EFFECTS OF THE ASPARAGUS RACEMOSUS ROOT EXTRACT ON LEARNING AND MEMORY IN OVARIECTOMIZED RATS

LADDAWAN LALERT

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Oral Defense Committee

.....Chair

(Assistant Professor Supang Maneesri le Grand, Ph.D.)

.....Advisor

(Assistant Professor Onrawee Khongsombat, Ph.D.)

.....Co-Advisor

(Hathairat Kruevaisayawan, Ph.D.)

.....Co-Advisor

(Patcharada Amatyakul, M.D.)

.....Internal Examiner

(Associate Professor Sutisa Thanoi, Ph.D.)

.....Internal Examiner

(Taksanee Mahasiripanth, Ph.D.)

Approved

.....

(Professor Rattana Buosonte, Ed.D.)

Dean of the Graduate School

15 July 2014

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Author	Laddawan Lalert
Advisor	Assistant Professor Onrawee Khongsombat, Ph.D.
Co-Advisor	Hathairat Kruevaisayawan, Ph.D.
	Patcharada Amatyakul, M.D.
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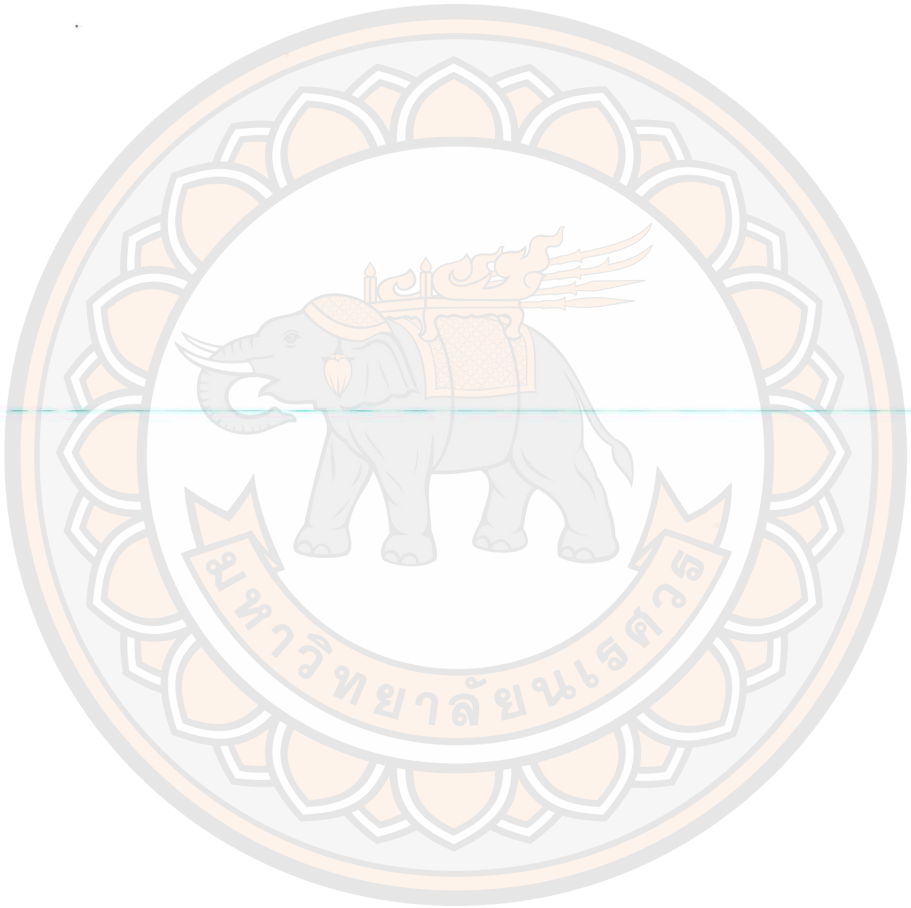
ABSTRACT

Estrogen decline in menopausal women impairs the cognitive function. This change can be ameliorated by estrogen replacement therapy but it causes serious side-effects. *Asparagus racemosus* (AR) is well known for its phytoestrogenic properties while neuroprotective effects of AR in ovariectomized (OVX) model are unknown. This study aimed to investigate effects and mechanisms of the AR root extract on learning and memory in OVX rats. Adult female Wistar rats were divided into five groups and gavaged for 90 days with vehicle (propylene glycol) for sham and OVX groups and another 3 groups of OVX rat were gavaged with 100 and 1000 mg/kg B.W./day of AR root extract or 0.1 mg/kg B.W./day of 17α-ethynylestradiol, respectively. Novel object recognition test was used to assess learning and memory. Serum estradiol concentration and morphological changes were determined using electrochemiluminescence immunoassay and hematoxylin and eosin staining, respectively. In addition, the expression of brain-derived neurotrophic factor (BDNF), estrogen receptor α and β (ERα and ERβ) subtypes were detected by using western blot analysis. In comparison to sham rats, the recognition index, serum estradiol level and intact neuronal density in hippocampus and medial prefrontal cortex significantly decreased in OVX rats. These alternations associated with the decreasing of protein expression of BDNF, ERα and ERβ in hippocampus and frontal cortex. AR could reverse the recognition memory impairment, morphological lesion as well as

expression of BDNF, ERα and ERβ proteins without change the serum estradiol concentration. This present study suggests that neuroprotective effects of AR root extract in ovariectomy may involve BDNF and ER subtypes up-regulation.



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ABBREVIATIONS

°C	=	Degree Celsius
ACh	=	Acetylcholine
AD	=	Alzheimer's disease
ANOVA	=	Analysis of variance
AR	=	*Aspapragus racemosus*
A.M.	=	Ante meridiem
BCA	=	Bicinchoninic acid
BDNF	=	Brain-derived neurotrophic factor
CA	=	Cornu Ammonis
CRE	=	cAMP response element
CREB	=	cAMP response element-binding protein
DAB	=	Diaminobenzidine
DMBA	=	7, 12 dimethylbenzαanthracene
DNA	=	Deoxyribonucleic acid
KA	=	Kainic acid
kg	=	Kilogram
ECLIA	=	Eletrochemiluminescence immunoassay
EE	=	Ethynylestradiol
ER	=	Estrogen receptor
EREs	=	Estrogen response element
ERT	=	Estrogen replacement therapy
ERs	=	Estrogen receptor subtypes
E2	=	Estradiol
GPx	=	Glutathione peroxidase
GSH	=	Glutathione
HRP	=	Horseradish peroxidase
HRT	=	Hormone replacement therapy
h	=	Hour
IgG	=	Immunoglobulin

ABBREVIATIONS (CONT.)

LTP	=	Long-term potentiation
MAR	=	Methanolic extract of AR roots
mg	=	Milligram
min	=	Minute
mm	=	Millimeter
mPFC	=	Medial prefrontal cortex
mRNAs	=	Messenger ribonucleic acid
MWM	=	Morris water maze
NaCl	=	Sodium chloride
NGF	=	Nerve growth factor
NOR	=	Novel object recognition
NT-3	=	Neurotrophin-3
NT-4/5	=	Neurotropin-4/5
OVX	=	Ovariectomized
PG	=	Propylene glycol
PVDF	=	Polyvinylidene difluoride
rpm	=	Revolutions per minute
SDS	=	Sodium dodecyl sulfate
S.E.M.	=	Standard errors of mean
TBS-T	=	Tween-20 Tris-buffered saline
TrkB	=	Tyrosine kinase receptor B
V	=	Volt
µg	=	Microgram

CHARPTER I

INTRODUCTION

Rational and significant of the study

Menopause is defined as the permanent cessation of menstruation resulting from the depletion of ovarian function (Josep and Mayo, 1999). It is marked by sudden change in the hormonal balance and estrogen hormone is decreased. It was suggested that the menopause may be a biological marker of aging (Snowdon, 1990). Thai women go through menopause when they are between 49 to 52 year old (Chompootweep, et al., 1993). Hill's study had predicted that the population of menopausal women throughout the word will be increased to 1.2 billion in 2030. (Hill, 1996).

Estrogen deprivation in menopausal women causes several physical changes including insomnia, hot flushes, vaginal atrophy and drying. These changes may along with osteoporosis and cardiovascular disease. Furthermore, menopausal women may experience behavioral changes such as mood disorders and cognitive dysfunction. Menopause associated with the cognitive changes which characterized by forgetfulness, loss of concentration and memory. Some study reported that menopausal women were much more likely to complain of memory loss than the women in the comparison group (Devi, et al., 2005). Furthermore evidence suggested that the deprivation of estrogen can increase risk for neurodegerative disease associated with learning and memory impairment, Alzheimer's disease (AD) (Gao, et al., 1998).

Brain-derived neurotrophic factor (BDNF) is a member of neurotrophin family. BDNF can mediate neuronal plasticity, differentiation, growth and survival in central and peripheral nervous system (Chao, 2003). There were evidences suggested that decrement of BDNF level associated with neurodegenerative disorder relating to learning and memory impairment (Lindsay, et al., 1991; Sohrabji and Lewis, 2006). Estrogen exerts its neuroprotective effect via the classical estrogen receptor subtypes (ERs), ER alpha (ERα) and ER beta (ERβ) which these receptors wildly distribute

throughout the hippocampus and cerebral cortex (Shughrue, et al., 1997). More evidences also indicated that estrogen affect on the BDNF expression in the hippocampus and cerebral cortex suggesting the crucial role of estrogen on the BDNF regulation (Spencer, et al., 2008; Luine and Frankfurt, 2013).

Estrogen replacement therapy (ERT) has demonstrated long-term benefits by improving unpleasant menopausal symptoms and protecting against menopause-related cognitive dysfunction; however, unopposed ERT is associated with serious side-effects such as endometrial cancer, breast cancer and venous thromboembolic events (Barrett-Connor and Grady, 1998).

Phytoestrogens are non-steroidal compounds obtained form plant and has estrogen-like properties. It was recognized that phytoestrogen can exert its effect similar to endogenous estrogen by binding with estrogen receptors (Rietjens, et al., 2012). Recently, phytoestrogens attracted interest as a potential alternative to the ERT.

Asparagus racemosus (AR) Willd or Shatavari, is a traditional medicine plant, locally known as Samsip in Thai (Boonsom, et al., 2012). It has phytoestrogenic properties and has been thought to be useful for female rejuvenation (Mayo, 1998). The major active compound presented in the AR root are steroidal saponins namely shatavarins (Hayes, et al., 2008). Thai traditional medicine has used the AR root to reduce menopausal symptoms. In addition, in Ayurvedic texts have recognized that AR can improve several diseases such as gastric ulcers, dyspepsia including has a galactogogue activity (Bopana and Saxena, 2007) and decreasing tumor (Rao, 1981). The neuroprotective properties of AR have been demonstrated by enhancing memory and protecting scopolamine-induced amnesia in rodents (Ojha, et al., 2010), reversing neuronal damage induced by kainic acid (KA) in mice (Parihar and Hemnani, 2004) and showing adaptogenic activity against different kinds of stressors in animals (Rege, et al., 1999). Although AR is well known for phytoestroginic and neuroprotective effects, the beneficial effects of AR on learning and memory impairment induced by ovariectomized (OVX) rat are unknown.

Purposes of the study

A general experiment objective

This experiment is designed to investigate the effects and mechanisms of AR root extract on learning and memory impairment induced by ovariectomy in rats.

Specific objectives

1. To investigate the effect of AR root extract on learning and memory in OVX rats.

2. To study the effect of AR extract on intact neuronal density in OVX rats.

3. To determine the effect of AR root extract on BDNF protein expression in OVX rats.

4. To evaluate the effect of AR root extract on ERs protein expression in OVX rats.

Scope of the study

This study used the adult sham-operated and ovariectomized female Wistar rats to investigate the effects of AR root extract on learning and memory. AR root extract at the dose of 100 and 1000 mg/kg B.W. were gavaged for ninety days before determining learning and memory abilities, serum estradiol levels, numbers of intact neuron as well as expression of BDNF and ERs protein. Learning and memory abilities were evaluated by using a novel object recognition (NOR) test. The serum estradiol levels were measured by using electrochemiluminescene immunoassay (ECLIA). The intact neuronal density in hippocampus and medial prefrontal cortex (mPFC) were examined by using a histological analysis. Finally, the expression of BDNF and ERs protein in the hippocampus and frontal cortex were evaluated by using a western blot analysis.

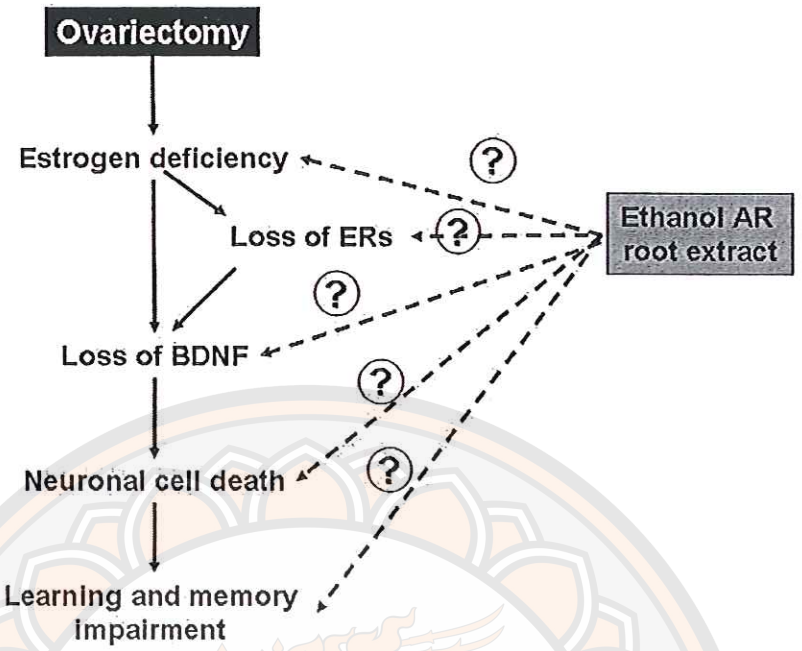

Figure 1 Conceptual research framework

Hypotheses

If AR has phytoestrogenic and/or neuroprotective effects, the animals receiving AR should improve their learning and memory associating with the increment of BDNF and ERs protein expression as well as number of intact neuronal cells.

Keywords

Asparagus racemosus, learning and memory, BDNF, ERs, ovariectomized rat

The anticipated outcomes of the study

The results from this study will show the effects and mechanisms of AR on learning and memory impairment induced by OVX. It may provide a basis for clinical studies as a new potential memory enhancing dietary supplement for menopausal women.

CHARPTER II

LITERATURE REVIEWS

Menopause

Menopause is a status resulted from ovarian function decline in women followed by termination of menstrual cycle. It is determined by disappear for consecutive 12 months of menstrual periods without pathologic or physiologic causes (Notelovitz, 1989). Menopause can be divided into 4 stages: premenopause, perimenopause, menopause and postmenopause. Premenopause often referred to as perimenopause, is the phase before the beginning of menopause. The average age for a woman to begin experiencing premenopausal symptoms is 45 year old. Perimenopause is the time leading up to menopause, characterized by normal change which often lead to menopausal symptoms and usually occurs between 45 and 60 years of age. Menopause is the final menstrual bleed and it is generally considered to have occurred after 12 months of amenorrhea. This stage has a wide starting range, but can usually be expected in the age range of 45 to 55 year. In Thai women, the average age at menopause is about 50.13 years (Punyahotra, et al., 1997). The last stage of postmenopause is the time after the last episode of menstrual bleeding. During this stage, there are risks for numerous diseases such as osteoporosis and heart disease.

Menopausal signs and symptoms associated with physical and psychological alterations. Physical signs and symptoms may include hot flushes, vaginal dryness, and risk of osteoporosis and cardiovascular disease. Psychological signs and symptoms may include anxiety, difficulty concentrating, depression and forgetfulness (Miquel, et al., 2006). Chronic deficiency of estrogen in menopause has many effects in non-reproductive functions. There is the possibility that the estrogen deprivation could worsen the age-related impair in memory (Maki, et al., 2001; Henderson, et al., 2003).

Numerous evidences revealed that loss of gonadal function in aging females reduced metabolic function (Alonso, et al., 2008), increased oxidative stress and acetylcholinesterase activity in brain (Martins, et al., 2012) leading to alteration of brain homeostasis and cognitive function. Moreover, clinical studies have shown that

ovarian hormone deprivation after menopause can increase the risk of Alzheimer's disease (AD) (Gao, et al., 1998).

There were several studies had been used murine experimental models. For menopausal models, ovariectomy in rodent became a good model for mimicking human ovarian hormone loss. Additionally, there had been increase in the number of publication, which focuses on the obtaining results of ovariectomy in rats such as physiological difference of nervous system, cardiovascular function, hepatocytes, bone, skin (Castillo, et al., 2005; Tresguerres, et al., 2008) as well as immune system (De la Fuente, et al., 2004; Baeza, et al., 2009).

Cognition

Cognition is the mental process that may be described as an experience of knowing, including learning, memory, reasoning and performance of effective executive action. Learning is attainment of new information and refers to the behavior adaptation caused by experiences while memory is the retention of learning information. Memory formation is processed by three main stages; encoding (receiving, processing and combining of obtained information), storage (recording of the encoded information) and retrieval (recalling the stored information to responding some cue for use in process or activity) (Carlson, 2004; Bear, et al., 2007). Memory is classified into three different types based on the duration of memory retention. At first, sensory memory is the shortest-term element of memory and decays very quickly, typically in the region of 200 - 500 milliseconds after the perception of an item, and certainly less than a second, such as visual information is detected by photoreceptor cells in the eyes. The second type, short-term memory is an immediately memory for stimuli that have just been perceived and the temporary storing limited amount of information. It allows recall from many seconds to minute without rehearsal. Finally, long-term memory is storage of an unlimited amount of information for potentially unlimited duration. The maintaining in stable and permanent changes of neural connections throughout the brain is the critical function to processes the long-term memory (Carlson, 2004; Bear, et al., 2007).

Long-term memory can be divided into two classifications. First is declarative memory which involves events and facts about the people, places, and things. This

memory requires conscious recollection and association of information. Second is non-declarative memory which involves training reflexive motor and skill consequences from direct experience. This memory is formed tending to require repetition and practice over a longer period of time, but it is less likely to be forgotten (Kandel, et al., 2000).

Recognition memory is the ability to identify information after experiencing it again. This memory were documented that it depend on the hippocampus and prefrontal cortical input (Ennaceur, et al., 1997). Some study demonstrated that lesions 30–50% of the dorsal hippocampus could disturb spatial memory, while lesions 75–100% in these area could impair the performance in object recognition (Broadbent, et al., 2004). Additionally, lesion in ventromedial prefrontal cortex displayed recognition memory impairment (Bachevalier and Mishkin, 1986; Meunier, et al., 1997).

Amnesia

The resulting of memories loss prominent in cognitive function decline described in the term "Amnesia". There are two main types of amnesia. The first type is anterograde amnesia which is characterized as an impairment or loss of ability to learn new information. People who have this illness can remember events that have occurred in the past but cannot retain information received after the brain damage. The second type is retrograde amnesia that is characterized by an inability to remember events that happened before the brain damage occurred. Usually, pure anterograde amnesia is rare, there is also a retrograde amnesia for events that occurred for a period of time before the brain damage has occurred (Carlson, 2004; Bear, et al., 2007).

Hippocampus and medial prefrontal cortex associated with cognition

The hippocampus and cerebral cortex are the main structures of the brain that participate critical role to process learning and memory. There were several reports indicated that recognition memory impairment was occurred following the lesion appearing of hippocampus and ventromedial prefrontal cortex (Drachman and Ommaya, 1964; Correll and Scoville, 1965; Sidman, et al., 1968; Bachevalier and Mishkin, 1986; Meunier, et al., 1997).

The hippocampal formation plays an important role in the long-term memory consolidation. It mediates the initial steps of long-term storage and then slowly transforms into neocortical storage system which permits the new data to be stored without disrupting the existing information (Martin, 1991). The mPFC is suggested to mediate the decision making which involved in the retrieval of long-term memory that associates context, locations, events, and corresponding adaptive responses (Euston, et al., 2012).

Hippocampus is subdivided into four longitudinal zones, the CA1, CA2, CA3 and CA4 (CA stands for Cornu Ammonis, Latin for the Ammon's horn) (Brodal, 1998). The composition of hippocampus, dentate gyrus as well as subiculum is called the hippocampal formation (Martin, 1991). The perforant pathway which originates from entorhinal cortex is the major input to the hippocampus by the way of pyramidal cell axons and synapses on granule cells of the dentate gyrus. After that, the granule cell axons which are called "Mossy fibers" synapse on pyramidal cells of CA3 which send their axons in termed "Schaffer collaterals" to pyramidal cells of CA1. Output from the CA1 sends the information to subiculum which sends back to the entorhinal cortex (Martin, 1991) (Figure 2).

The association between hippocampus and prefrontal cortex was shown in the figure 3. The major cortical afferents to the hippocampus initiates from the lateral and medial entorhinal cortex. Input from perirhinal cortex directs to terminate the lateral entorhinal cortex where the information is sent to the hippocampus by the lateral perforant pathway which synapes the granule neurons in dentate gyrus (Witter, et al., 1989; Witter, et al., 2000; van Strien, et al., 2009). The major outputs from hippocampus are subiculum and CA1 where neuronal axons project to the prefrontal cortex (Jay and Witter, 1991; Verwer, et al., 1997). Additionally, there are also the directly projections from the sub-region in the mPFC, infralimbic and prelimbic region, to the hippocampus (Burwell and Amaral, 1998).

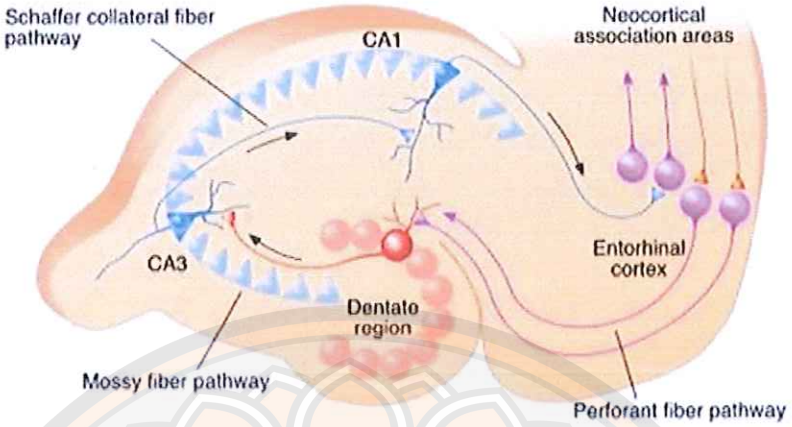

Figure 2 Connection and component of hippocampal formation

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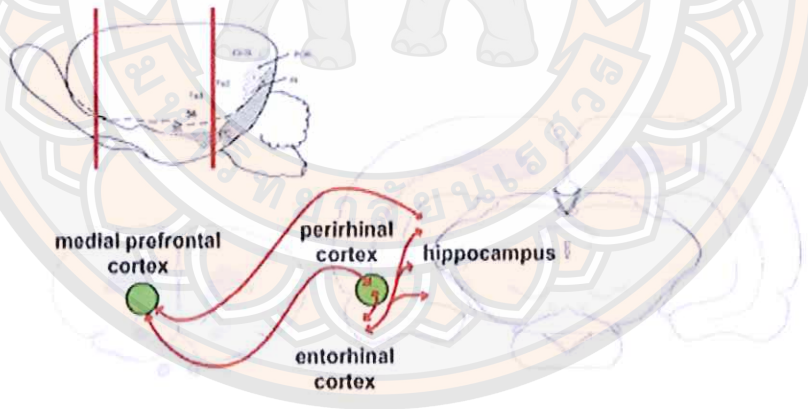


Figure 3 Association between the hippoacampus and prefrontal cortex

Source: http://www.bris.ac.uk/synaptic/research/projects/memory/recognition-memory/connections.jpg

Estrogen and Cognition

Estrogen is a steroid gonadal hormone that is mostly produced by ovarian follicles. Some is also produced in smaller amounts by other tissues such as liver, adrenal glands, adipose tissue and the breasts. Estrogen has not only critical impact on reproductive system and reflects sexual behavior in mammals but also has effects on non-reproductive behaviors such as anxiety and depressive-like behaviors along with cognitive function. It had been reported that OVX rats administrated with 17β-estradiol exhibited decreases in anxiety and depressive behaviors (Walf and Frye, 2006). However, estrogen affects on cognitive function which rely on type of task and area of brain. For instance, the administration of estrogen in female rats diminished the ability on a striatum-dependent task (Korol, 2004) while it enhanced the ability of prefrontal cortical-dependent learning (Luine, et al., 2003). Estrogen affects on several brain areas to mediate the cognitive function such as hippocampus, striatum, basal forebrain as well as prefrontal cortex (Spencer, et al., 2008). It had been suggested that estrogen fluctuation manipulates on the test of memory across estrus cycle (4-6 days), when serum estradiol (E2) peaks in proestrus, the rats that could solve a plus maze task (Korol, et al., 2004). Accordingly, the OVX rats treated with E2 it showed a similar result when using same task (Korol and Kolo, 2002). In women, some cognitive abilities are fluctuated through the menstrual cycle and related with the serum estrogen levels (Rosenberg and Park, 2002; Sherwin, 2003). It had also been accepted that estrogen fluctuation across of menstrual cycle associates with the activation of hippocampal formation (Dreher, et al., 2007). These studies suggested that ovarian steroid hormones affect on cognitive function requiring the hippocampal formation. Moreover, the suppression of ovarian hormones showed the impairment of hippocampal- and prefrontal-cortical-dependent memory but this effect was relieved by the exogenous E2 administration. For instance, OVX rats impaired ability on a task dependant on hippocampal and prefrontal cortical faculties, the object recognition memory task (Wallace, et al., 2006) was reversed by E2 administration (Korol, 2004).

Estrogen has been documented that it can mediate the brain function through cholinergic neurotransmission by reverse the decreasing in choline, the precursor of acetylcholine (ACh) synthesis, which induced by ovariectomy (Singh, et al., 1994). Furthermore, it was recognized that estrogen can process learning and memory by

mediate the morphology, physiology and chemical change of hippocampal neurons (Mukai, et al., 2010).

A current study has showed that the cognitive impairment in aging humans is the results from the change in neuronal morphology and neuronal chemistry such as the disturbance of neuron connections, loss of neurons and changes in neuronal transmission (Juraska and Lowry, 2012). Accordingly, these evidences were supported by prior investigation in rodents and human, which found cognition decline correlated with age and estrogen deficit by menopause, but it was improved by estrogen therapy (Sherwin and Tulandi, 1996; Markowska and Savonenko, 2002). Over all evidences suggest that estrogen has an important role for preventing cognitive abilities impairment induced by aging and ovarian hormone loss in women.

The effects of estrogen to be a neuroprotective and neurotropic abilities are recognized by improving and enhancing in cognitive function (Shughrue and Merchenthaler, 2000; Wise, et al., 2001). The previous investigation suggested that estrogen replacement therapy (ERT) can decrease risks and delay the onset of AD after menopause by increase cerebral blood flow, reduce neuronal inflammatory as well as enhance neuronal plasticity (Toran-Allerand, et al., 1999). In addition, estrogen is also crucial role to optimal brain function which were explained by exposure to estrogen *in vivo* and *in vitro* causes neurobiological changes in hippocampus that included dendritic spines increasing, neurotransmitter systems modulation, synaptic plasticity enhancing and cell signaling events controlling (Dohanich, 2002).

Notably, ERT is suggested to relive the menopausal symptoms and prevent cardiovascular disease and osteoporosis including reduce the risk for neurodegenerative disorder. However, there are several concern for using the ERT, because it produces serious side-effects such as increase the risks for endometrial cancer, breast cancer and venous thromboembolic events (Barrett-Connor and Grady, 1998).

Estrogen receptor

Estrogen receptors are divided into two classical subtypes, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). There are several distinct pathways

which estrogen and ERs regulate biological process. The first is the classical (genomic) pathway, estrogen binds to nuclear receptor and this complex binds to estrogen response element (EREs) to activate gene transcription. Second, it is known as "tethered pathway". This pathway involves protein-protein interaction with other transcription factor after ligand activation and thereby gene transcription is regulated by indirect DNA binding. The third pathway is also known "nongenomic" which produced by binding of estrogen to membrane receptors that leads to trigger the phosphorylation cAMP response element-binding protein (CREB), and consequently bind to DNA promoter. Finally, estrogen exerts it effects through the ligand-dependent pathway that involves activation through other signaling pathway such as growth factor signaling. In this pathway, the activated kinases phosphorylate ERs and thereby activate them to form dimerization and regulates gene following DNA binding (Heldring, et al., 2007; Cui, et al., 2013).

Both ERα and ERβ are distributed in many brain areas associating learning and memory process such as cerebral cortex and hippocampus (Shughrue, et al., 1997). ERα is the predominant subtype in hypothalamus and amygdale suggesting that involves the regulation of neuroendrocine system and emotional reactions. ERβ expression is highest in the hippocampus and cerebral cortex suggesting this subtype is importance for learning and memory process (Osterlund, et al., 2000). Several studies have suggested that ERs implicate in neuronal functions such as synaptic potentiation, synaptic depression and synapse formation (Day, et al., 2005; Szymczak, et al., 2006; Liu, et al., 2008). Some studied revealed that significant attenuation of both ERα and ERβ expression in spine synapse complex in hippocampal CA1 decreased ability of E2 in promoting spine density in hippocampus during aging (Adams, et al., 2001). Previous study has also shown that ERβ agonist, diarylpropionitrile (DPN), could attenuate neuronal damages in ischemic-induced mice, but not ERα agonist, propyl pyrazole triol (PPT). It was considered that a significant estrogen receptor-induced neuroprotective effect in a global ischemia involving ERβ (Carswell, et al., 2004). In the case of central nervous system (CNS) inflammation, it has been revealed that ERα could mediate to be a neuroprotection by signaling through astocytes (Spence, et al., 2013). These data suggest that ERα or ERβ can signal through distinct intracellular pathways to mediate the neuroprotective

activities, while the distinct molecular pathway by which ERα and ERβ affect neuronal functions are poorly understood.

Recent investigation revealed that subcellular distribution and activities of ERα and ERβ also change in aging and lead to differential response to estrogen in aging brain (Navarro, et al., 2012). The effects of low estrogen levels have been controversial for ERs expressions. Recent study had shown a decrement of the ERα in telencephalon and hippocampus of long-term OVX rats (6-24 months) which was reversed by E2 administration (Navarro, et al., 2012). Meanwhile, shot-term estrogen declined in OVX rats referred to up-regulation of ERα in the hippocampus and this effect would be absence when theses animals were immediately treated with estrogen after surgery (Cardoso, et al., 2010). Furthermore, there was some study showed a significant decrease in the expression of ERβ in the brains in three months OVX rats (Rose'Meyer, et al., 2003). These evidences have been demonstrated that estrogen might affect to regulate the expression of their receptor subtypes in the brain.

Brain-derived neutotrophic factor

Brain-derived neurotrophic factor (BDNF) is member in neurotrophins family which comprises nerve growth factor (NGF), neurotrophin-3 (NT-3) as well as neurotropin-4/5 (NT-4/5). BDNF exerts its effect by binding with its specific receptor, tyrosine kinase receptor B (TrkB), and few binding with p75 neurotrophin receptor (P75NTR). These binding lead to mediate the neuronal growth, survival, and plasticity in both central and peripheral nervous system (Chao, 2003).

BDNF was extensively presented throughout the brain with highest levels in the hippocampus followed by cerebral cortex (Hofer, et al., 1990). Previous reports explained that BDNF plays a critical role in learning and memory formation (Lindsay, et al., 1991) by enhancing the cholinergic system in the basal forebrain (Nonomura, et al., 1995). Furthermore, BDNF also produces LTP formation in the hippocampus (Patterson, et al., 1996), encourages activity-dependent dendritic growth (Ma, et al., 2002) as well as protects cell apoptosis (Courtney, et al., 1997).

Age-related decrease BDNF expression impacted on learning and memory ability by using the task of memory in animals (Croll, et al., 1998). In addition, aging could also diminish the expression of BDNF associating neurodegenerative disease,

such as AD. Numerous documents have demonstrated that AD patients showed decrease of BDNF in several brain areas such as hippocampus (Hock, et al., 2000), entorhinal cortex (Narisawa-Saito, et al., 1996) and cortex (Connor, et al., 1997; Michalski and Fahnestock, 2003).

Previous study has shown that hormonal status can greatly influence the expression of BDNF. It was reported that BDNF mRNA and protein levels were changed throughout the estrous cycle and the level of BDNF protein was highest during proestrus phase in female rats (Scharfman, et al., 2003). Moreover, ovarian hormones can regulate the BDNF synthesis. Spencer et al. evaluated effects of ovarian hormones on the hippocampus and found that fluctuation of these hormones could mediate the TrkB activation (Spencer, et al., 2008). These results are consistent with other studies that established the expression of BDNF was increased when administrated with exogenous estrogen in the olfactory bulb (Jezierski and Sohrabji, 2000), hippocampus (Allen and McCarson, 2005), cortex (Sohrabji, et al., 1995), amydala (Zhou, et al., 2005) and septum (Gibbs, 1998) in OVX rats.

Furthermore, extensive literature gathered evidences to decide the exactly role of ERs on BDNF expression. Previous study considered that estrogen exerts its effects on BDNF expression via binding nuclear ERs and brings to estrogen response element (ERE) formation followed by stimulating BDNF gene transcription in the pyramidal neurons of hippocampus (Sohrabji, et al., 1995; Luine and Frankfurt, 2013). In addition, other studies have also proposed that binding of estrogen to extranuclear ERs activate the CREB which trigger the CRE in DNA followed by promoting the BDNF gene transcription (Tao, et al., 1998; Luine and Frankfurt, 2013). Previous reports have considered that ERβ co-localize with GABAergic neuron and plays an indirect role to up-regulate BDNF in rats suggesting ERβ is an importance on BDNF regulation (Blurton-Jones and Tuszynski, 2002). The role of ERs to regulate the BDNF expression has been observed by the recently study, ERβ knockout mice (BERKO) showed a significant decreasing of BDNF mRNA, not ERα knockout mice (AERKO), suggesting ERβ affect on the regulation of BDNF transcription (Spencer-Segal, et al., 2012). Although ERα had no affect on BDNF transcription, it has been proposed that ERα involved the regulation of posttranscription and releasing of BDNF (Jezierski and Sohrabji, 2003; Sato, et al., 2007). These data have indicated that both

ERα and ERβ subtypes have a crucial role on the BDNF regulation. Moreover, some study has also concluded that both ERα and ERβ are associated with hippocampal neurogenesis by enhancing cell proliferation in the dentate gyrus (Mazzucco, et al., 2006). Theses findings suggested that ERs may modulate brain function in partial regions of the brain which associated learning and memory.

Phytoestrogens

Phytoestrogens are non-steroid compounds obtained from plants and have estrogenic-like effects (Patisaul, et al., 2001), which has the proven ability to attach to ERs in humans. It were referred to in literature as "plant estrogens" (Glazier and Bowman, 2001).

Phytoestrogens are divided into three main classes: isoflavones, comestans, and lignans (Thompson, et al., 2006). Among various phytoestrogens, isoflavones had been extensively studied while few had been studied for lignans and comestans. Isoflavones was found predominately in soybeans and soybean products. It was interested to useful prevent several diseases such as cancers, cardiovascular disease and oseteoporosis in menopausal women (Duncan, et al., 2003). Recently, phytoestrogens attracted interest as a potential alternative to the estrogen supplements which produce side-effects when use it for long time (Cornwell, et al., 2004).

Since several literatures indicate that ERT can improve the cognitive abilities decline and reduce the risk for neurodegenerative disorder in post-menopausal women, phytoestrogens may play a positive role in these conditions. Several studies have determined beneficial effects of soy phytoestogen on brain function. They showed that soy phytoestrogens regulated choline acetyltransferase, NGF and BDNF in brain areas such as the frontal cortex and hippocampus of female rats (Pan, et al., 2010). Moreover, OVX rats treated with phytoestrogens had been shown a dose-dependent improvement of visual spatial memory (Pan, et al., 1999). Additionally, study of Kim et al. found that dietary phytoestogens attenuated tau protein phosphorylations. They established conclusively that phytoestrogen may be effective to improve AD (Kim, et al., 2000). Previous study have considered that isoflavones was estrogen mimics which bound to ERβ with higher affinity than ERα. The estrogenic potency of phytoestrogens might trigger many biological responses on

brain function, which were evoked by the physiological estrogens (Kuiper, et al., 1998).

Phytoestrogens might able to substitute of estrogen that followed increase BNDF levels and bring to improve cognitive function. Pan et al. had shown that administration with soy germ phytoestrogen in OVX rats could increase BDNF and improve spatial memory without side-effects on reproductive organs in OVX rats. They have considered that phytoestrogen effects on hippocampal function by increase BDNF expression which bring to activate the synaptic formation and results to enhance learning and memory in circulating estrogen deficit (Pan, et al., 2010).

***Asparagus racemosus* Willd.**

Asparagus racemosus (AR) or Willd is known as Asparagaceae family. It is well known as "Shatavari" in the Ayurveda drug of India. AR is herbaceous plant which distinguished by has numerous roots (Figure 4). Its roots are used as herbal medicine and called as "Rak Sam Sip" in Thai (Boonsom, et al., 2012). The major active compounds (Figure 5) which presented in the root of AR are steroidal saponins or shatavarin and other constituents such as sarsasapogenin, racemosol and asparagamine (Bopana and Saxena, 2007).

AR was recognized for its neuroprotective properties in the animal models. Parihar and Hemnani found that the AR root methanolic extract could reverse the neuronal injury in the mice hippocampus and striatum by kainic acid (KA) injection. The neuroprotective effects of the AR root methanolic extract were revealed by increasing of glutathione peroxidase (GPx) activity and glutathione (GSH) content and lead to improve memory. They have demonstrated that AR root methanolic extract has potential effects to reduce oxidative stress by its anti-oxidant activity (Parihar and Hemnani, 2004). Numerous studies have been reviewed on the potential benefits of AR in cerebroprotective effects. It has been suggested that administration of AR methanolic extract 400 mg/kg B.W. protected the rats from ischemic-induced brain injury may be due to reduction of oxidative stress which occurs by alternation in levels of antioxidants, neurotransmitters and the AR extract had the potential to use in treatment of ischemia (Nandagopal, et al., 2011). It has been found that administration of AR methanolic root extract 150 mg/kg B.W. for 7 days could reverse learning and

memory deficits in mice (Ashwlayan and Singh, 2011). AR had also been documented for its nootropic and anti-amnesic activities by Ojha and co-workers. They have considered that AR root extract could mediate the augmentation of cholinergic system which brought to improve memory impairment induced by scopolamine (Ojha, et al., 2010). Furthermore, Mentat™ is an herbal psychotropic AR preparation that could improve the withdrawal symptoms caused by alcohol abstinence such as tremors, convulsions, hallucinations and anxiety in rats (Kulkarni and Verma, 1993).

Beside the saponin-rich fraction is extracted form AR, there are another active compounds that derived form AR. Wiboonpun and colleagues have found DPPH (α, α –diphenyl- β-picrylhydrazyl) which was decided to be a new antioxidant namely 'racemofuran' (Wiboonpun, et al., 2004). AR was also used in traditional ayurvedic formulations and known as "Shatavari kalpa" (Unnikrishnan, 1998). Furthermore, there were the several herbal medicine which were prepared from AR such as Abana® (containing 10 mg Stavari root extract per table) and Diabecon® (containing 20 mg Stavari root extract per table) by Himalaya Herbal Healthcare, India (Bopana and Saxena, 2007). There was report found the active constituent namely "Asparagamine", which was isolated form AR, showed anti-oxytocic properties (Sekine, et al., 1994). Moreover, isolation of AR root extract established the new compound called 'Racemosol' (Sekine, 1997).

For pharmacological applications of AR, it has been reported that AR was used for galactagogue, diuretic, anti-spasmodic as well as nerve tonic (Sharma, 2000). This herbal is one significant remedial plant in rasayana drugs which was report that it could increase cellular vitality and resistance to verity insults (Goyal, et al., 2003) which was recognized in Indian and British Pharmacopoeias (Bopana and Saxena, 2007).

The phytoestrogenic properties of AR was widely known and used to be a hormonal modulator to stimulant health tonic for women (Mayo, 1998), which has effects and structure similar to estrogen (Saxena, et al., 2010) (Figure 6). In vivo studies revealed that AR could inhibit the mammary carcinogenesis induced by DMBA (Rao, 1981) and showed inhibitory effect on uterine contractions induced oxytocin (Gaitonde and Jetmalani, 1969). It was suggested that "U-3107" or EveCare®, the herbal preparation containing 32 mg AR per 5 ml syrup, was used to

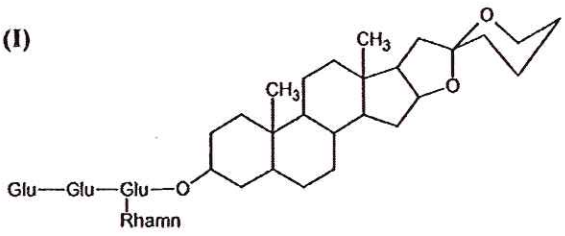
improve several menstrual disorders and defense abortion as well as increased wet and dry uterine weights accompanied by increasing of estrogen levels (Mitra, 1999). Furthermore, previous investigation of Neverkar had provided that EveCar capsules could improve uterine breeding and menstrual cycle in the 63 volunteer women (Nevrekar, 2002). In another study had provided that EveCare also showed its effect to correct the suffering from dysmenorrheal and pre-menstrual syndrome in 40 patients (Swarup, 1998).

(A) **(B)**

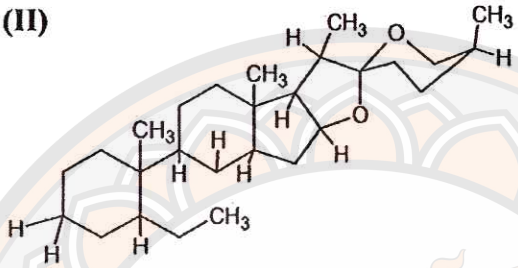
Figure 4 The characteristic of *Asparagus racemosus* (AR) Willd
The leaves and fruits of AR (A) and the root of AR (B)

Source: http://www.twinlotus.com/asp-bin/pic_HL/000095_HL.jpg
http://www.thaigoodview.com/files/u72739/025.jpg

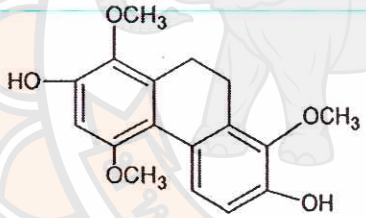
(I) **Shatavarin**



(II) **Sarsasapogenin**



(III) **Racemosol**



(IV) **Asparagamine**

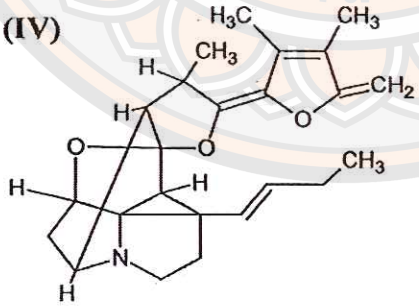
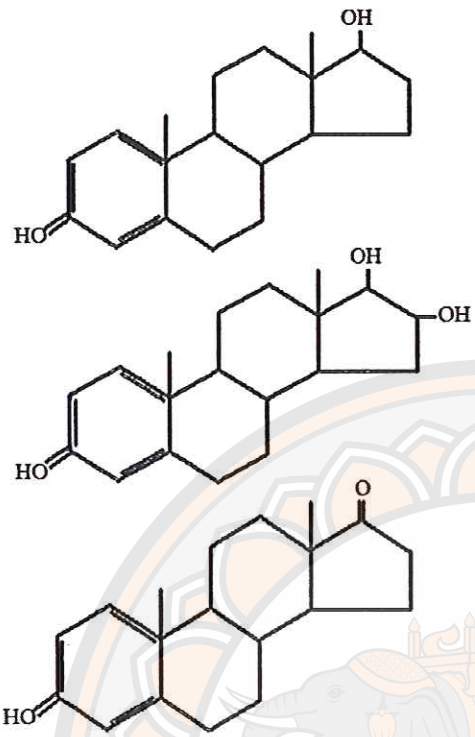


Figure 5 Active principles of AR (I) Shatavarin, (II) Sarsasapogenin, (III) Racemosol and (IV) Asparagamine

Source: Bopana and Saxena, 2007



Estradiol

Estriol

Estrone

Figure 6 Chemical structure of estrogens

Source: http://www.the-hormonal -nightmare.com/images/ Human_ Estrogen_ and_ Bioidentical_Estrogen.gif

CHAPTER III

RESEARCH METHODOLOGY

Instrument and materials

1. Instruments

Autocentrifuger (Biofuge fresco)

Electrophoresis instrument (miniVE, Amersham Biosciences)

Microplate reader (Labsystems iEMS Reader MF)

Microtome (Leica RM 2235)

Novel object recognition test equipment

Tissue embedding machine (Leica EG 1160)

Tissue processing machine (Leica TP 1020)

Video camera (LYD-8080, LYD)

2. Materials

17α-Ethynylestradiol powder (Sigma, St. Louise, MO)

40% formalin (RCI Labscan)

96 well plate (Greiner bio-one)

Acrylamide (Sigma-Aldrich Inc, Louis, USA)

Ammonium Persulfate (APS) (Amresco Inc, Solon, USA)

Anti-BDNF antibody (Santa Cruz Biotecnology Inc, California, USA)

Anti- β-actin antibody (Santa Cruz Biotecnology Inc, California, USA)

Anti-ERα antibody (Abcam Inc, Cambrige, UK)

Anti-ERβ antibody (Abcam Inc, Cambrige, UK)

Anti-rabbit anyibody (Vector Laboratories Inc, Burlingame, USA)

Atropine sulfate

Avidinbiotinylated horseradish peroxidase complexes (ABC) kit (Vector Laboratories Inc, Burlingame, USA)

Bicinchoninic acid protein assay reagent (BCA) kit (Pierce Biotecnology Inc, Rockford, USA.)

Biotinylated anti-rabbit IgG (Vector Laboratories Inc, Burlingame, USA)

Biotinylated anti-rabbit IgG (Vector Laboratories Inc, Burlingame, USA)

Bovine serum albumin (BSA) blocking buffer (Amresco Inc, Solon, USA)

Cover slips (Menzel-glaser)

ImmPACT™ diaminobenzidine (ImmPACT™DAB) (Vector Laboratories Inc, Burlingame, USA)

Distilled water

Embedding cassette

Eosin (C.V. Laboratories CO., Ltd)

Ethanol (Vidhyasom Co., Ltd.)

Hematoxyline (C.V. Laboratories CO., Ltd)

Heparin

HRP-conjugated chicken anti-goat IgG (Millipore, Billerica, USA)

Liquid nitrogen

Loading dry (Amresco Inc, Cambrige, UK)

Lysis buffer (Invitrogen Corporation, Camarillo, USA)

N, N'-Methylenebisacrylamide (Sigma-Aldrich Inc, Louis, USA)

Paraplast (Leica microsystems)

Pentobarbital (Nembutal®)

Permount (Fisher)

Polyvinylidene difluoride (PVDF) membrane (Pall Corporation, BioTrace)

Propylene glycol (Vidhyasom Co.,Ltd.)

Proteinase inhibitor cocktail (Sigma-Aldrich Inc, Louis, USA)

Protein marker (Gene Direx)

Sodium chloride (MERCK)

Sodium dodecyl sulfate (Sigma-Aldrich Inc, Louis, USA)

TMED (Amresco Inc, Cambrige, UK)

Tris (Amresco Inc, Solon, USA)

Tris-base buffer saline (Amresco Inc, Cambrige, UK)

Tween 20 (Biotect)

Xyline (Zen point)

Plant material and preparation of crude extract

The AR roots were collected from Rayong, Thailand. The voucher specimen of the plant was kept at Faculty of Pharmaceutical Science, Naresuan University, Phitsanulok, Thailand.

The roots of AR were dried by hot air oven at 45 °C for 24 h before milled into coarse powder. After that the dried powdered roots of AR was macerated at room temperature with hexane for 3 days. Then residue was macerated with 95% ethanol for 3 day, and was filtered and extracted again. The crude of AR ethanolic extract was mixed with propylene glycol to stock suspension in a dose of 100 and 1000 mg/kg B.W. The suspension was orally administered to the rats during 7:00 – 8:00 A.M.

Animals

Eight-weeks-old female Wistar rats were purchased from the National Laboratory Animal Center Mahidol University, Nakhon Prathom, Thailand. The rats were acclimatized for at least one week before starting the experiment. They were housed in group of 4-5 animals under a standard light/dark (12:12 h) at constant temperature of 24 ± 1 °C. The animals were allowed free access to food and tab water *ad libitum*. The experiment protocol was approved by the Ethical committee for the Use of Animal, Naresuan University.

Methods

1. Experimental protocols

The rats were examined the estrous cycle by using vaginal cornification assay for three consecutive cycles before treating. All animals were bilaterally ovariectomized or sham-operated under pentobarbital (50 mg/kg B.W.) in aseptic technique during a diestrus phase. Fifteen days after operation, the animals were randomly divided into five groups:

1. Sham group (n = 7), the rats were gavaged with propylene glycol (PG) as a vehicle control.

2. OVX group (n = 7), the OVX rats were gavaged with PG.

3. OVX+AR100 group (n = 7), the OVX rats were gavaged with AR 100 mg/kg B.W.

4. OVX+AR1000 group (n = 7), the OVX rats were gavaged with AR 1000 mg/kg B.W.

5. OVX+17α-ethynylestradiol (EE) group (n = 7), the OVX rats were gavaged with EE 0.1 mg/kg B.W. as a positive control.

All experimental groups were gavaged once daily for 90 days and weighed once a week.

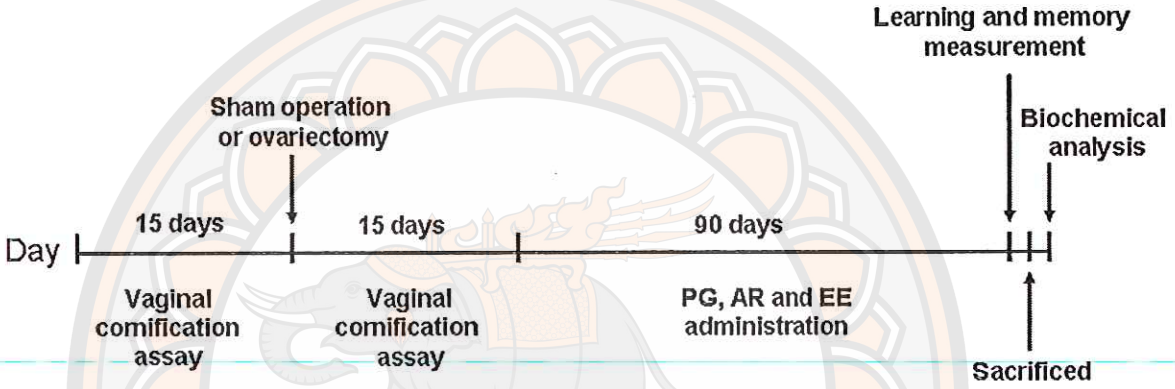


Figure 7 Experimental schedule

2. Preparation of 17α-Ethynylestradiol solution

The 17α-Ethynylestradiol powder was dissolved with small absolute ethanol volume, followed by the addition of deionized water and leave for evaporation of ethanol at room temperature. The solution was kept in the dark bottles at 4 °C (Urasopon, et al., 2007).

3. Vaginal cornification assay

All rats were checked vaginal epithelium daily between 8:00-9:00 A.M. by using disposable pipette which contained 0.9% NaCl solution, and then pushing and injecting 0.9% NaCl solution that was gently into the vagina approximately 2-5 mm depth. After that the fluid in vaginal lumen was sucked up into the pipette and smeared on the microscopic slide to determine the vaginal epithelial cell under a light microscope.

4. Novel object recognition test

The novel object recognition (NOR) test was induced by Ennaceur and Delacuor in 1998. This is a test of dorsal hippocampal function and prefrontal cortex and base on the promise that rodent will explore a novel object more than a familiar one, but only if they remember the familiar one by learning (recognition) and memory process (Ennaceur and Delacour, 1988). This test was performed without external motivation, reward or punishment but a little training or habituation is required (Silvers, et al., 2007). Moreover, it is a widely used model for the investigation of effects on memory and also has been used to test the effects of various pharmacological treatments and brain damage (Goulart, et al., 2010).

4.1 Equipment

The equipment consists of a cubic chamber (100 cm x 100 cm with 50 cm high walls) and three different objects. Object A, B and C was the cylinder, pyramidal and cuboidal shape, respectively. The position of two objects was placed in the centre of the area, 30 cm from each other and 35 cm from the nearest wall of the chamber.

4.2 Acclimatization, training trial and retention test

Before training, each rat was introduced to get acclimatized to the testing environment which is an empty space for 10 min. After acclimatization sessions, the rat was ready for the training session. This session, object A and B were placed in a symmetric position (Figure 8). Each rat was allowed to explore in the box for a total 10 min and considered to be exploring the object when the rat's nose pointed toward the object at a distance ≤ 1 cm (Dodart, et al., 2002). The amount of time spent exploring object A and B (T_A and T_B) was recorded manually and calculated as a preference index $[(T_A \times 100)/(T_A + T_B)]$. Following the training period, the rat was removed from the environment for a delay period (10 min). After the delay, the rat was returned to the chamber again, where one of the original object (B) was replaced by new object (C). The recognition index of each rat was calculated from the following formula $(T_C \times 100)/(T_A + T_C)$, where T_A and T_C are the time spent exploring the object A and C, respectively. After each session, the arena and objects were cleaned by 70% ethanol to ensure that behavior of animals was not guided by odor cues.

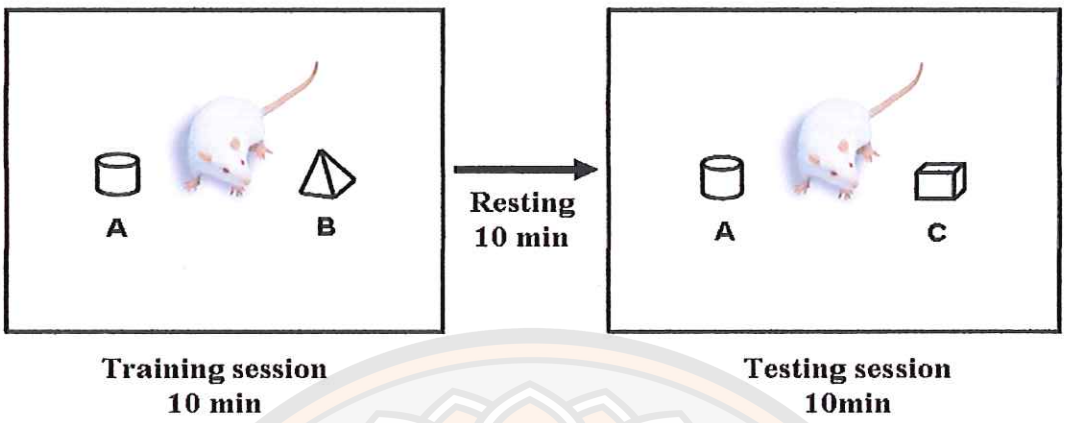


Figure 8 Illustration of the novel object recognition (NOR) test

5. Blood and tissue collection

After study of learning and memory abilities, all experimental animals were sacrificed with pentobarbital (50 mg/kg B.W., intraperitoneal injection). Then, the blood was collected by using a cardiac puncture method and added with heparin to prevent blood clotting. Serum was separated from the blood by centrifugation at 1000 rpm for 10 minutes at room temperature and kept at -80 °C until analysis. The brain was quickly removed out of the skull after blood collection. The hippocampus and frontal cortex were dissected from left brains and kept at -80 °C to determine the expression of BDNF and ERs proteins and right brains were fixed in buffered 4% formadehyde to investigate the histological changes.

6. Serum estradiol levels evaluation

Serum estradiol levels was measured using eletrochemiluminescence immunoassay (ECLIA) with Elecsys 2010 automate analyzer (Roche Diagnostics GM6H, Mannhein, Germany) at Faculty of Medicine, Chiang Mai University Hospital, Chiang Mai, Thailand.

An ECLIA was performed according to the procedure described by the manufacturer. Briefly, sample was first incubated with estradiol-specific biotinylated antibody and estradiol-specific antibody labeled with ruthenium to form an immune complex. Next, the streptavidin-coated microparticles were added. The biotin and streptavidin was bound to become a solid phase. After that, sample was drawn to the

surface of the electrode with the help of a magnet and held there temporarily. Unbound substances were removed with Procell. The electrochemiluminescent was generated by voltage application and the resulting light emission was measured by the photomultiplier.

7. Western blot analyses of BDNF and ERs expression

The hippocampus and frontal cortex including rat uterus tissue, a positive tissue for ER subtypes detection, were homogenized in ice-cold lysis buffer with protease inhibitor cocktail. Then the homogenized sample was centrifuged at 10,000 rpm for 10 minutes at 4 °C. After that, the supernatant were transferred to a new tube. The protein content in each sample was measured by using BCA kit.

7.1 Analysis of BDNF expression by western blot technique

Aliquots of sample containing 50 µg of proteins were separated by electrophoresis on 12.5% SDS–PAGE at 100 V for 3 h is followed by electrophoretic transfer to a PVDF membrane at 20 V for 2 h. Next, the blotted membrane were treated with 0.1% tween-20 Tris-buffered saline pH 7.4 (TBS-T) and 5% skim milk for 1 h at room temperature followed by the addition of 1:200 diluted rabbit polyclonal anti-BDNF antibody in TBS-T with 5% skim milk for overnight at 4 °C. The membranes were washed three times for 5 min each in TBS-T and were incubated 30 min at room temperature with 1:200 diluted biotinylated anti-rabbit antibody in TBS-T with 5% skim milk. After washing three times for 5 min each in TBS-T, the blotted membranes were incubated 30 min at room temperature with avidin-biotinylated horseradish peroxidase complexes (ABC kit) to enhancing signal, followed by washing in TBS-T. The immune complexes were visualized using the ImmPACT™ DAB development and followed by rinsed in distilled water for terminate the reaction.

7.2 Analysis of ERs expression by western blot technique

Aliquots of sample containing 70 µg of proteins were separated by electrophoresis on 10% SDS–PAGE at 90 V for 4 h. The separated proteins were eletrophoretically transferred to a PVDF membrane at 20 V for 2 h. The membranes were treated with rapid blocking solution for 30 min at room temperature and followed by the incubation of 1:400 diluted rabbit polyclonal anti- ERα antibody in TBS-T and 1:500 diluted rabbit polyclonal anti-ERβ antibody in TBS-T with 5% skim

milk for overnight at 4°C. Then the membranes were washed three times for 5 min each in TBS-T and were treated with 1:1000 diluted goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody in TBS-T with 5% skim milk for 2 h at room temperature. After washing, the immune complexes were visualized by using the ImmPACT™ DAB development and followed by rinsed with distilled water for terminate the reaction. The illustration of ERs protein band for each sample was shown in the figure 9.

The intensity of each protein band, BDNF and ERs were normalized to their respective loading control, β-actin protein. For the step detection, the blotted membranes were treated with 5% skim milk in TBS-T for 1 h at room temperature. The 1:500 diluted goat polyclonal anti- β-actin antibody in TBS-T with 5% skim milk were added to incubate for overnight at 4°C and followed by washing three times for 5 min each in TBS-T. After that, the blots were incubated with 1:1000 diluted chicken anti-goat HRP-conjugated secondary antibody in TBS-T with 5% skim milk for 2 h at room temperature and washed three times before immune complexes visualizing. The results of protein expression were processed by developing with ImmPACT™ DAB substrate.

The intensity of protein bands were quantified by using Image J software for densitometric analysis and normalized to its respective loading control.

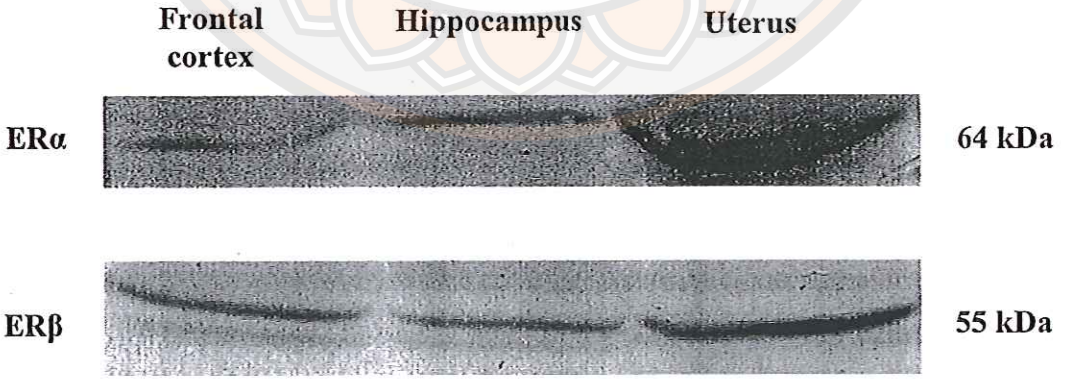
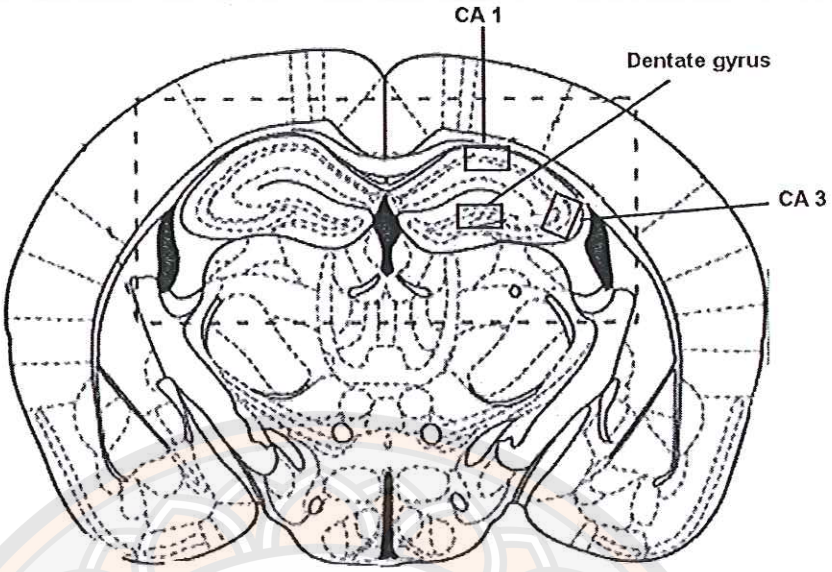


Figure 9 Illustration of ERs protein band of each sample by using western blotting. Uterus tissue was used to indicate actually protein band for ER subtypes expression.

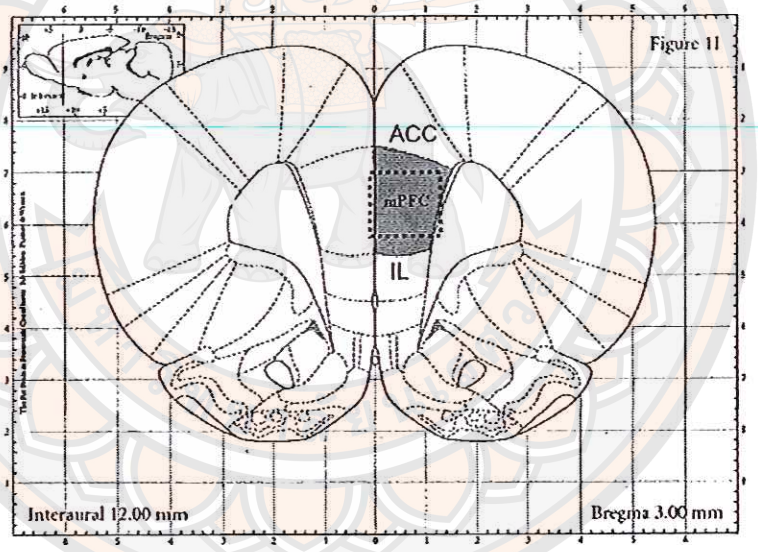
8. Histological analysis

Four brains per group were used to determine the morphological changes. Formalin-fixed brains were dehydrated in ethanol series and embedded in paraffin wax. Each brain block was coronal sectioned at 5 μm thickness and used one out of every 5 sections to determine the density of intact neurons. After allowing tissue slides to dry, the tissue slides were stained with hematoxylin and eosin (H&E) according to standard protocol. The slides were mounted after staining and covered with slide cover slips and analyzed by light microscope (Nikon Eclipse 80i, Nikon) for histological change. All images were photographed and captured with a digital photo camera that attached to the Nikon Eclipse 80i microscope.

The examination of intact neuronal number was performed by using image analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, USA). The number of intact neurons in the dentate gyrus, CA1 and CA3 sub-regions of hippocampus (approximately -3.14 to 3.30 from bregma) were counted (Figure 10 (A)) in square area 80 x 80 μm^2 under 40x magnifications (Uysal, et al., 2005) and the number of neuron in mPFC (approximately 2.70 to 3.20 from bregma) were counted (Figure 10 (B)) in square area 500 x 500 μm^2 under 20x magnifications (Ni, et al., 1995). The intact neuronal cells were defined as round-shaped, cytoplasmic membrane-intact cells, without any nuclear condensation or distorted aspect (Liu, et al., 2010).



(A)



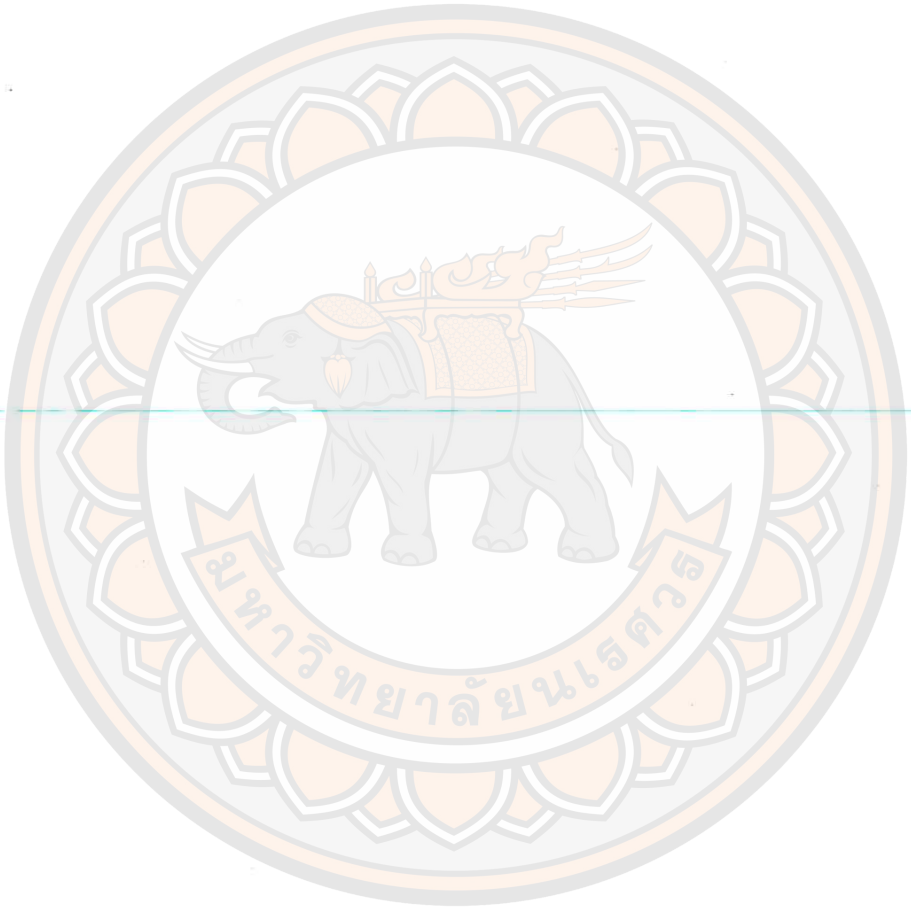
(B)

Figure 10 Schematic representations of coronal sections illustrating the regions selected for quantification of neuronal viability in the CA1, CA3 and dentate gyrus area of hippocampus (A) and mPFC (B)

Source: Paxinos and Watson, 1998

9. Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM) and analyzed using a Statistical Package for the Social Sciences (SPSS) software. Comparison among experimental groups was performed by one-way ANOVA with post hoc LSD test. The statistical significance was determined as P-value less than 0.05.



CHAPTER IV

RESULTS

Effect of AR root extract on NOR test

Learning and memory of animals were investigated after 90 days of administrations using NOR test. The results were expressed as percentage index of preference and recognition index. Statistical analysis revealed that there was not significantly different in the preference index among experimental groups (P=0.808) (Figure 11 (A)) while the recognition index in OVX group was significantly decreased compare to the sham group (P<0.001). However, the OVX received AR root extract 100 and 1000 mg/kg including EE 0.1 mg/kg showed significantly increased the recognition index compared to OVX group (P<0.001, P=0.026 and P<0.001, respectively) (Figure 11 (B)).

Effect of AR root extract on serum estradiol

The effects of AR on estradiol in serum were determined by ECLIA. The results were expressed as mean ± SEM (Figure 12). The serum estradiol concentration was significantly decreased in OVX group (13.13 ± 1.95 pg/ml) compared to sham group (22.91 ± 5.63 pg/ml, P=0.035). However, the significant difference of the serum estradiol were not observed among OVX, OVX+AR100 (12.71 ± 1.97 pg/ml, P=0.924), OVX+AR1000 (13.77 ± 1.51 pg/ml, P=0.887) and OVX+EE (16.09 ± 2.55 pg/ml, P=0.507).

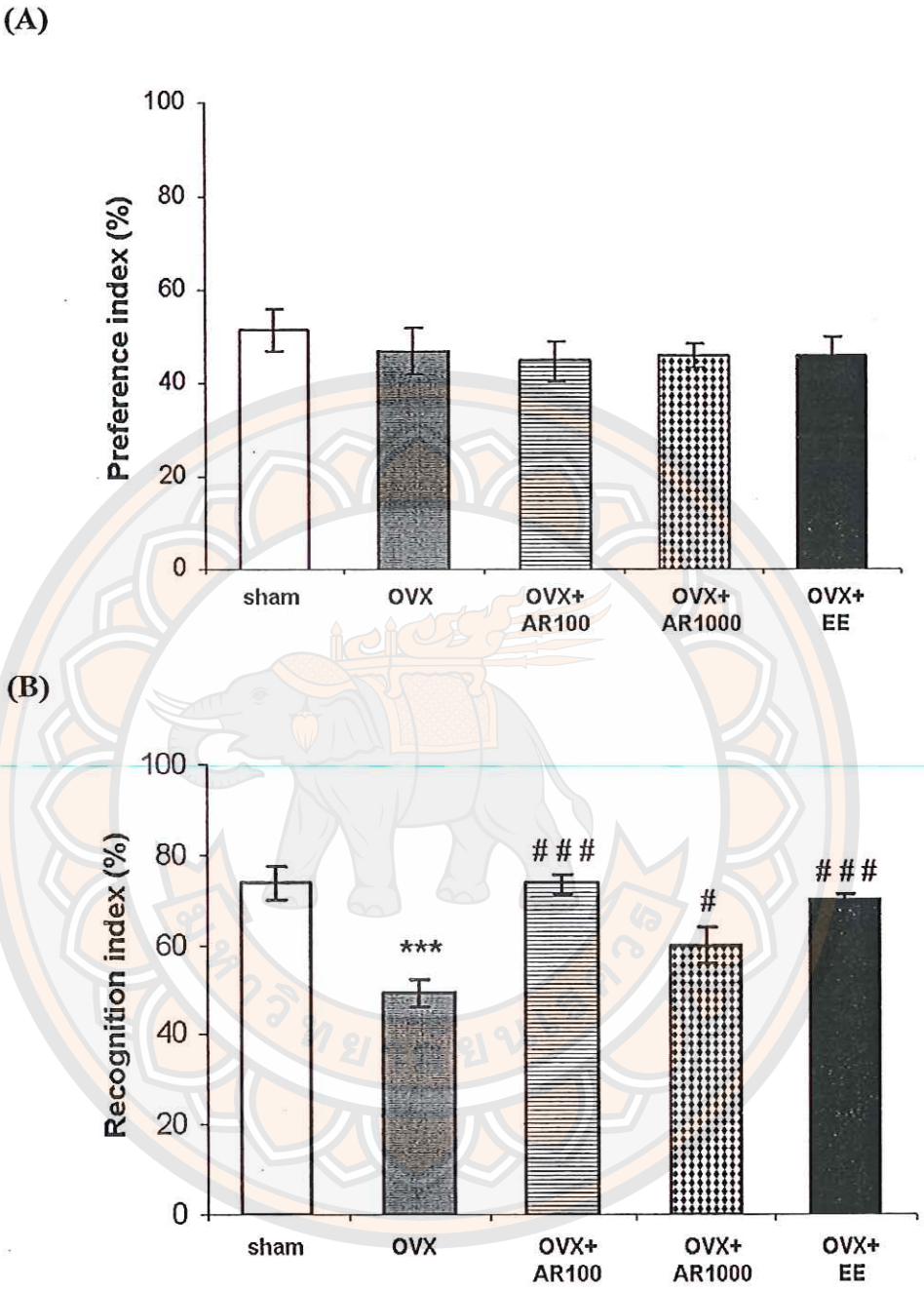


Figure 11 Effects of the AR root extract and EE on recognition memory of OVX rats were assessed by NOR test after 90 days of administrations. The data are expressed as percentage of preference index (A) and recognition index (B). Each histogram bar is expressed as mean ± S.E.M. $^{***}P < 0.001$ compared to sham group, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared to OVX (one-way ANOVA with LSD post-hoc test).

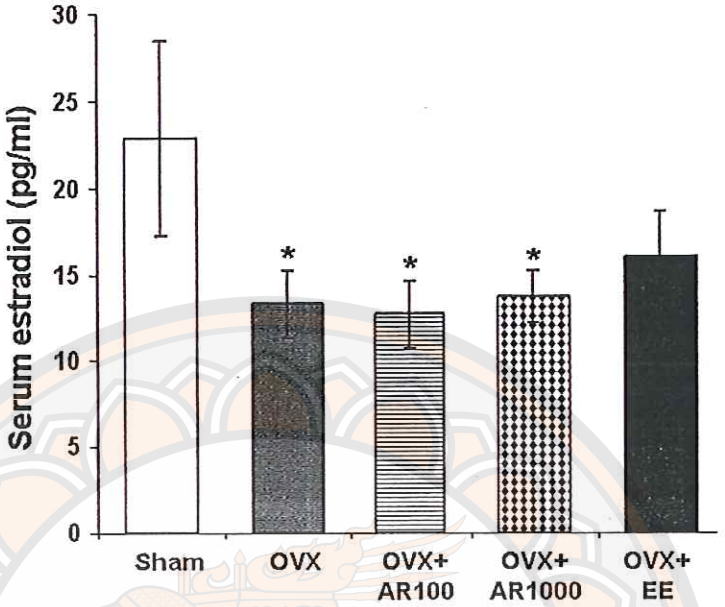


Figure 12 Effects of the AR root extract and EE on serum estradiol concentrations were assessed by ECLIA system after 90 days of administrations. Each histogram bar is expressed as mean ± S.E.M. *$P < 0.05$ compared to sham (one-way ANOVA with LSD post-hoc test).

Effect of the AR root extract on ER subtypes and BDNF protein expression

To investigate the effects of AR root extract on ER subtypes and BDNF protein expression, brain hippocampus and frontal cortex were determined by western blot analysis. Protein expression was determined by normalized the band intensity with β-actin. The control level of protein expression was considered as 100 % and the treated levels were calculated as relative percentages for each experiment.

Effect of the AR root extract on ER subtypes protein expression

The expression of hippocampal ERα protein in the OVX group was significantly reduced (36.84 ± 6.53%) when compared to the sham group (99.98 ± 25.86%, P=0.029) (Table 1 and Figure 13). The data were revealed that hippocampal ERα protein in the OVX+AR1000 and OVX+EE groups were 94.91 ± 22.65% and 131 ± 22.18%, respectively. In comparison with OVX, administration of AR 1000 mg/kg B.W. (P=0.043) and EE 0.1 mg/kg B.W. (P=0.002) were significantly increased the hippocampal expression of ERα protein. The level of ERα protein was higher in the hippocampal tissue of OVX+AR 100 (85.20 ± 9.47%), although not significant, than that OVX (P=0.087).

In the frontal cortex, ERα protein expression in OVX (36.70 ± 12.38%) was significantly decreased when compared with sham group (100.00 ± 9.17%, P=0.007). The ERα protein in OVX+AR1000 (100.22 ± 26.49%) was significantly higher than that in OVX group (P=0.006) although a significant increasing in the ERα protein was not detected in OVX+AR100 (48.91± 6.44%, P=0.566) and OVX+EE (72.55 ± 10.52%, P=0.102) compared to OVX group (Table 1 and Figure 14).

For the quantitative analysis of ERβ protein in the hippocampus, the data showed a significant differences among experimental groups (P=0.006). Post hoc analysis revealed that ERβ protein expression in OVX (48.54 ± 8.66%) was significantly lower than in sham (100.00 ± 9.88%, P=0.001) while the protein expression obtained form OVX+AR100 (100.20 ± 9.06%, P=001), OVX+AR1000 (83.66 ± 9.50%, P=0.019) as well as OVX+EE (95.17 ± 11.26%, P=0.003) were significantly increased in the ERβ protein compared with those in OVX group (Table 2 and Figure 15).

The quantitative analysis of ERβ protein expression in the frontal cortex, was also shown a significant difference among experimental groups (P=0.008). The

data revealed that the ERβ protein level in OVX was significantly reduced to 72.50 ± 4.51% compared to sham group (99.98 ± 7.85, P=0.002). However, the expression of ERβ protein in OVX+AR100 (101.38 ± 5.99%, P=0.001), OVX+AR1000 (90.93 ± 3.92%, P=0.025) as well as OVX+EE (92.51 ± 3.30%, P=0.015) were significantly increased when compared with the OVX group (Table 2 and Figure 16).

Table 1 Effect of the AR root extract on ERα protein expression

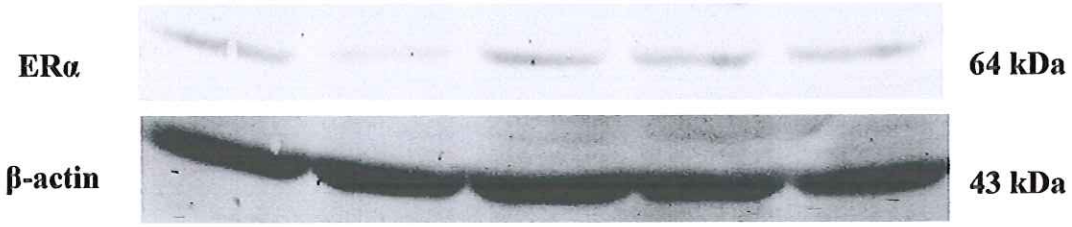
Group	Relative protein level of ERα/β-actin	
	Hippocampus	Frontal cortex
Sham	0.48 ± 0.12	0.80 ± 0.07
OVX	0.17 ± 0.03*	0.29 ± 0.10**
OVX+AR100	0.41 ± 0.04	0.39 ± 0.11
OVX+AR1000	0.43 ± 0.10$^{\#}$	0.80 ± 0.21$^{\#\#}$
OVX+EE	0.63 ± 0.10$^{\#\#}$	0.57 ± 0.08

Note: The data are represented as mean ± S.E.M. *$p < 0.05$ and **$p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$compared to OVX (one-way ANOVA with LSD post-hoc test).

Table 2 Effect of the AR root extract on ERβ protein expression

Group	Relative protein level of ERβ/β-actin	
	Hippocampus	Frontal cortex
Sham	1.79 ± 0.17	2.33 ± 0.18
OVX	0.87 ± 0.15**	1.69 ± 0.10*
OVX+AR100	1.79 ± 0.16$^{\#\#}$	2.36 ± 0.13$^{\#}$
OVX+AR1000	1.50 ± 0.17$^{\#}$	2.12 ± 0.09$^{\#}$
OVX+EE	1.70 ± 0.20$^{\#\#}$	2.16 ± 0.07$^{\#}$

Note: The data are represented as mean ± S.E.M. *$p < 0.05$ and **$p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$compared to OVX (one-way ANOVA with LSD post-hoc test).



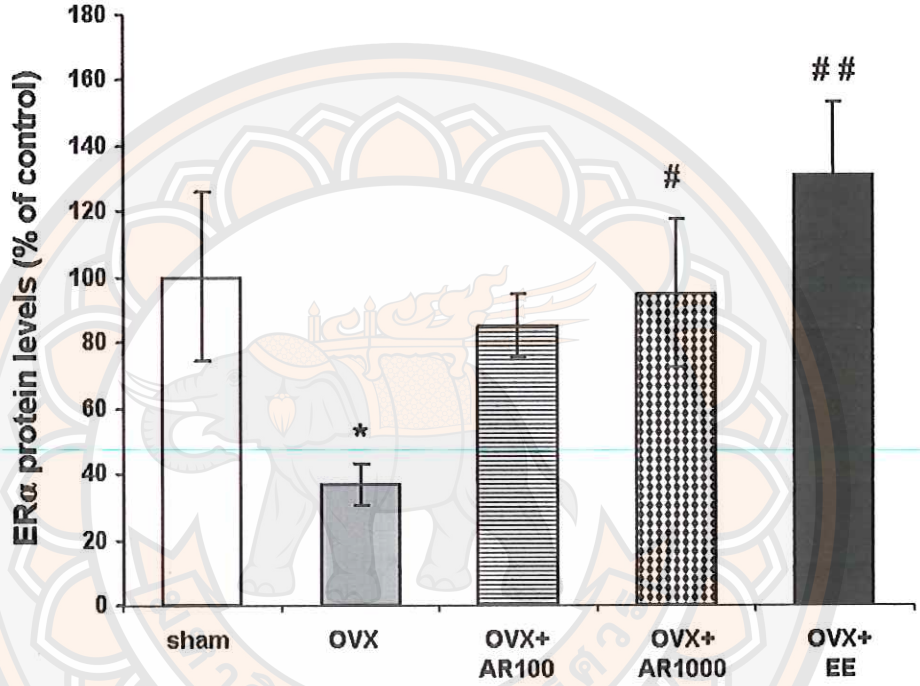


Figure 13 Effect of the AR root extract on ERα protein levels in the hippocampus were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. *$p < 0.05$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).

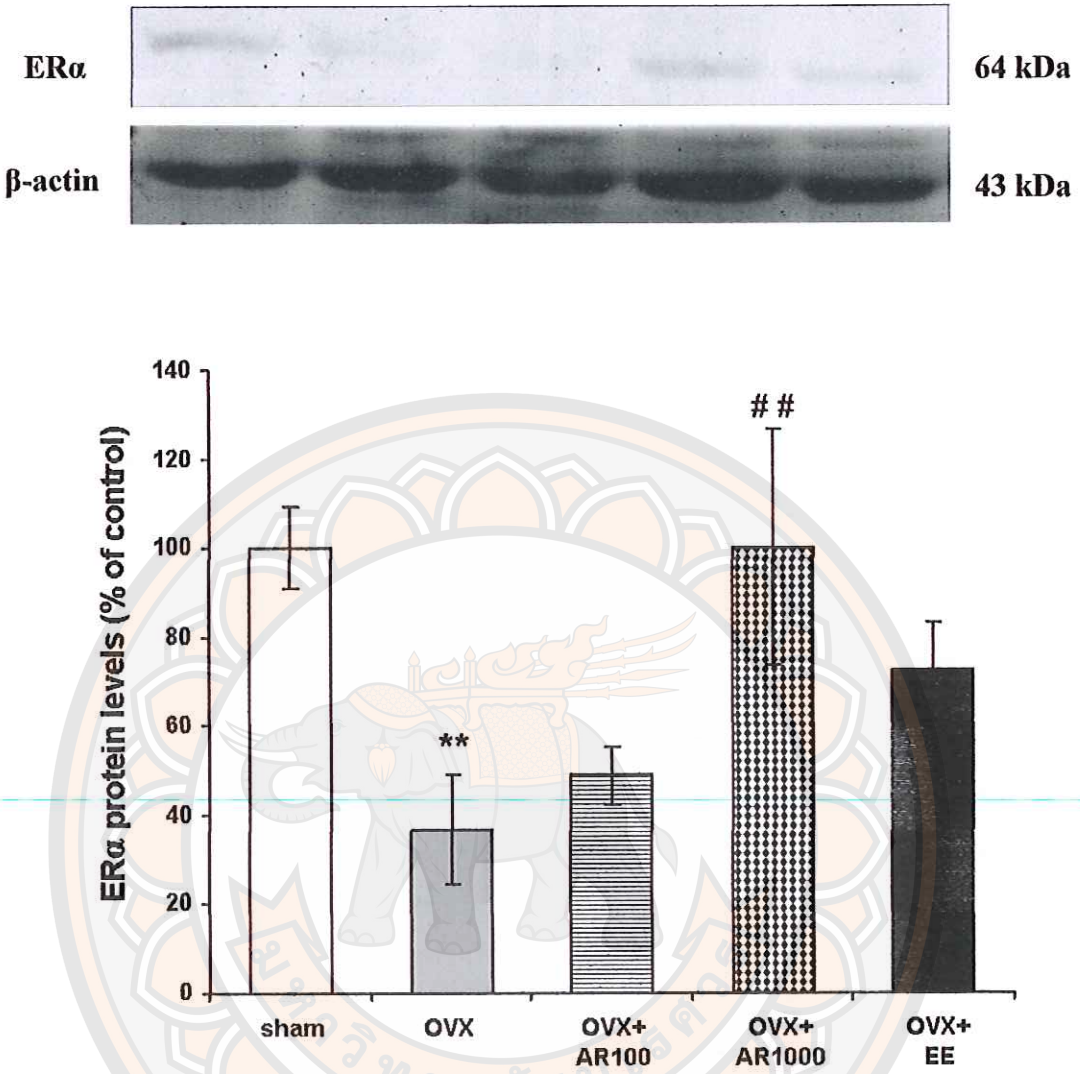


Figure 14 Effect of the AR root extract on ERα protein levels in the frontal cortex were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. **$p < 0.01$ compared to sham, $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).

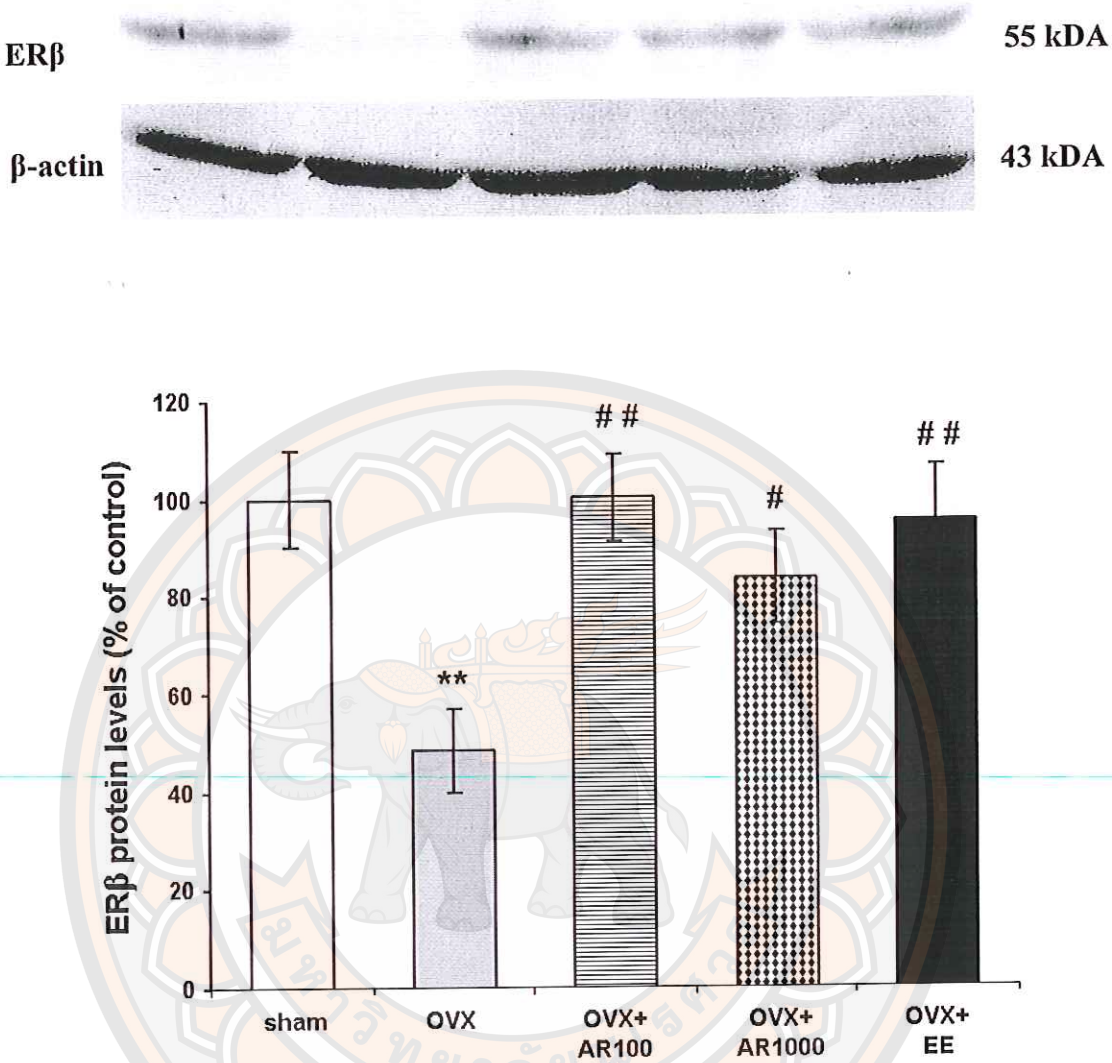
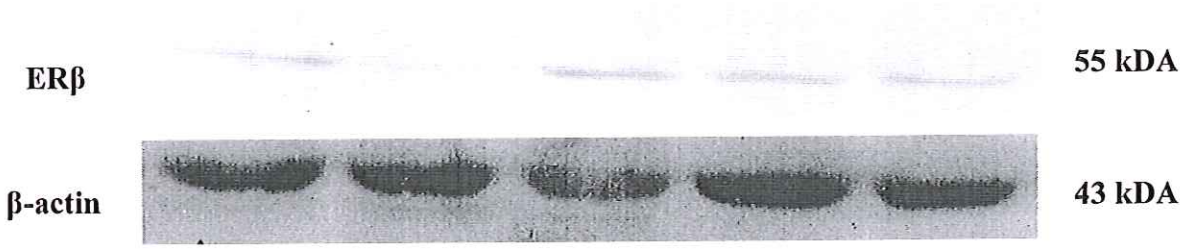


Figure 15 Effect of the AR root extract on ERβ protein levels in the hippocampus were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. **$p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).



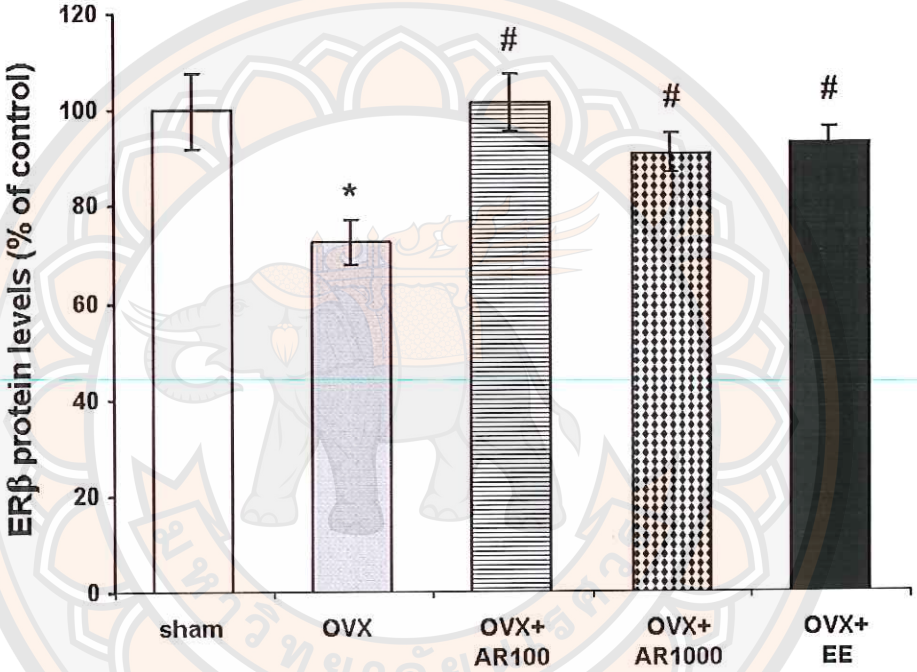


Figure 16 Effect of the AR root extract on ERβ protein levels in the frontal cortex were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. *$p < 0.05$ compared to sham, $^{\#}p < 0.05$ compared to OVX (one-way ANOVA with LSD post-hoc test).

Effect of the AR root extract on BDNF protein expression

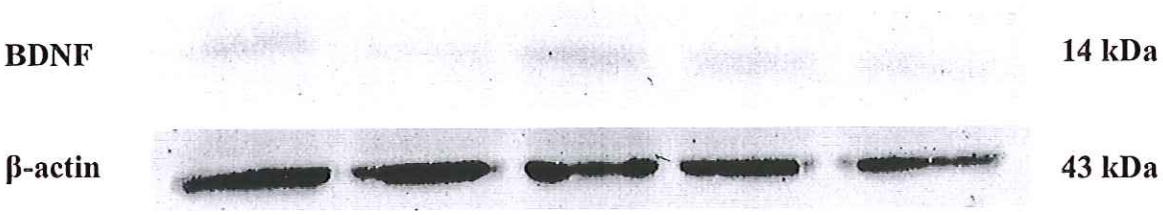
In the hippocampus, the results demonstrated that BDNF protein level in the OVX was significantly reduced to 23.17 ± 8.94% compared with the sham group (100.00 ± 20.40%, P=0.001). However, the BDNF protein level in OVX+AR100 (84.65 ± 12.16%, P=0.005), OVX+AR1000 (67.54 ± 16.06%, P= 0.040) OVX+EE (71.40 ± 8.78%, P=0.036) were significantly increased when compared with those in the OVX group (Table 3 and Figure 17).

For the expression of BDNF protein in the frontal cortex, the statistical analysis showed that there were significant differences among experimental groups (P=0.009) (Table 3 and Figure 18). The data were revealed that BDNF protein was significantly decreased in the OVX to 38.51 ± 7.99% compared with the sham (99.99 ± 5.53%, P=0.001). The levels of BDNF protein in OVX+AR100, OVX+AR1000 and OVX+EE were 53.61 + 7.4%, 70.76 + 6.59% and 86.02 + 21.74%, respectively. The comparison analysis showed that a significant increasing in the BDNF protein levels was observed in the OVX+EE (P=0.009), although there was no significant differences in OVX+AR100 (P=0.366) and OVX+ AR1000 (P=0.062) compared to those in the OVX group.

Table 3 Effects of the AR root extract on BDNF protein expression

Group	Relative protein level of BDNF/β-actin	
	Hippocampus	Frontal cortex
Sham	0.96 ± 0.19	1.02 ± 0.05
OVX	0.22 ± 0.08**	0.39 ± 0.08**
OVX+AR100	0.81 ± 0.11$^{\#\#}$	0.51 ± 0.07
OVX+AR1000	0.64 ± 0.15$^{\#}$	0.68 ± 0.06
OVX+EE	0.67 ± 0.07$^{\#}$	0.88 ± 0.22$^{\#\#}$

Note: The data are represented as mean ± S.E.M. $^{**}p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$compared to OVX (one-way ANOVA with LSD post-hoc test).



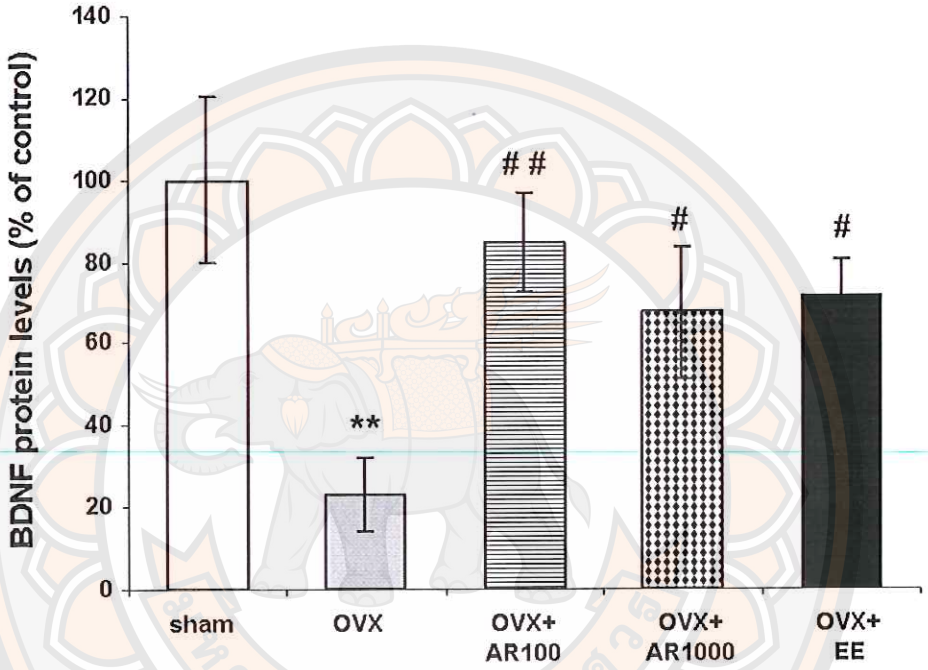


Figure 17 Effect of the AR root extract on BDNF protein levels in the hippocampus were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. **$p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).

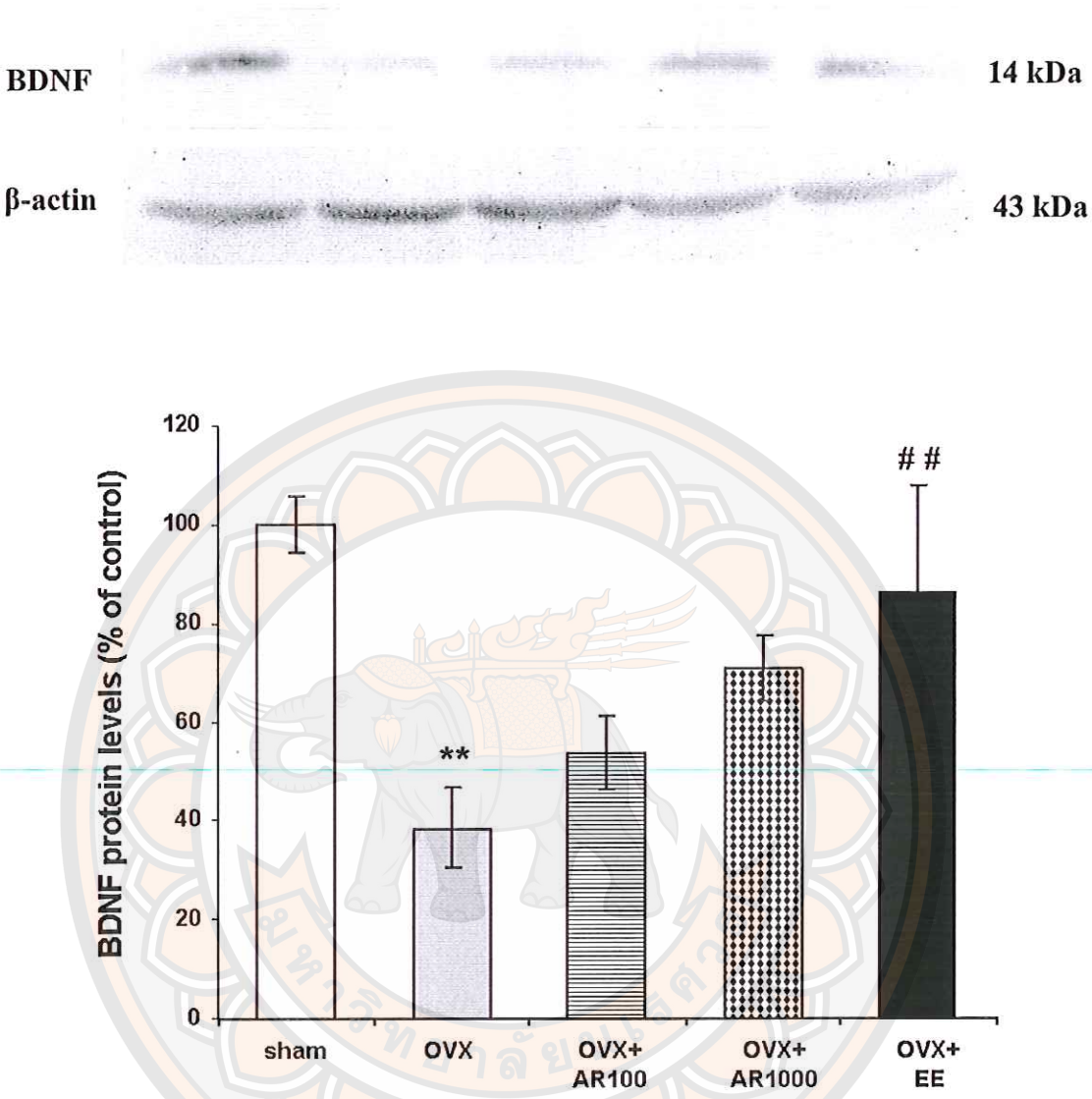


Figure 18 Effect of the AR root extract on BDNF protein levels in the frontal cortex were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. **$p < 0.01$ compared to sham, $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).

Effect of the AR root extract on histological changes

The photomicrograph of H&E staining sections revealed the morphology of intact neurons in the CA1 (Figure 19), CA3 sub (Figure 20) and dentate gyrus sub-regions (Figure 21) of hippocampus as well as the mPFC (Figure 22). The data were expressed as mean ± SEM within an area 6,400 μm^2 of hippocampal CA1, CA3 and dentate gyrus region and 0.25 mm^2 in mPFC (Table 4).

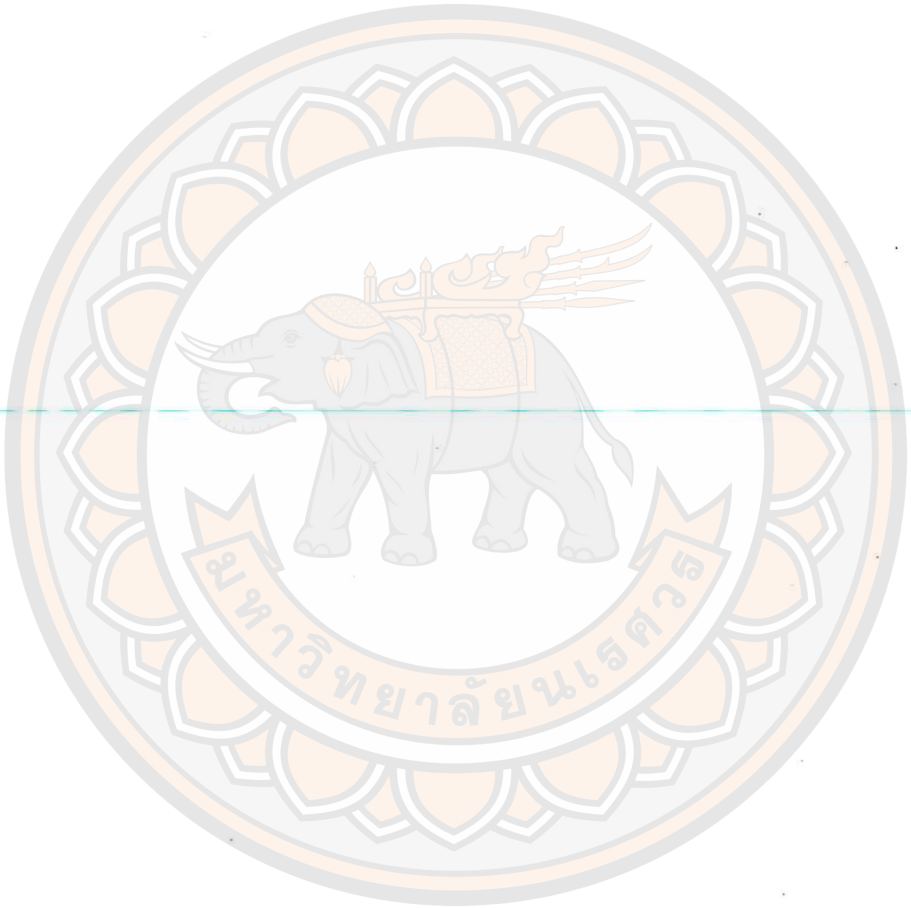
The histological changes of neuronal cells in hippocampal CA1 region were showed no morphological lesion in sham group. Neuronal loss, shrinkage and dark staining of neurons were observed in OVX group. Number of the intact neurons in OVX (17.42 ± 0.31 cells) was significantly reduced compared with the sham (23.47 ± 2.29 cells, P=0.018). The intact neurons in OVX+AR100, OVX+AR1000 and OVX+EE were 22.78 ± 1.1, 19.18 ± 1.28 and 21.8 ± 2.83 cells, respectively. The statistical analysis revealed that administration of AR 100 mg/kg B.W. and EE 0.1 mg/kg B.W. attenuated neuronal loss caused by OVX (P=0.027 and P=0.03, respectively). There was no significant different between OVX+AR1000 and OVX groups (P=0.442).

In the hippocampal CA3 region, the results showed that number of intact neurons in the OVX group (12.6 ± 0.81 cells) was significantly decreased when compared with the sham (18.7 ± 2.02 cells, P=0.004). The intact neurons in the OVX+AR100, OVX+AR1000 and OVX+EE groups were 18.46 ± 0.7, 18.43 ± 0.23 and 17.20 ± 1.52 cells, respectively. These results established a significant increasing the number of intact neurons in the OVX+AR100 (P=0.006), OVX+AR1000 (P=0.01) as well as OVX+EE (P=0.023) when compared with OVX group.

In dendate gyrus, the intact neurons were counted within area 6,400 μm^2. The statistical analysis revealed that there was not significant differences in the number of intact neurons among experimental groups (P=0.57). The intact neurons in the sham, OVX, OVX+AR100, OVX + AR1000 and OVX + EE groups were 48.87 ± 9.28, 39.96 ± 3.56, 51.83 ± 7.04, 49.4 ± 6.21 and 40.6 ± 1.94 cells, respectively.

For evaluation the effects of the AR root extract on the number of neurons in mPFC, these cells were counted within an area 0.25 mm^2. The neurons in the OVX group (75.9 ± 15.76) was significantly decreased when compared with the sham (161.47 ± 13.24, P<0.001). The intact neurons in the OVX+AR100, OVX+AR1000

and OVX+EE were 124.43 ± 9.62, 154.8 ± 11.54 and 191.86 ± 11.92 cells, respectively. The number of intact neurons in OVX+AR100 ($P=0.014$), OVX+AR1000 ($P<0.001$) as well as OVX+EE ($P<0.001$) was significant higher than OVX.



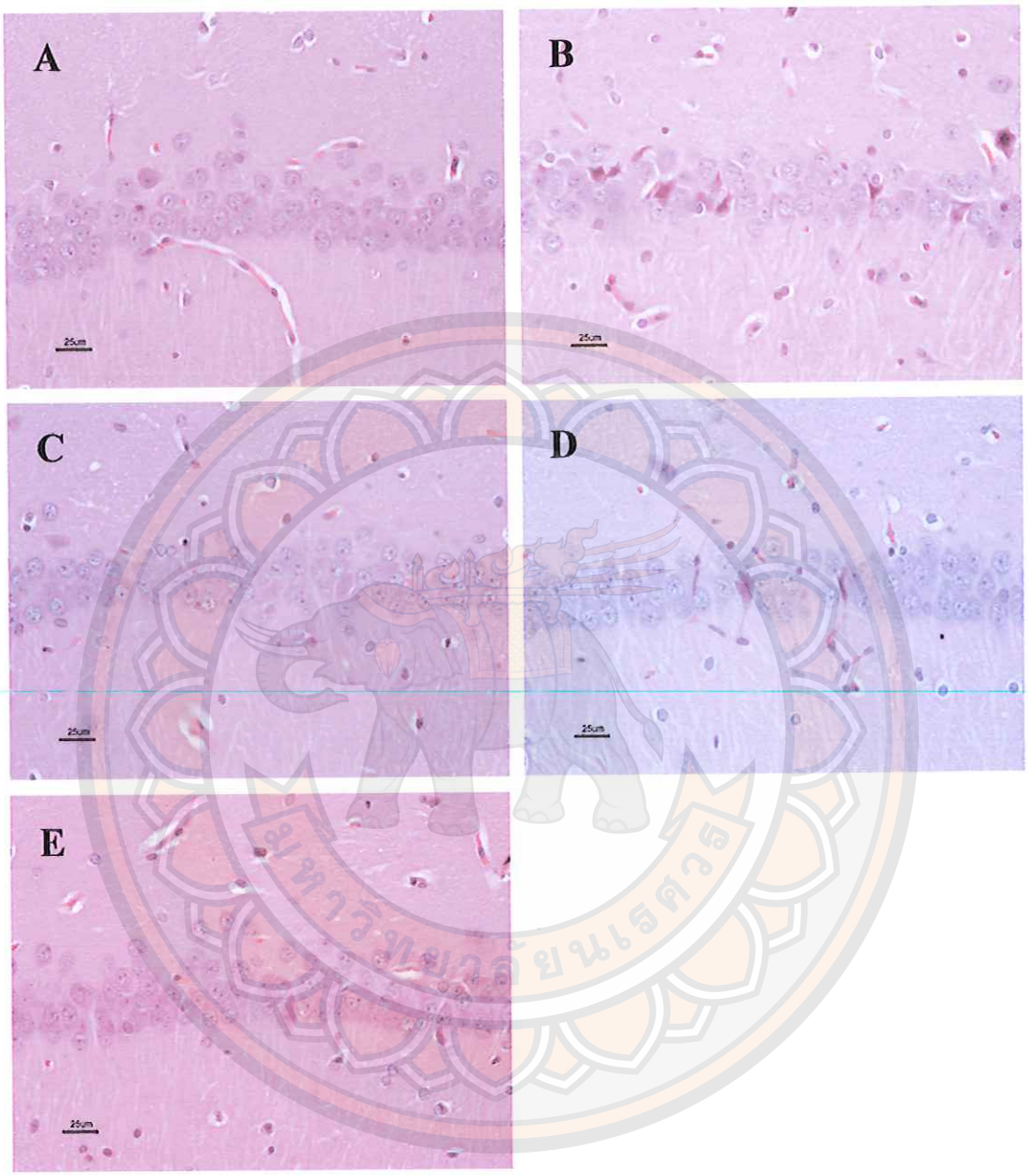


Figure 19 Microscopic photographs of neuronal densities in the hippocampal CA1 sub-region (40X)

Note: Scale bar = 25 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

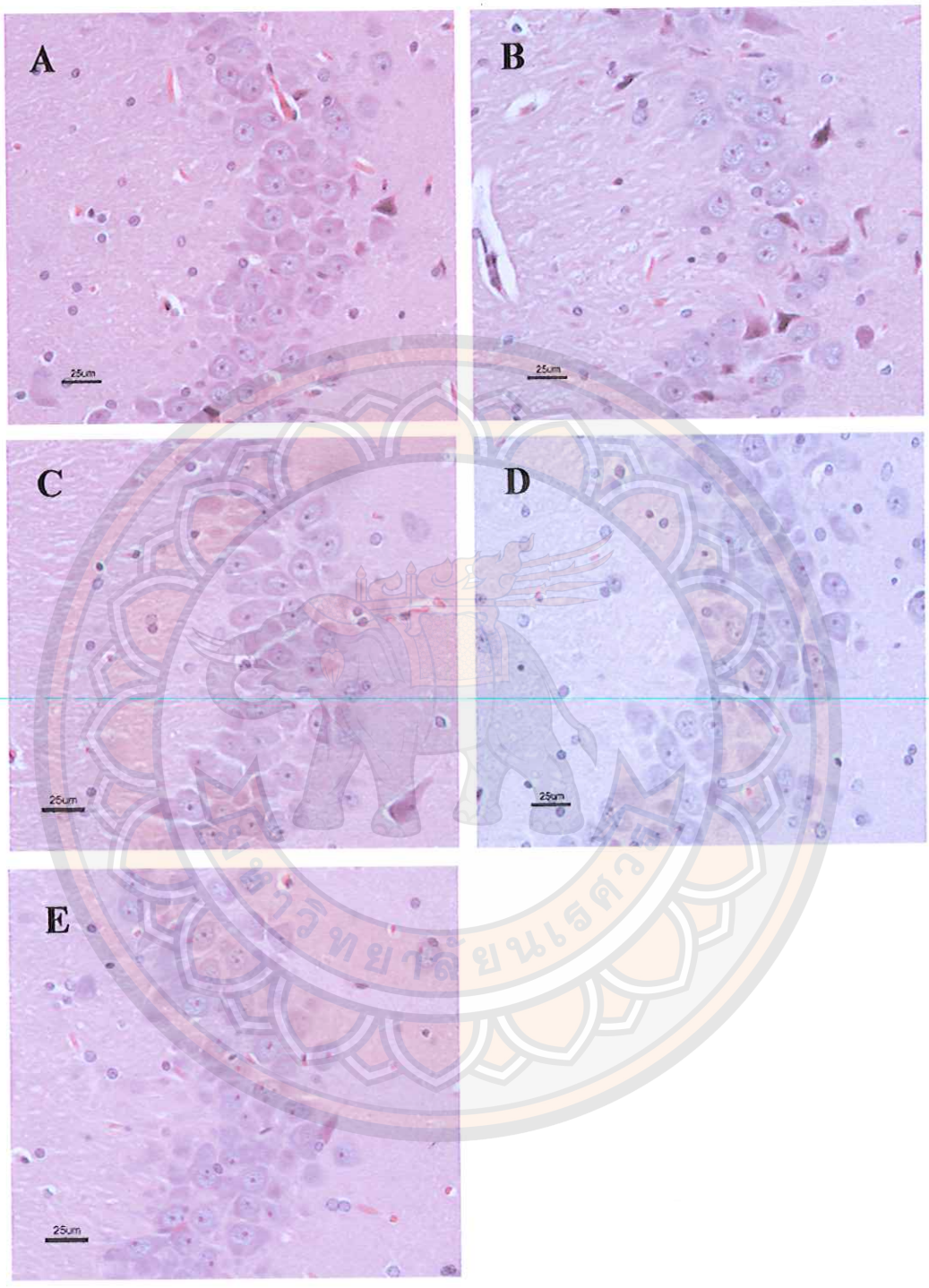


Figure 20 Microscopic photographs of neuronal densities in the hippocampal CA3 sub-region (40X)

Note: Scale bar = 25 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

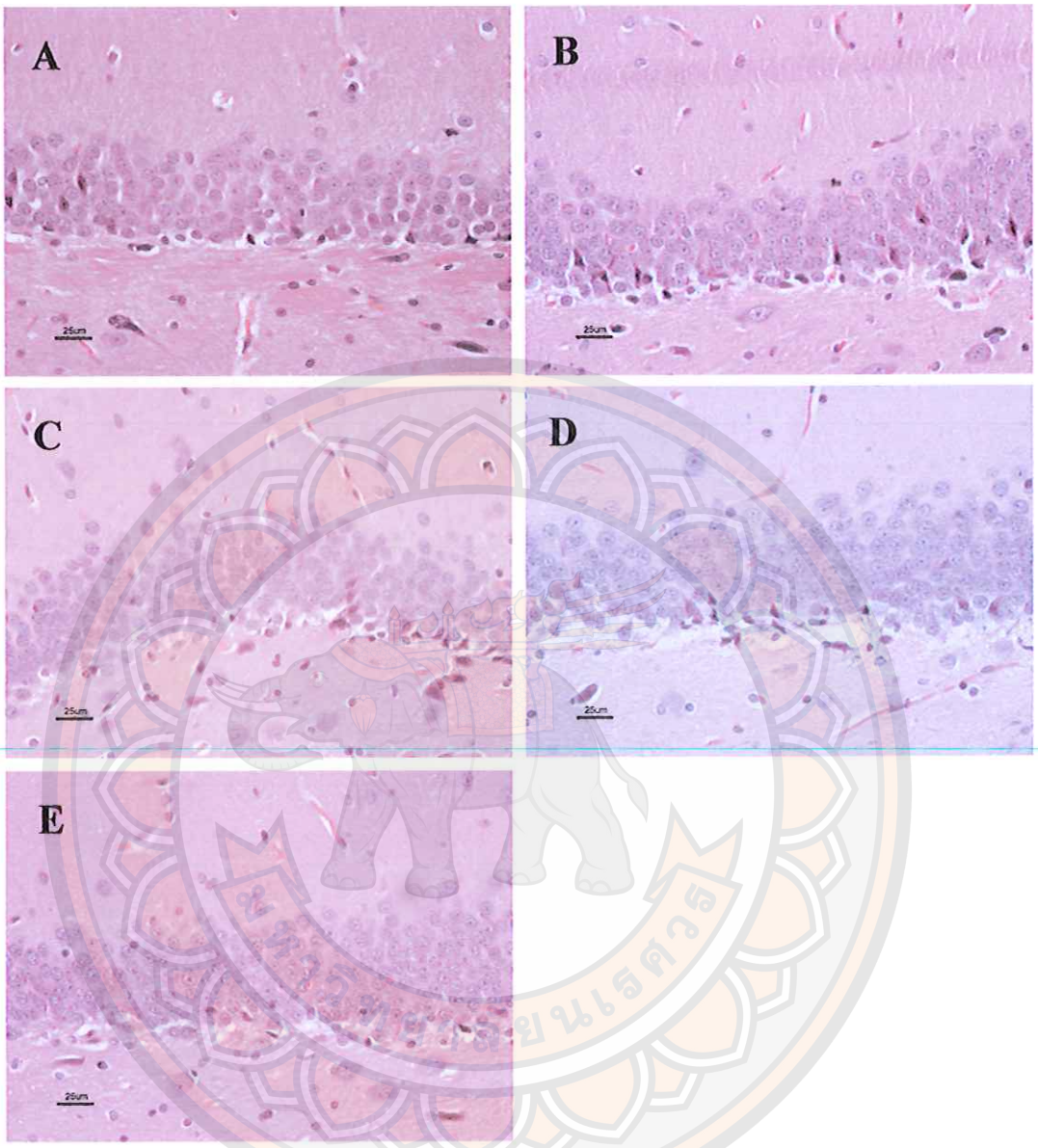


Figure 21 Microscopic photographs of neuronal densities in the dentate gyrus sub-region of hippocampus (40X)

Note: Scale bar = 25 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

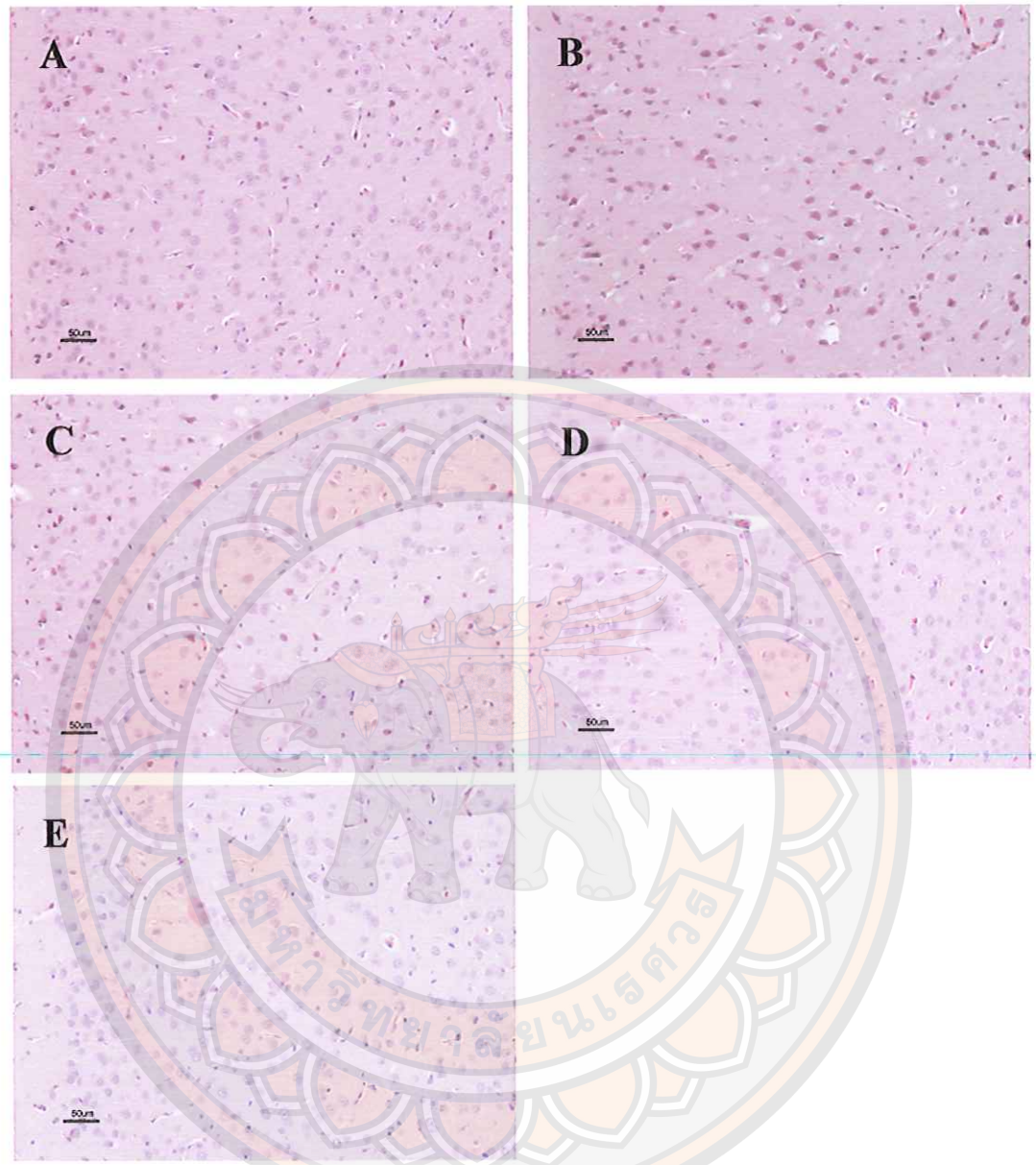


Figure 22 Microscopic photographs of neuronal densities in the medial prefrontal cortex (mPFC) area (20X)

Note: Scale bar = 50 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

Table 4 Effects of the AR root extract on intact neuronal densities

Group	Intact neuronal densities (cells/unit area)			
	CA1	CA3	DG	mPFC
Sham	23.47 ± 2.29	18.70 ± 2.02	48 ± 9.28	161.47 ± 3.24
OVX	17.42 ± 0.31*	12.60 ± 0.81**	39.96 ± 3.56	75.90 ± 15.76***
OVX+AR100	22.78 ± 1.10$^{\#}$	18.46 ± 0.70$^{\#\#}$	51.83 ± 7.04	124.43 ± 9.62$^{\#}$
OVX+AR1000	19.18 ± 1.20	18.43 ± 0.23$^{\#}$	49.40 ± 6.21	154.80 ± 11.54$^{\#\#\#}$
OVX+EE	21.80 ± 2.83$^{\#}$	17.20 ± 1.52$^{\#}$	40.60 ± 1.94	191.86 ± 11.92$^{\#\#\#}$

Note: The numbers of intact neuron were counted within area 6,400 μm^2 for hippocampal CA1, CA3 and dentate gyrus (DG) and 0.25 mm^2 for mPFC. The data are represented as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to sham, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to OVX (one-way ANOVA with LSD post-hoc test).

CHAPTER V

DISCUSSION AND CONCLUSION

Estrogen deprivation in menopause could produce learning and memory impairment including increased risk for neurodegenerative disease such as AD (Gao, et al., 1998). Although ERT has a neuroprotective activity and can reduce undesired condition from menopause in women, it has increased risk of serious side-effects such as endometrial cancer, breast cancer and venous thromboembolic events that limit the usefulness of this agent. (Barrett-Connor and Grady, 1998). Therefore, many studies have looked for the new substances which have higher safety and phytoestrogens are attracted interest as a potential alternative to ERT. AR has phytoestrogenic properties and has been thought to be useful for female rejuvenation (Mayo, 1998). The present study, we considered it is interesting to investigate the neuropretective effects of the AR root extract on the animal model of menopause.

Estrogen has neuroprotective effects in several models such as preventing neuronal cell injury, improving performance in memory task of ischemic-induced behavioral deficit in OVX animals (Wappler, et al., 2010; Raval, et al., 2013) and recovering memory deficit by attenuate brain inflammation in rats (Sun, et al., 2013). Earlier document was also revealed that 7 days post-ovariectomy disturbed the performance of object recognition and post ovariectomy for 4 weeks declined spatial memory in rats (Wallace, et al., 2006). The present study found that after ovariectomy for 3 months induced learning and memory impairment indicated by decreasing the recognition index in the NOR test. This finding provides the supporting evidence that ovariectomy affects on recognition memory. Interestingly, our study found that ethanol AR root extract 100 and 1000 mg/kg B.W. as well as EE 0.1 mg/kg B.W. could reverse OVX-induced learning and memory deficit suggesting that AR root extract has neuroprotective properties against recognition memory deficit caused by ovariectomy. Many studies have shown that AR has neuroprotective effects in several animal models. Ojha, et al. (2010) found that AR showed nootropic and anti-amnesic activities via mediating through augmentation of the cholinergic system by anti-

cholinesterase activity (Ojha, et al., 2010). In addition, a rasayana drug containing AR had adaptogenic activity in animals exposed to different kinds of stressors (Rege, et al., 1999).

Loss of memory is associated with neuronal morphological changes have been recognized by several studies. The depletion of neurons and synapses in brain areas mediating memory, such as hippocampus and prefrontal cortex, is characterization of neurodegenerative disease associated with memory deficit, AD. Some studies suggest that estrogen depletion in brain may be a significant risk factor for AD neurophatology (Yue, et al., 2005; Pompili, et al., 2012). Several earlier information have shown that estrogen is a neuromodulator, which mediates the physiological and neuronal function leading to facilitate memory processing through alternations in spine density (Luine and Frankfurt, 2012). The hippocampus and mFPC have been recognized to implicate the recognition memory in the NOR test (Ennaceur and Delacour, 1988). Our results found that 3 months post OVX induced neuronal cells loss in hippocampus (CA1 and CA3 subfields) and mPFC area. These results are consistent with the previous study showing that the Nissl-positive cells staining of three months OVX displayed the reduction of the neuronal density in rats CA3 and dentate gyrus (Takuma, et al., 2007) as same as the results of Su et al. that reported the reduction of neuronal density in CA3 region including loss the volume of hippocampus and neocortex in 4 months post-ovariectomy which were accompanied by spatial memory deficit (Su, et al., 2012). These finding suggested that recognition memory impairment in ovariectomy rats might be associated with neuronal loss in the brain areas of hippocampus and mFPC.

As discussion above, OVX could decline the percentage of recognition index as well as induced neuronal cell loss in the hippocampus and mPFC area. However, administration for 3 months with AR root extracted (100 and 1000 mg/kg B.W.) could reverse OVX-induced morphological loss in hippocampus and mPFC similar to the EE administration. Similar finding was previously observed, an administration of AR root extract (18 ml/kg, 2 weeks) could improve kainic acid-induced neuronal damage and memory loss in mice suggesting that AR enhanced GPx activity and GSH content which resulted in a protective effect (Parihar and Hemnani, 2004). Moreover, neuroprotective effect of the AR methanolic root extract has also been discussed by

Nandagopal et al. and considered that 200 and 400 mg/kg B.W. AR methanolic root extract administration for 7 days rescued the ischemic-induced neuronal damage in rats by its antioxidant activity (Nandagopal, et al., 2011). However, effects of phytoestrogens are not clearly understood but there is several evidences suggest that phytoestrogens could act through estrogen receptors (Kuiper, et al., 1997; Kuiper, et al., 1998; Matthews, et al., 2000). In our study, AR root extract improved memory impairment and prevented neuronal loss induced by ovariectomy as the effect of EE administration. Therefore, it is considered that AR may exert theses effect by its phytoestrogenic activity to be a neuroprotective effect like an exogenous estradiol. These results provide evidence that ability of AR root extract to reduce neuronal morphological changes associating the improving of recognition memory impairment induced by ovariectomy.

It was mentioned above that estrogen available to mediate morphology and physiology of hippocampus and cortex, enhanced cognitive function, and protect neurons from a various kinds of brain insults (McEwen and Alves, 1999; Takuma, et al., 2007; Pompili, et al., 2012). Estrogen has also been recognized for its roles by involvement of BDNF modulating (Solum and Handa, 2002; Scharfman and MacLusky, 2006; Pluchino, et al., 2013). BDNF is widely known to be a member of neurotrophin family, which exerts its effects due to binding with high affinity receptor, tyrosine kinase receptor B (TrkB), and low affinity receptor, p75 neurotrophin receptor (p75NTR). Binding of BDNF to TrkB activates phosphatidylinositol-3-kinase/ protein kinase B (PI3K/Akt) signaling leading to promote neuronal plasticity and neuronal survival essential to process learning and memory (Thoenen, 2000; Yamada, et al., 2005; Driscoll, et al., 2012; Li, et al., 2012).

Based on our results, three months post OVX decreased BDNF protein levels both in hippocampus and frontal cortex. These results were consistent with the previous studies that revealed ovariectomy attenuated BDNF mRNA in the hippocampus (Takuma, et al., 2007) and cortex (Sohrabji, et al., 1995). As previous mentioned above, estrogen has been recognized for its effective in cognitive function via an involvement of BDNF. The evidences suggesting estrogen affects on BDNF was revealed by *in vitro* study that demonstrated 17β-estradiol increased protein levels of BDNF in hippocampal slice cultures (Aguirre and Baudry, 2009). In addition, the

in vivo studies has also been reported that BDNF mRNA levels are significantly reduced in rat hippocampus and cortex after ovariectomy 28 weeks whereas this effect was abolished by estradiol replacement (Singh, et al., 1995). The extensive evidence has shown that estrogen act on ERs to induce BDNF gene transcription. It is understood for mechanism that binding of estrogen on nuclear ERs triggers ERE in the BDNF promoter on DNA to activate gene transcription. Another way, estrogen acts on extranuclear ERs which may trigger signaling pathways leading to phosphorylation of the CREB protein, resulting to BDNF transcription through a cAMP response element in the BDNF promoter (Luine and Frankfurt, 2013). Effects of estrogen on facilitate the neuronal function associating learning and memory improvement was also confirmed by study of Sato and colleagues. They revealed that estradiol increased the postsynaptic density protein-95 (PSD-95) where the PSD-95 is protein associating synaptic formation and plasticity, and also increased the spine density at proximal sites in hippocampal CA3 area. These results have been suggested that estrogen promotes the synaptogenesis by enhancing BDNF synthesis (Sato, et al., 2007). The findings of our study may indicate that estrogen insufficiency produces neuronal morphological lesion associating the reduction of BDNF protein level leading to learning and memory loss.

In addition, we investigated the effects of AR comparative to EE on the expression of BDNF protein in OVX. We found that AR (100 and 1000 mg/kg B.W.) and EE administration for 3 months significantly prevented the following of BDNF protein level induced by ovariectomy in hippocampus while it tended to increase this protein in frontal cortex. However, our results are consistent with the recent studies that the enhancement of the BNDF mRNA level has occurred after administration of soy germ phytoestrogen (1.6 g/kg) for 12 weeks in hippocampus (Pan, et al., 2010) and 150 mg/kg soy phytoestrogens for 8 weeks in frontal cortex in ovariectomy model (Pan, et al., 1999). Previous studied had reported that isoflavones were actives constituent of AR roots (Saxena and Chourasia, 2001). Moreover, the major active constituent of AR root extract is steroid saponins (Shatavarin I-IV) which has phytoestrogenic effects (Bopana and Saxena, 2007). AR root extract in the present study had been evaluated the total saponins by using enzyme-linked immunosorbent assay (ELISA) which revealed the total saponin was 7.42%. Other study has reported

that ginsenoside Rg1, a steroidal saponin of high abundance in ginseng, were also known for its phytoestrogenic effects (Chan, et al., 2002; Lee, et al., 2003) and it could reverse the corticosterone-induced changes in mRNA levels of BDNF in hippocampal mice (Chen, et al., 2014). In the present study, AR root extract and EE increased BDNF protein levels in the hippocampus and frontal cortex of OVX rats suggesting that AR might exert these effects by depending on phytoestrogenic activity to increase BDNF protein expression. These effects leaded to prevent the morphological changes associating attenuation learning and memory impairment induced by ovariectomy.

Several investigations have extensively considered that fluctuation of circulating estrogen levels have an affect on learning and memory ability. They found that enhancing memory was occurred during highest levels of estrogen in proestrus in rodent and fluctuated across menstrual cycle correlating positively with serum estrogen levels (Frick and Berger-Sweeney, 2001; Rosenberg and Park, 2002; Korol, et al., 2004; Spencer, et al., 2008). Although AR root extract had shown neuroprotective effects similar to exogenous estradiol, it had no effect on serum estradiol concentration in OVX in the present study. Similar to previous study, Menosan, a herbal preparation containing AR and used to improve symptoms of menopause, had uterotrophic activity by increasing the uterine weight without an effect on serum estradiol in OVX rats (Gopumadhavan, et al., 2005). Moreover, our findings also reliable with the previous studies that investigated the effects of U-3107 (the herbal formulation containing AR 6.4%) on uterine weight and serum estradiol level. They found that U-3107 (1 mg/kg) administration for 21 days increased uterine weight in normal rats but not in OVX animals. It has been considered that U-3107 possesses uterotrophic activity only in the presence of a functional ovary (Mitra, 1999).

The present study did not find AR affected on serum estradiol concentration in the OVX but AR could improve recognition memory associating the changes of BDNF protein and neuronal morphology. These results were considered that AR may process its effect to prevent the memory loss in estrogen insufficiency due to another mechanism. Estrogen processes its neuroprotective effect by binding with classical ER subtypes, ERα and ERβ. These receptors are presented in the hippocampus and frontal cortex where these regions specifically associated with recognition memory

(Shughrue, et al., 1997; Towart, et al., 2003). Previous investigation showed that BDNF and TrkB including PSD-95 mRNA were decreased in ERα and ERβ knockout mice (Spencer-Segal, et al., 2012). Therefore, it was considered that both of ERα and ERβ played an important role on hippocampus functions.

The present study demonstrated that down-regulated ERs protein expression in both the hippocampus and frontal cortex was found after 90 days of OVX. We have been obtained several evidences indicating that estrogen affects on the expression of ER subtypes. One of them is the investigation of Romeo and co-workers that showed ERα in the hippocampal dendritic spines in proestrus is higher than in diestrus phase of rats (Romeo, et al., 2005). In addition, the study in the changes of ERs expression in rat brain has considered that ERβ in cortex significantly declined with aging (Wilson, et al., 2002). The mechanisms of estrogen to mediate their receptor status remain unclear. However, there was a suggesting one conserved function of steroid hormone receptors are autoregulation of their own gene expression (Schmidt and Meyer, 1994).

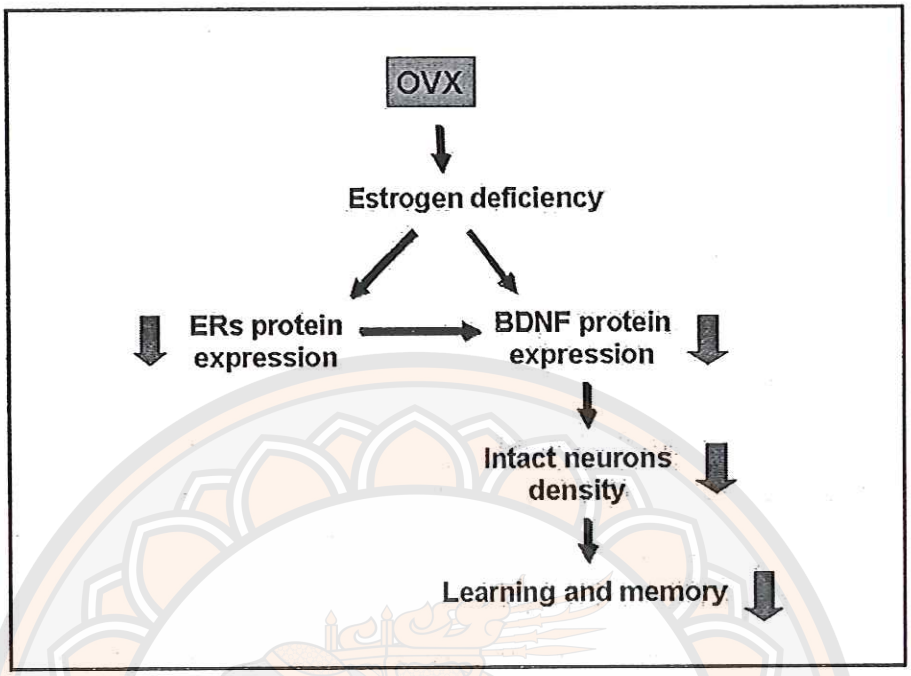
Our result of down-regulation of ERs proteins in OVX was not consistent with the study of Cardoso and colleagues that OVX 15 days produced ERα up-regulation in the rat hippocampus (Cardoso, et al., 2010). However, our findings in accordance with the recently study that demonstrated a decrement of ERα in rat cortex and hippocampus after long-term of ovariectomy (6-24 months) (Navarro, et al., 2012) and an amelioration of ERβ in the brain of 3 months OVX rats (Rose'Meyer, et al., 2003). These results may be considered that a short-term of ovariectomy causes a short period for a lack of circulating estrogen which results in a compensatory up-regulation of ERs for physiological responses. On the other hand, the lack of estrogen for 3 months has supported the idea that long-term of estrogen insufficiency leads to down-regulation their receptors in the brain. Accordingly, Navarro et al also reported that E2 administration abolished OVX-induced ERα decline similar to our findings which EE treatment inhibited the decreasing of ERs. It is reasonable to support that estrogen play an important role against ERs down-regulation in brain.

In the present study, ethanol AR root extract (1000 mg/kg B.W.) could prevent the decreasing of ERα protein in hippocampus and frontal cortex as the effect of EE. Furthermore, it was also revealed that ethanol AR root extract (100 and 1000 mg/kg B.W.) restored a down-regulation of ERβ in OVX as same as EE in both hippocampus

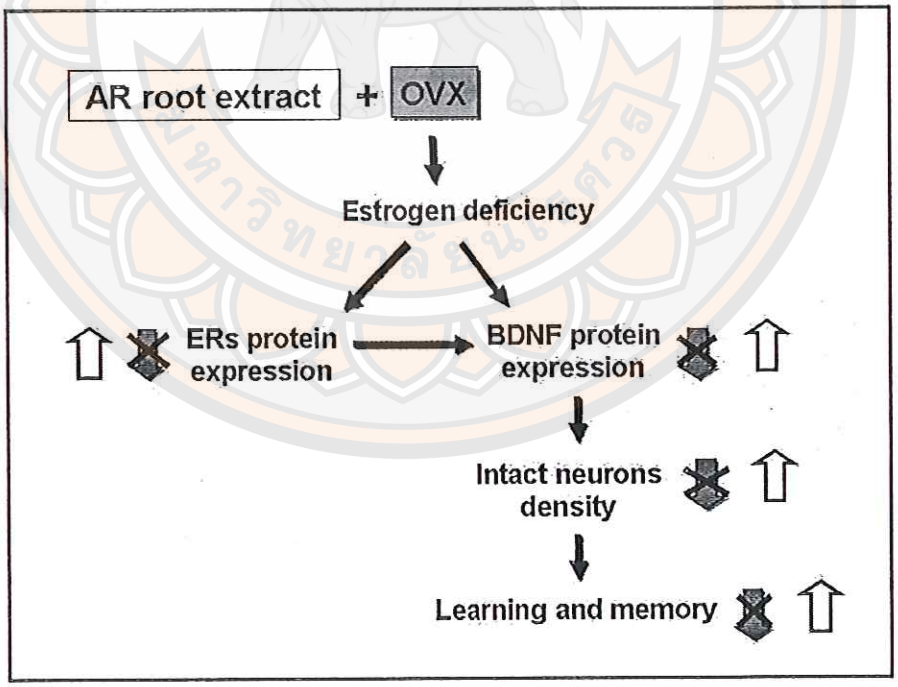
and frontal cortex. Phytoestrogenic properties of AR are wildly known and used as a hormonal modulator in a herbal stimulant health tonic for women (Saxena, et al., 2010). Although, phytoestrogenic effects are poorly understood, it could act through estrogen receptors (Kuiper, et al., 1997; Kuiper, et al., 1998; Matthews, et al., 2000). Previous study has reported that coumestrol, one class of phytoestrogens, could mediate the expression of ERβ in the paraventicular nuclease of hypothalamus (Patisaul, et al., 1999). In addition, previous study exhibited that dietary intake of phytoestrogens up-regulated ERα in breast tumor cell in premenopausal women (Touillaud, et al., 2005). As our results, ethanol AR root extract protected down-regulation of ERs. It was explained that AR may mediate its activities through ERs binding and trigger the ERs expression to process the protective effects in the OVX.

Summary and conclusion

As the results, the present study demonstrated that OVX produced learning and memory impairment associated neuronal cell loss. These changes related to ameliorate of estradiol levels including the expression of BDNF and ERs proteins. These results in the present study were considered that OVX induced the downregulation of ERs proteins which resulted in reduction of BDNF protein expression. This decrement might cause neuronal injury and death in the hippocampus and mPFC where associated with recognition process (Figure 23 (A)). However, the administration of the ethanol AR root extract could obviate the OVX-induced learning and memory loss as well as neuronal damage in hippocampus and mPFC. Although, AR had no effect on estradiol levels, it was found to ameliorate the reduction of ERs and BDNF protein expression induced by OVX. Therefore, these results suggest that protection of ethanol AR root extract against OVX-induced learning and memory loss was accounted by its ability to increase the expression of ERs and BDNF proteins which were diminished by OVX (Figure 23 (B)). However, further investigations are required to understand the possible involvement of another mechanism for improving property of AR.



(A)



(B)

Figure 23 The mechanism of OVX on learning and memory (A) and the protective effect of ethanol AR root extract against learning and memory impairment (B)

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APPENDIX

APPENDIX

List of solutions and buffers

30% Monomer

29.2%	Acrylamide
0.8%	Bisacrylamide

Separating gel buffer

1.5 M	Tris-HCl pH 8.8

Stacking gel buffer

0.5 M	Tris-HCl pH 6.8

2X-Laemmli sample buffer

0.5 M	Tris-HCl pH 6.8
0.3%	Bromophenol blue
20%	Glycerol
0.4%	SDS
20 mM	DL-Dithiothreitol (DDT)

Running buffer

25 mM	Tris base
192 mM	Glycine
0.1%	SDS

Transfer buffer

25 mM	Tris base
192 mM	Glycine
0.1%	SDS
20%	Methanol

Coomassie blue staining solution

45%	Methanol
10%	Acetic acid
0.25%	Coomassie Blue R-250

Destaining coomassie blue solution

25%	Methanol
10%	Acetic acid

Tris-buffer saline pH 7.4

25 mM	Tris base
140 mM	NaCl
3 mM	KCl

10% Separating gel (15 ml)

5 ml	30% Monomer
6 ml	Distilled water
3.75 ml	Separating gel buffer
0.15 ml	10% SDS
0.15 ml	10% APS
0.015 ml	TEMED

12.5% Separating gel (15ml)

6.24 ml	30% Monomer
4.74 ml	Distilled water
3.75 ml	Separating gel buffer pH 8.8
0.15 ml	10% SDS
0.15 ml	10% APS
0.015 ml	TEMED

4% Stacking gel (5 ml)

0.668 ml	30% Monomer
3.58 ml	Distilled water
0.625 ml	Stacking buffer pH 6.8
0.05 ml	10% SDS
0.05 ml	10% APS
0.005 ml	TEMED

4% Formaldehyde (1000 ml)

100 ml	35-40% Formaldehyde
4 g	NaH_2PO_4
6.5 g	$Na_2HPO_4.H_2O$