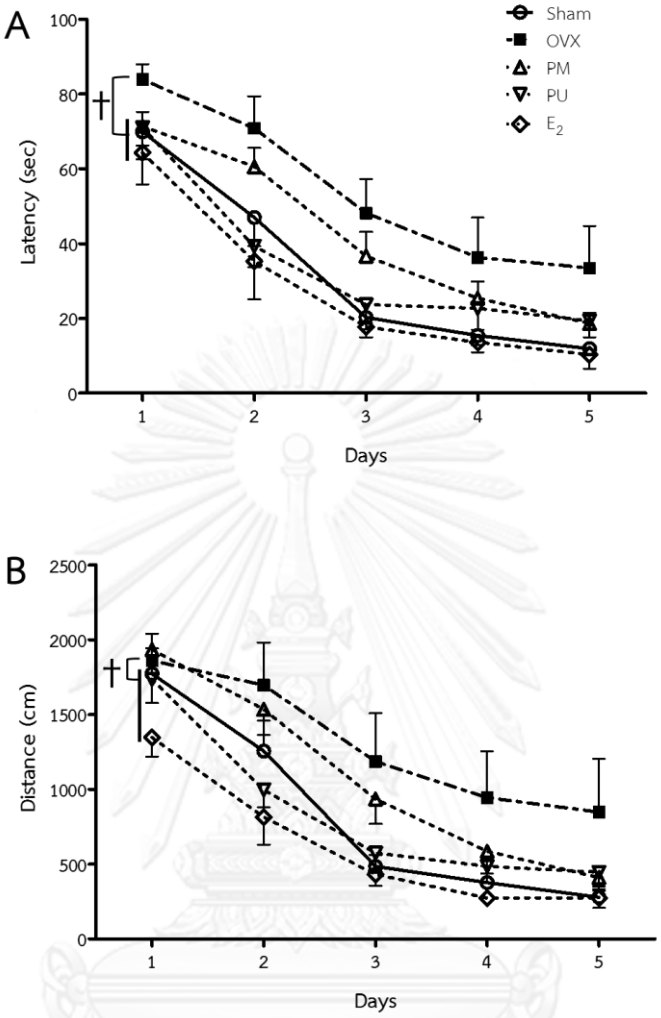

Figure 7.5 The latency period (A) and distance (B) of searching for the hidden platform during the 5-day Morris water maze test in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). † $p < 0.05$ compared to the OVX group.

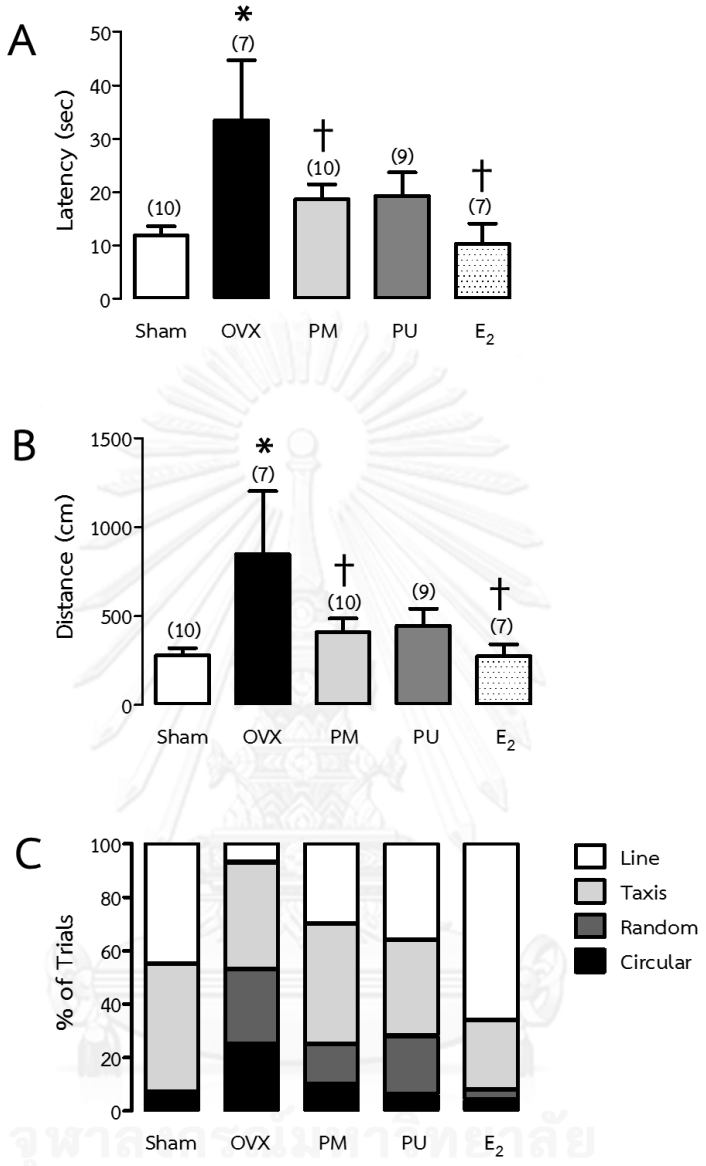

Figure 7.6 The latency period (A), distance (B) and strategy of movement patterns (C) of searching for the hidden platform at the 5th day of the Morris water maze test in the ovary-intact rats (SH) and ovariectomized rats treated with distilled water (OVX), 100 mg/kg BW/day of *P. mirifica* extract (PM), 7 mg/kg BW/day of puerarin (PU), or 80 μg/kg BW/day of 17β-estradiol (E_2). ** $p < 0.005$ compared to the sham control, and † $p < 0.05$ compared to the OVX rats, respectively. The number in parenthesis indicates the number of rats used in each group.

Discussion

The low relative weights of uterus, vagina and serum E_2 level and high serum LH and FSH levels confirmed the successful complete ovariectomy in all OVX rats. Unexpectedly, the serum E_2 level in the sham control rats was also low and comparable to that of the OVX rats. This can be explained by two possible reasons. Firstly, the sham control rats were euthanized during the time of diestrus when the serum E_2 level was low. Secondly, the sham control rats were in the transferring period from the middle age (12 months old for the time of blood collection) to the pre-acyclic age (13-15 months old) (Fernandez et al., 2013), which indicated a decline in the ovary function in the pre-acyclic period. However, because the low serum LH and FSH levels and high relative weights of uterus and vagina could be observed in the sham control rats, the first reason has higher possibility. The significantly increased relative weights of uterus and vagina in the *P. mirifica* treated rats confirmed the estrogenic activity of phytoestrogens in the plant as reported previously (Malaivijitnond et al., 2004).

Post-menopausal women are in an estrogen-deprived state and are at risk for Alzheimer's disease with a decline in cognitive function (Farrag et al., 2002; Sherwin, 2006). Unfortunately, several studies show that ERT after the disease process has started was generally unsuccessful in ameliorating the decline in cognitive function (Brenner et al., 1994; Henderson, et al. 2000; Mulnard et al., 2000). Besides, nobody verified why and how this phenomenon happens. The common pathology of neurodegenerative diseases is characterized by deposition of Aβ peptide and NFTs (Maccioni et al., 2001). Therefore, the scientific community is divided over the pathogenesis of the disease between "baptists" and "tauists". Baptists suggest that Aβ, the peptide deposited in neuritic plaques, is the cause of all damage, while tauists suggest that the hyperphosphorylated tau, the cytoskeleton protein that forms NFTs, is the culprit for the disease (Racchi & Govoni, 2003).

Aβ is generated from APP by β-secretase (Bace1) and γ-secretase activity (De Strooper et al., 2010). The Aβ plaque was increased during 3 months after ovariectomy in 18 months old triple-transgenic mice (Palm et al., 2014) and Bace1 activity was elevated together with brain estrogen depletion in transgenic mice model of Alzheimer's disease (Yue et al., 2005). In this study, 8 months after ovariectomy in 12 months old rats showed a tendency of high *App*, *Bace1* and *Adam10* mRNA expression,

in comparison with the sham control rats, which indicates the possibly increased Aβ production. Interestingly, no alterations of the expression of those three genes were observed after *P. mirifica*, puerarin and E_2 treatment, and only *P. mirifica* treatment decreased the expression of *APP* mRNA level. Non-response to E_2 treatment in these OVX rats might be explained by the shortage of brain estrogen which alters the sensitivity of response to E_2 treatment (Li et al., 2013a, 2013b). Additionally, this also indicates that *P. mirifica* plant should be a better neurotherapeutic agent on recovering the pathology of neurodegeneration via Aβ pathway compared with the synthetic estrogens.

Tau pathology is not only a prominent feature of Alzheimer's disease, but it is also seen in a variety of other related neurodegenerative diseases (Skovronsky et al., 2006). Tau is a microtubule-associated protein that binds to and stabilizes microtubules. There are now more than 30 distinct mutations or pathogenic nucleotide substitutions in the gene for *Tau* that have been shown to cause frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) in more than 50 different kindreds (Goedert & Jakes, 2005). In previous study, the *Tau3* and *Tau4* mRNA expression were significantly increased at 4 months after ovariectomy (Anukulthanakorn et al., 2013), however, only the tendency of increase, in comparison to the sham control rats, was observed in the OVX rats after 8 months of ovariectomy in this study. Besides, no alterations of the expression were detected in the *P. mirifica*, puerarin and E_2 treated rats. This is different from the results reported previously in Chapter V and VI for the neuroprotective actions of those chemicals in the OVX rats. Taken together, it can be concluded that estrogens and phytoestrogens could not cure the occurred neurodegeneration via Tau pathway. However, other than the condition of estrogen deficiency in rats, the age of the animals (12 months old for the time of hippocampus collection) used in this study needs to be considered.

ERα and ERβ share similar domain organization and show substantial sequence homology but have differences in their binding affinities for ligands. Both ERs have been shown to be differentially expressed throughout the rodent brain (Shughrue et al., 1997a). *ERα* and *ERβ* are both expressed in the hippocampus, a region of the brain involved in cognitive function (Bliss & Collingridge, 1993). *ERβ* is expressed in the CA3 stratum lucidum, CA1 stratum radiatum (Mitra et al., 2003) and dentate gyrus regions (Herrick et al., 2006) and at the subcellular level is found in axons, dendrites and

dendritic spines (Milner et al., 2005). In the hippocampus of rats, the expression of *ERs* decreases with age (Mehra et al., 2005; Yamaguchi-Shima & Yuri, 2007) and similar decrease occurs in human brain (Zandi et al., 2002). Interestingly, it was reported that expression of *ER*α and *ER*α/*ER*β ratio was increased during the time of low estrogen level together with the low memory performance (Foster, 2012) which is in the same line with the results of OVX rats in this study. Treatment with puerarin and E_2 had no effect on the mRNA levels of either *ER*α or *ER*β compared to the OVX rats and the 12 months old sham rats. Contrarily, *P. mirifica* treatment decreased the *ER*α mRNA level, but not the *ER*β. Previous studies indicated that the ERα mediates many of the effects of estrogenic chemicals on sexual behavior, learning, and memory (Rissman et al., 1999) and the ERβ enhances cognitive performance of female mice in the object recognition and placement tasks (Walf et al., 2008b). This might help to explain why only the *P. mirifica* treated rats showed the reduction of *App* expression together with the recovery of cognitive performance tested by Morris water maze.

The Morris water maze is a device to investigate spatial learning and memory in laboratory rats and it has become one of the most frequently used laboratory tools in behavioral neuroscience. The lesions in distinct brain regions like hippocampus, striatum, basal forebrain, cerebellum and cerebral cortex induced the cognitive impairment in rodents tested by Morris water maze (D'Hooge & De Deyn, 2001). In the previous studies, the cognitive impairment in rats was found 4 months after ovariectomy (Feng et al., 2004; Anukulthanakorn et al., 2013), and 4 months daily injection of E_2 (80 μg/kg) could alleviate the cognitive impairment by decrease in Aβ and NFT production (Feng et al., 2004; Anukulthanakorn et al., 2013) and increase in choline acetyltransferase activity (Feng et al., 2004). Interesting, 80 μg/kg E_2 injection for 4 months to rats, after the cognitive impairment appeared, did not change expression of any genes associated with Aβ and NFT production, although the cognitive performance was improved. Other pathway for the mechanism of neurotherapeutic action of E_2 such as via the cholinergic pathway should be tested in the future.

In conclusion, *P. mirifica* could improve the memory deficit in the OVX rat (or neurotherapeutic action) when the treatment is initiated at the time of cognitive impairment has occurred. The mechanism of action partially passes through the suppression of amyloid plaque production. This result corroborates the high potential of development of *P. mirifica* plant as therapeutic agent for Alzheimer's disease.

CHAPTER VIII
GENERAL DISCUCSSION AND CONCLUSIONS

In the present study, the neuroprotective and neurotherapeutic effects of *P. mirifica*, phytoestrogens containing plant, were determined and compared with synthetic estrogen (E_2) and synthetic phytoestrogen (puerarin) in OVX rat model. Firstly, the sequence of changes in expression of genes associated with NFTs (*Tau4* and *Tau3*) and amyloid plaques (*App*, *Adam10*, and *Bace1*) during the different time course (0-4 months) after ovariectomy was determined together with the serum levels of reproductive hormones, and cognitive ability. Then, the neuroprotective effects of *P. mirifica* extract in OVX rats during the time of estrogen depletion or estrogen depletion with LH peak, but without cognitive impairment, were determined. Lastly, the neurotherapeutic effect of *P. mirifica* extract on cognitive impairment, in OVX rats was determined. The estrogenic activity of phytoestrogens in *P. mirifica* extract at the concentration of 100 mg/kg BW/day which was used in the above mentioned experiments was determined and equated with synthetic E_2. The estrogenic activity of 100 mg/kg BW of *P. mirifica*, indicated by a decrease in body weight and increases in uterus weight and %Co was approximately equivalent to 14.32 mg/kg BW of E_2. The neuroprotective and neurotherapeutic actions of *P. mirifica* examined in the OVX rats are summarized as fellows;

1. Estrogen deficiency in rats induced consequent events; increases in serum LH and FSH levels after 1 and 2 months of ovariectomy, upregulation of *Tau4* and *Tau3* expression within 1 month after ovariectomy, and an increase in mRNA levels of *App*, *Adam10*, and *Bace1* at 4 months after ovariectomy, while the cognitive impairment was found at 3 and 4 months after ovariectomy (Figure 8.1).

2. If 100 mg/kg BW/day of *P. mirifica* extract was fed to rats for 4 months after two days of ovariectomy, it could clearly elicit the neuroprotective effect by marginally decreasing serum LH level, significantly decreasing *Tau4*, *App* and *Bace1* mRNA levels, and significantly lower latency and distance travelled in search of the hidden platform during the 5-day Morris water maze test compared to the OVX rats (Figure 8.2).

3. If 100 mg/kg BW/day of *P. mirifica* extract was fed to rats at 2 months after ovariectomy for 4 months during the time of estrogen deprivation and LH peak, but without cognitive impairment, it also shows the neuroprotective effect by significantly

decreasing serum LH level and *Bace1* mRNA level, while it has no effect on Tau pathway, however, the latency and distance travelled in search of the hidden platform during the 5-day Morris water maze test are lowered compared to the OVX rats (Figure 8.2).

4. If 100 mg/kg BW/day of *P. mirifica* extract was fed to rats for 4 months at the time of cognitive impairment (or 4 months after ovariectomy), it partially shows neurotherapeutic effect by significantly decreasing serum LH level and *App* mRNA level, while it has no effect on Tau pathway, however, the cognitive impairment was improved (Figure 8.4).

5. Regarding the expression of *ERs* measured, underlying mechanism of *P. mirifica* on neuroprotective and neurotherapeutic effects on hippocampus of rats seems to pass through ERα by ablating the increased *ERα* mRNA levels induced by ovariectomy.

6. Judging the estrogenic activity of chemicals based on the reduction of serum LH and FSH levels, E_2 at concentration of 80 μg/kg BW/day exhibits the estrogenic activity greater than 100 mg/kg BW/day of *P. mirifica* extract, and 7 mg/kg BW/day of puerarin exhibits no estrogenic activity.

7. The 80 μg/kg BW/day of E_2 and 7 mg/kg BW/day of puerarin exhibit the neuroprotective and neurotherapeutic effects comparable to those of 100 mg/kg BW/day of *P. mirifica* extract. However, the mechanism of action of puerarin was mainly via the reduction of intracellular NFTs (reduction of *Tau4* and *Tau3* expression) (Figure 8.3), while it was via the reduction of extracellular senile plaques of Aβ (reduction of *App*, *Adam10*, and *Bace1* expression) for E_2.

8. This corroborates the high potential of *P. mirifica* as an alternative drug to prevent and cure the Alzheimer's disease. Enhancing cognition with phytotherapy could reduce the cost and is safer, cheaper and easily to access by locals in the developing countries.

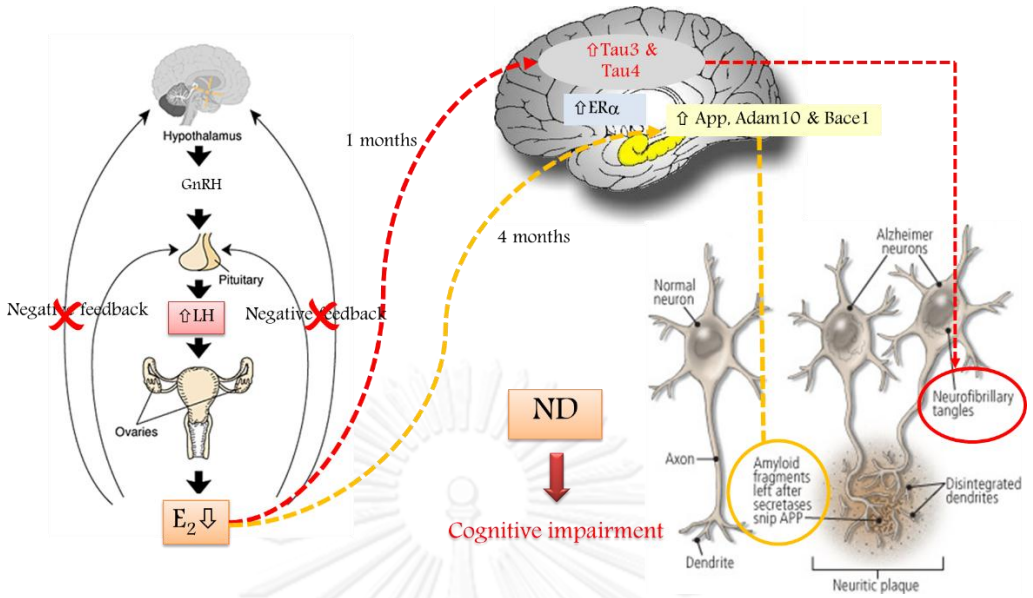

Figure 8.1 The relationship between estrogen deficiency, increased serum LH level and neuronal dysfunction in the hippocampus of rats.

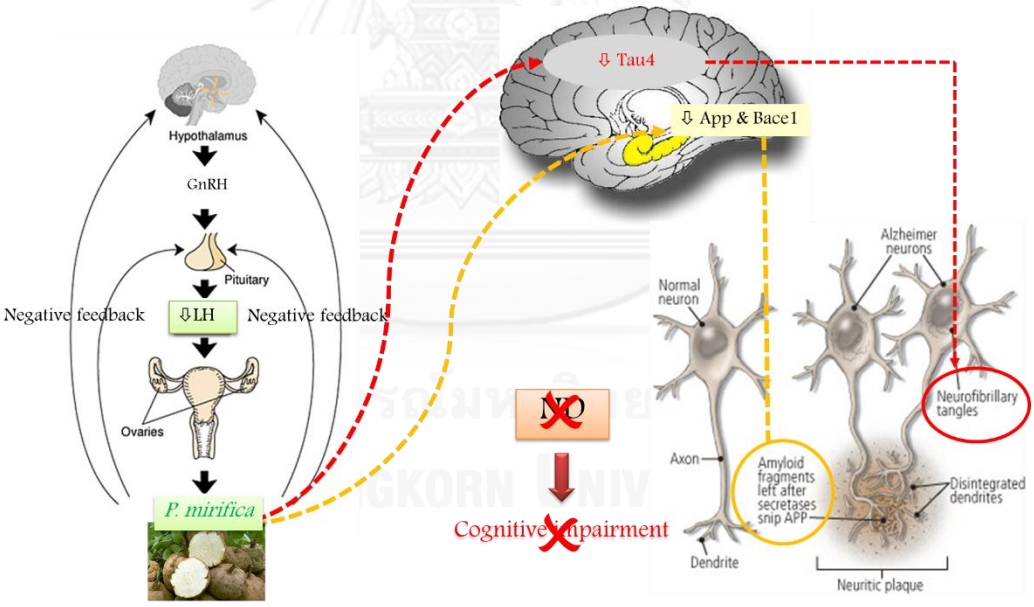

Figure 8.2 The neuroprotective effects of *P. mirifica* on neuronal dysfunction in the hippocampus of estrogen deficient rats. The treatment was initiated at 2 days after ovariectomy.

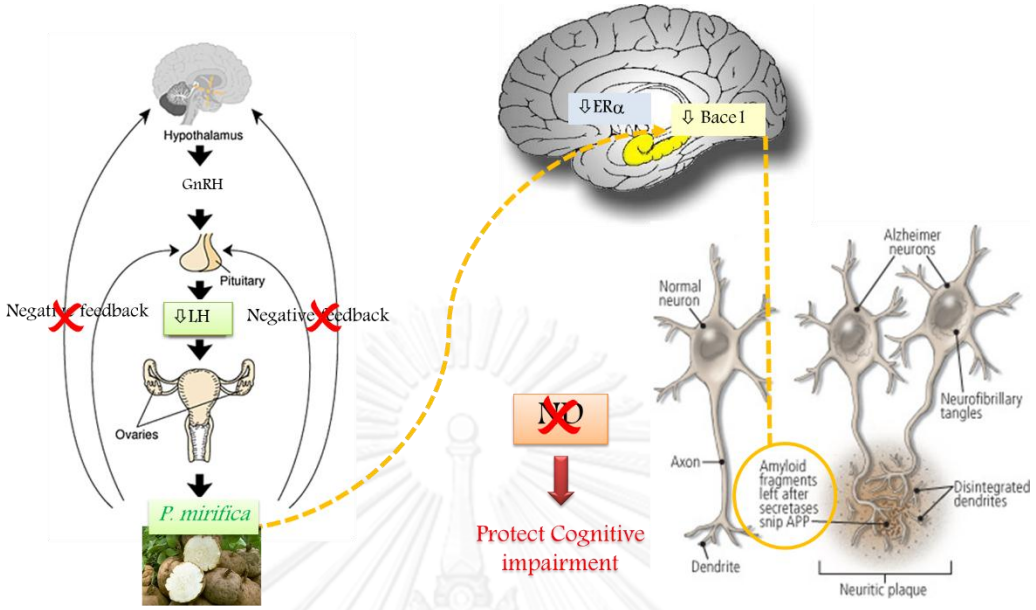

Figure 8.3 The neuroprotective effects of *P. mirifica* on neuronal dysnfunction in the hippocampus of estrogen deficient and LH-surge rats. The treatment was initiated at 2 months after ovariectomy.

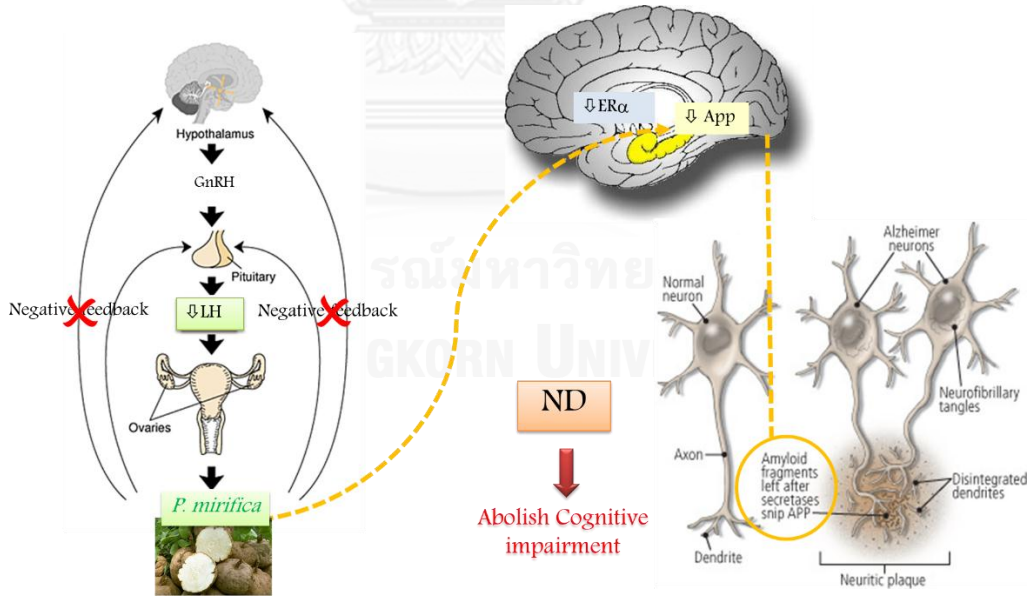

Figure 8.4 The neurotherapeutic effects of *P. mirifica* on neuronal dysfunction of estrogen deficient, LH-surge and cognitive impairment rats. The treatment was initiated at 4 months after ovariectomy.

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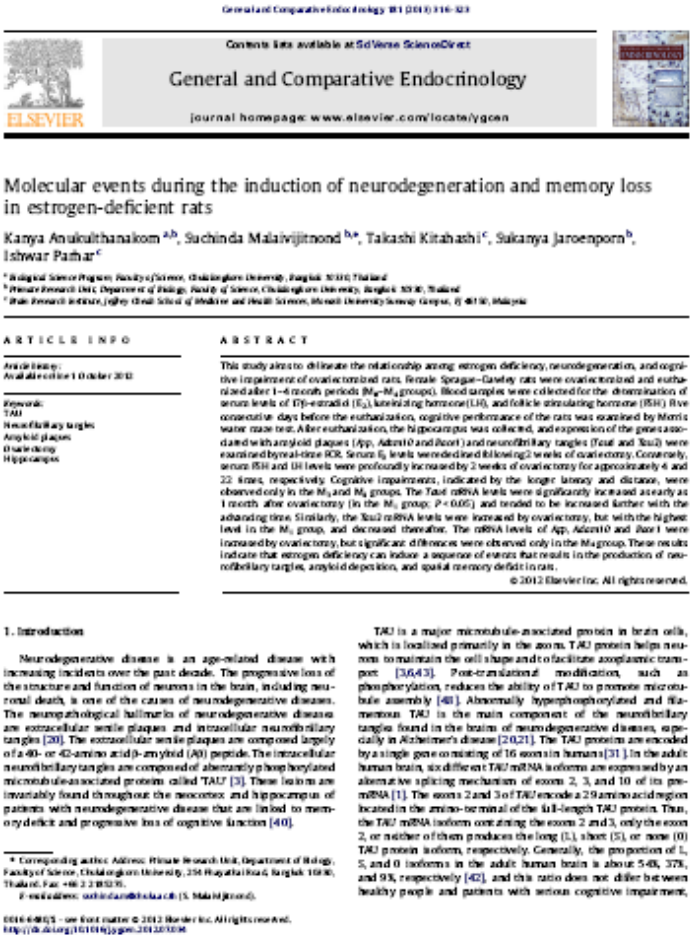
APPENDIX

APPENDIX A
LIST OF PUBLICATIONS

1. Anukulthanakorn, K., Malaivijitnond, S., Kitahashi, T., Jaroenporn, S. & Parhar, I. (2013). Molecular events during the induction of neurodegeneration and memory loss in estrogen-deficient rats. *General and Comparative Endocrinology*, 181, 316-323.



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Molecular events during the induction of neurodegeneration and memory loss in estrogen-deficient rats

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ABSTRACT

This study aims to delineate the relationship among estrogen deficiency, neurodegeneration, and cognitive impairment of ovariectomized rats. Female Sprague–Dawley rats were ovariectomized and euthanized after 1–6 month periods (M_1–M_6 groups). Blood samples were collected for the determination of serum levels of 17β-estradiol (E_2), luteinizing hormone (LH), and follicle stimulating hormone (FSH). Five consecutive days before the euthanization, cognitive performance of the rats was examined by Morris water maze test. After euthanization, the hippocampus was collected, and expression of the genes associated with amyloid plaques (*App*, *Adam10* and *Bace1*) and neurofibrillary tangles (*Tau4* and *Tau3*) were examined by real-time PCR. Serum E_2 levels were declined following 2 weeks of ovariectomy. Conversely, serum FSH and LH levels were profoundly increased by 2 weeks of ovariectomy for approximately 4 and 22 times, respectively. Cognitive impairments, indicated by the longer latency and distance, were observed only in the M_3 and M_6 groups. The *Tau4* mRNA levels were significantly increased as early as 1 month after ovariectomy (in the M_1 group; $P < 0.05$) and tended to be increased further with the advancing time. Similarly, the *Tau3* mRNA levels were increased by ovariectomy, but with the highest level in the M_3 group, and decreased thereafter. The mRNA levels of *App*, *Adam10* and *Bace1* were increased by ovariectomy, but significant differences were observed only in the M_6 group. These results indicate that estrogen deficiency can induce a sequence of events that results in the production of neurofibrillary tangles, amyloid deposition, and spatial memory deficit in rats.

1. Introduction

Neurodegenerative disease is an age-related disease with increasing incidents over the past decade. The progressive loss of the structure and function of neurons in the brain, including neuronal death, is one of the causes of neurodegenerative diseases. The neuropathological hallmarks of neurodegenerative diseases are extracellular senile plaques and intracellular neurofibrillary tangles [20]. The extracellular senile plaques are composed largely of a 40- or 42-amino acid β-amyloid (Aβ) peptide. The intracellular neurofibrillary tangles are composed of abnormally phosphorylated microtubule-associated protein called TAU [3]. These lesions are invariably found throughout the neocortex and hippocampus of patients with neurodegenerative disease that are linked to memory deficit and progressive loss of cognitive function [40].

TAU is a major microtubule-associated protein in brain cells, which is localized primarily in the axons. TAU protein helps neurons to maintain the cell shape and to facilitate axoplasmic transport [3,6,43]. Post-translational modification, such as phosphorylation, reduces the ability of TAU to promote microtubule assembly [48]. Abnormally hyperphosphorylated and filamentous TAU is the main component of the neurofibrillary tangles found in the brains of neurodegenerative diseases, especially in Alzheimer's disease [20,21]. The TAU proteins are encoded by a single gene consisting of 16 exons in humans [31]. In the adult human brain, six different *TAU* mRNA isoforms are expressed by an alternative splicing mechanism of exons 2, 3, and 10 of its pre-mRNA [1]. The exons 2 and 3 of *TAU* encode a 29 amino acid region located in the amino-terminal of the full-length TAU protein. Thus, the *TAU* mRNA isoform containing the exon 2 and 3, only the exon 2, or neither of them produces the long (L), short (S), or none (0) TAU protein isoform, respectively. Generally, the proportion of L, S, and 0 isoforms in the adult human brain is about 54%, 37%, and 9%, respectively [42], and this ratio does not differ between healthy people and patients with serious cognitive impairment,

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2. Anukulthanakorn, K., Jaroenporn, S. & Malaivijitnond, S. (2014). Simple, sensitive and reliable in vivo assays to evaluate the estrogenic activity of endocrine disruptors. *Reproductive Medicine and Biology*, 13, 37-45.

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ORIGINAL ARTICLE

Simple, sensitive and reliable in vivo assays to evaluate the estrogenic activity of endocrine disruptors

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Abstract
Purpose We compared three in vivo assays, determining changes of body weight, and uterotropic and vaginal cytology assays, for the evaluation of estrogenic activity of an estrogen disrupting compound, *Pueraria mirifica* (PM), in comparison with 17β-estradiol (E).
Methods Female rats were ovariectomized and gavaged with distilled water, 0.01, 0.1, 1, 10 and 20 mg/kg BW/day of E and 100 and 1,000 mg/kg BW/day of PM for 14 days. Body weights were measured weekly, and vaginal epithelium cells were monitored daily. The uterus was dissected at the end of the treatment period, weighed and examined for histomorphometry.
Results There were a decrease in body weight and an increase in uterine weight, uterine, endometrium and myometrium areas, uterine gland numbers, and percent of cornified cell which were dependent on doses of E and PM treatments.
Conclusion Of the three assays proposed, although all are reliable and had critical read-out, measurements of body and uterine weights is likely convenient and simple, but the uterotropic assay needs to kill the animals. Vaginal cytology assay appears most promising for sensitivity and shortening the duration of the assay. Compared to those of E, the estrogenic activity of PM at concentrations of 1.00 and 1,000 mg/kg BW was in the range of 1/4 to >1/20 mg/kg BW.

Keyword Body weight · Phytoestrogen · *Pueraria mirifica* · Uterotropic assay · Vaginal cytology assay

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Introduction

Estrogen disrupting compounds (EDCs) encompass a variety of chemical classes, including drugs, pesticides, industrial by-products, pollutants, and naturally produced botanical chemicals. Since these compounds are fat soluble, it is likely they accumulate from the environment in the fatty tissue of animals or humans exposed to them, and consequently generate outbursts on health effects. Generally, EDCs occur in a very low dose in the environment and are difficult to detect [1, 2].

There are two models in determining the estrogenic activity, in vitro and in vivo assays. In in vitro assays, various kinds of estrogen responsive cancer derived cell lines, e.g., MCF-7 human breast cancer cells [3, 4], HeLa cervical cancer cells [5], HepG2 hepatocarcinoma cells [6], CV-1 monkey kidney cells [7] or yeast cells [8] have widely been used. This assay is based on the estrogen receptor (ER) binding assay, such as the relative binding affinity assays, and an ER transcriptional activation assay, including cell proliferation assays. Although the in vitro assay for estrogenic activity in EDCs is rapid and convenient, and quite applicable for commercial scale assays, it has some disadvantages. For example, using the in vitro MCF-7 proliferation assay to determine estrogenic activity levels may not represent the estrogenic response in animals including humans due to the lack of bioavailability and biotransformation (absorption, distribution, metabolism,

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PUBLICATIONS

Anukulthanakorn, K., Malaivijitnond, S., Kitahashi, T., Jaroenporn, S. & Parhar, I. (2013). Molecular events during the induction of neurodegeneration and memory loss in estrogen-deficient rats. General and Comparative Endocrinology, 181, 316-323.

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