SCREENING OF THE CENTRAL NERVOUS SYSTEM ACTION OF AGARWOOD LEAVES EXTRACT IN FEMALE OVARIECTOMIZED RATS

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Thesis entitled "Screening of the central nervous system action of agarwood leaves extract in female ovariectomized rats"

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has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science Degree in Physiology Program of Naresuan University

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ABSTRACT

Agarwood leaves tea has been consumed among people in the Southeast Asia and Oceania countries as a medicinal herb for sedative, antinociceptive, antihyperglycemic and laxative purposes. Agarwood contain alkaloids, saponins, steroids, terpenoids, tannins, flavonoids and phenolic compounds. In addition, antioxidant properties of agarwood leaves extracts (AS) have been reported. We, therefore, interested in investigating actions of AS on the central nervous system (CNS) function particularly on learning and memory which has been shown to be enhanced by antioxidant-rich foods. In this study, sixty ovariectomized (OVX) rats were used as animal model of amnesia and were divided into 6 groups as 1) OVX (control-OVX) group, 2) sham control group, 3) OVX + AS (100 mg/kg B.W.), 4) OVX + AS (1000 mg/kg B.W.), and 5) OVX + diazepam (2.5 mg/kg B.W.), and 6) OVX + donepezil (5 mg/kg B.W). After 2 months period of AS administration, rats were subjected to behavioural testing including an open field locomotor activity test, the elevated plus maze test, the analgesic tests (i.e. hotplate analgesic and tail-flick analgesic tests), the barbiturate potentiation test, and the cognitive function tests (i.e., Novel Object Recognition (NOR) and Morris Water Maze (WMW) tests). We found that the AS-treated groups exhibited a) a significant reduction in spontaneous locomotion, b) increases in anti-anxiety and anti-nociceptive activities, c) a marked potentiation of barbiturate induced sleeping time and d) an improvement of recognition and spatial memories when compared to the control

group. These results suggest that agarwood is a promising potential natural source for treatment of cognitive deficits. Further investigation is warranted to uncover the mechanism(s) of action of agarwood leaves extract and its active ingredient(s) responsible for the effects on CNS.

LIST OF CONTENTS

Chapter | Page

I INTRODUCTION 1

Rational and significant of the study 1

Main objectives 4

Specific objectives 4

The scope of the study 4

Preliminary Agreement 4

Keywords 5

Hypotheses 5

The anticipated outcomes of the study 5

II REVIEW OF RELATED LITERATURE AND RESEARCH 6

Amnesia 6

Neuropathology of amnesia 6

Animal model of amnesia 7

Treatment of amnesia 9

Estrogen effect on learning and memory 11

Agarwood 14

III RESEARCH METHODOLOGY 17

Chemicals and reagents 17

Materials and research instrument 18

Method 20

Statistical analysis 46

LIST OF CONTENTS (CONT.)

Chapter **Page**

IV RESULTS 47

Screening of the CNS action of agarwood leaves extract 47

Acute effect of agarwood leaves extract on locomotor activity 47

Screening of acute anxiolytic effect of agarwood leaves extract 50

Screening of acute anti-nociceptive effect of agarwood leaves extract 51

Screening of barbiturate potentiation efficacy of agarwood leaves extract 53

Screening of cognitive effect of agarwood leaves extract 54

Serum estrogen analysis 57

Assay for brain neurotransmitters 58

V DISCUSSION AND CONCLUSIONS 65

Summary results of the present study 70

Behavior tests 70

Biochemical tests 71

REFERENCES 72

APPENDIX 87

BIOGRAPHY 99

LIST OF TABLE

Table		Page

1 % yields and % mangiferin contents in the different lots of *A. subintegra* leaves extract 21

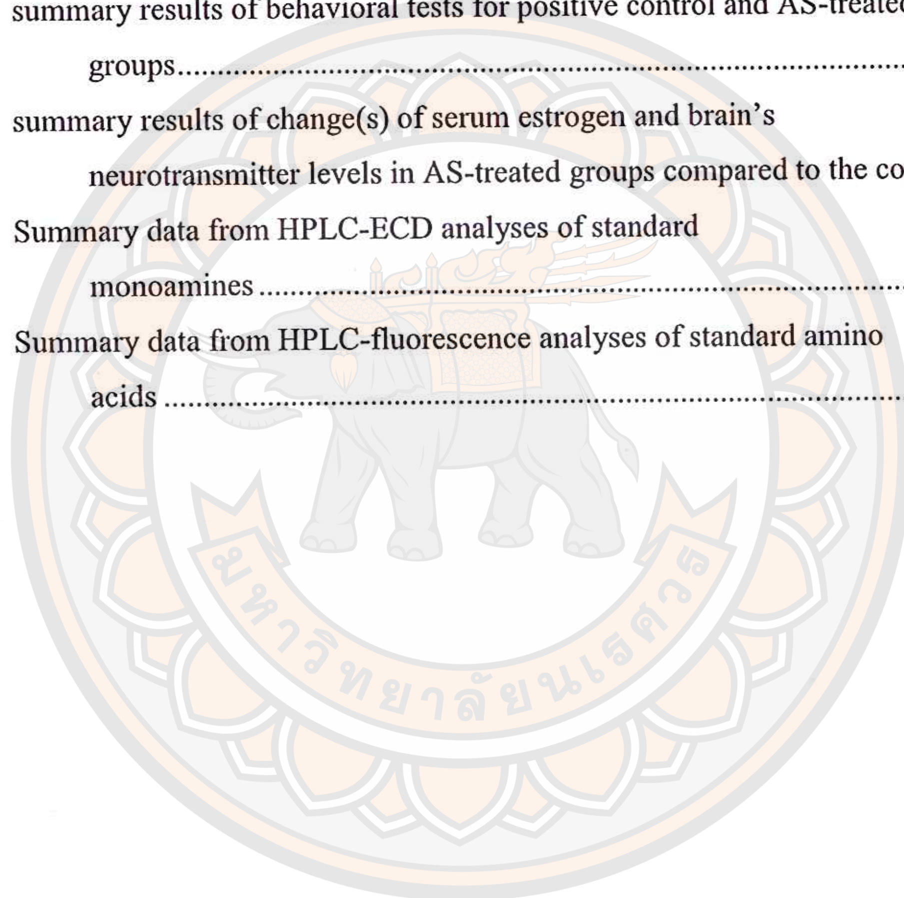
2 Preparation of diluted albumin standards 44

3 summary results of behavioral tests for positive control and AS-treated groups 70

4 summary results of change(s) of serum estrogen and brain's neurotransmitter levels in AS-treated groups compared to the control 71

5 Summary data from HPLC-ECD analyses of standard monoamines 95

6 Summary data from HPLC-fluorescence analyses of standard amino acids 96



LIST OF FIGURES

Figure **Page**

1 Molecular structure of (A) donepezil, (B) rivastigmine, (C) memantine, (D) galantamine 10
2 Chemical structure of estrogen 11
3 Estrogen synthesis in brain 12
4 Estrogen enhances memory of genomic and nongenomic pathways 13
5 Agarwood tree (A) and leaves (B) 14
6 HPLC chromatogram of 400 µg/ml *A.subintegra* crude extract 22
7 Group of animals and experimental schedule 24
8 Procedure for bilateral ovariectomy 25
9 The Locomotor activity apparatus 28
10 The Elevated plus maze apparatus 30
11 The hotplate analgesic test 32
12 Tail-Flick analgesic meter and tail-flick analgesic test in rat 33
13 The barbiturate potentiation effect test 35
14 The Morris's water maze set up 37
15 Video recording and plot of the rat swim by Anymaze software 38
16 Experimental scheme for a novel object recognition test 39
17 The process for brain tissue collection 40
18 Procedure for protein analysis 45
19 Standard calibration curve of BSA standard 45
20 Profile of rat's locomotor activity before (Pre) and 10 min epochs 48
21 Total distance of post administration 49
22 Screening of acute anxiolytic action of AS leaves extract by using an elevated plus maze test 50
23 Screening of acute antinociceptive action of AS leaves extract by using a hotplate analgesic test 52

LIST OF FIGURES (CONT.)

Figure		Page
24	Screening of acute antinociceptive action of AS leaves extract by using a tail-flick analgesic test	52
25	Screening of barbiturate potentiation on induction time and duration of anesthesia after administration for 30 minutes	53
26	Learning profile of each group of the rats within a 7-day training phase of a Morris water maze test	55
27	Comparison of spatial memory index of each group of the rats evaluated by using a probe trial test (on Day 8)	55
28.	Comparison of recognition index of each group evaluated by using a novel object recognition test	56
29	Comparison of serum estrogen levels of each group of the rats	57
30	DA levels in rat's brains analyzed by using a HPLC-ECD	59
31	5-HT levels in rat's brains analyzed by using a HPLC-ECD	59
32	Hippocampal aspartate (ASP) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector	62
33	Hippocampal glutamate (GLU) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector	62
34	Hippocampal serine (SER) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector	63
35	Hippocampal glutamine (GLN) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector	63
36	Hippocampal glycine (GLY) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector	64

LIST OF FIGURES (CONT.)

Figure		Page
37	Hippocampal GABA levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector	64
38	HPLC chromatogram of DA standard (A) and Standard calibration curve of DA (B)	88
39	HPLC chromatogram of 5-HT standard (A) and Standard calibration curve of 5-HT (B)	89
40	HPLC chromatogram of sample consisted of DA and 5HT	90
41	Standard calibration curve of ASP	91
42	Standard calibration curve of GLU	91
43	Standard calibration curve of SER	92
44	Standard calibration curve of GLN	92
45	Standard calibration curve of GLY	93
46	Standard calibration curve of GABA	93
47	HPLC chromatogram of standard consisted of ASP, GLU, SER, GLN, GLY and GABA	94
48	HPLC chromatogram of brain sample consisted of ASP, GLU, SER, GLN, GLY and GABA	94

ABBREVIATIONS

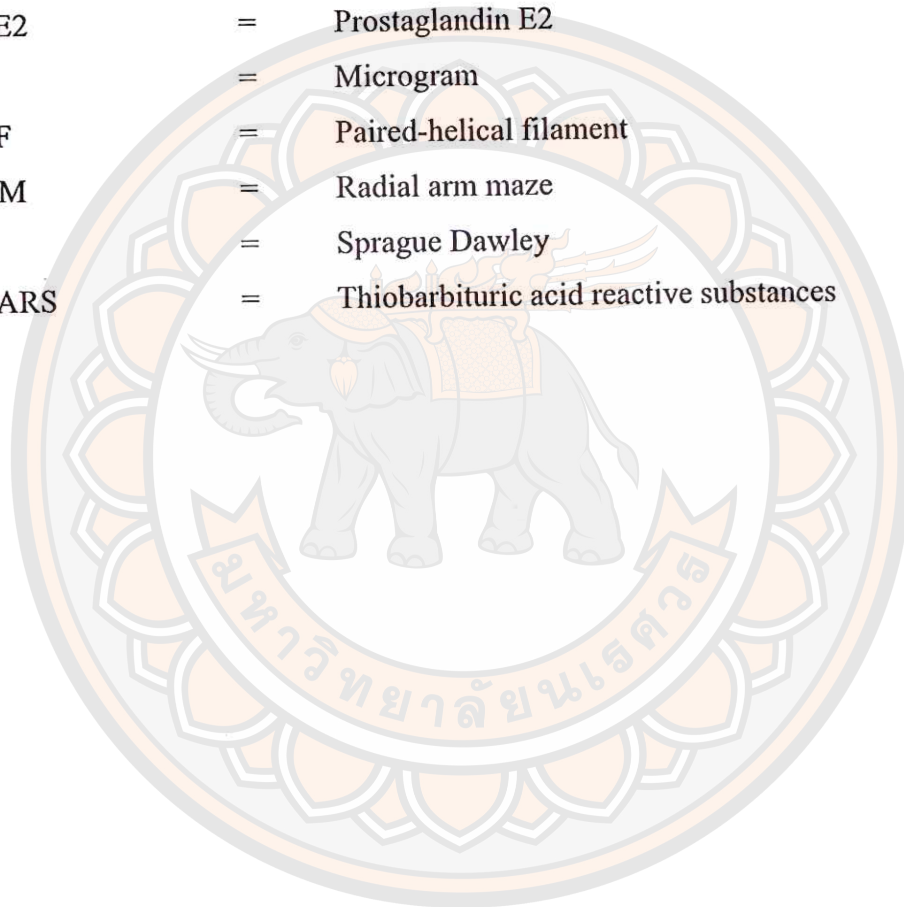
3β-HSD	=	3β-hydroxysteroid dehydrogenase
17β-HSD	=	17β-hydroxysteroid dehydrogenase
Aβ	=	Amyloid- β
ACh	=	Acetylcholine
AChE	=	Acetylcholinesterase
A. malaccencis	=	*Aquilaria malaccencis*
APP	=	Amyloid precursor protein
A. sinensis	=	*Aquilaria sinensis*
A. subintegra	=	*Aquilaria subintegra*
AD	=	Alzheimer's disease
Ag II	=	Angiotensin II
ALB	=	Albumin
ALP	=	Alkaline phosphatase
AST	=	Serum aspartate aminotransferase
ALT	=	Serum alanine transaminase
ACUC	=	Animal care and use committee
BBB	=	Blood brain barrier
BCA	=	Bicinchoninic acid
BDNF	=	Brain-derived neurotrophic factor
BuChE	=	Butyrylcholinesterase
ChAT	=	Choline acetyltransferase
cm	=	Centimeter
CMC-Na	=	Carboxymethylcellulose sodium
CNS	=	Central nervous system
CREB	=	cAMP response element binding protein
DHEA	=	Dehydroepiandrosterone
DPPH	=	2, 2-diphenylpicrylhydrazyl

ABBREVIATIONS (CONT.)

E2	=	Estradiol
ECD	=	Electrochemical detector
ECLA	=	Electrochemiluminescence immunoassay
EDTA	=	Ethylenediaminetetraacetic acid
ER	=	Estrogen receptor
ERα	=	Estrogen receptor alpha
ERβ	=	Estrogen receptor beta
ERE	=	Estrogen response element
HPLC	=	High performance liquid chromatography
iNOS	=	Inducible nitric oxide synthases
LDH	=	Serum lactate dehydrogenase
LPS	=	Lipopolysaccharide
LTP	=	Long term potentiation
m	=	Meter
mAChRs	=	Muscarinic acetylcholine receptors
MCI	=	Mild cognitive impairment
MGF	=	Mangiferin
mL	=	Milliliter
MWM	=	Morris water maze
nAChRs	=	Nicotinic acetylcholine receptors
NFT	=	Neurofibrillary tangles
NLAC	=	National laboratory animal center
nm	=	Nanometer
NMDA	=	N-methyl-D-aspartate
NO	=	Nitric oxide
NOR	=	Novel object recognition
NPH	=	Normal pressure hydrocephalus
NUACUC	=	Naresuan university animal care and use committee
NUCAR	=	Naresuan university center for animal research
OVX	=	Ovariectomy

ABBREVIATIONS (CONT.)

RH = Relative humidity
RO = Reverse osmosis
TBI = Traumatic brain injury
TP = Total protein concentration
TrkB = Tropomyosin receptor kinase B
PGE2 = Prostaglandin E2
μg = Microgram
PHF = Paired-helical filament
RAM = Radial arm maze
SD = Sprague Dawley
TBARS = Thiobarbituric acid reactive substances



CHAPTER I

INTRODUCTION

Rational and significant of the study

According to WHO 2015, by the year 2050, 30% of the total population will be aged more than 65 years old. As the aged population increased, the cost of care and treatment of health disorders also increases. Neurodegenerative diseases (i.e., stress, anxiety, insomnia, Alzheimer's disease (AD) and mild cognitive impairment (MCI)) are among the diseases that can be found in this aged population. With this reason, older population has been viewed as low efficiency and productivity worker. In addition, population ageing may hinder achievement of economic and development goals. So, good health throughout life make them feel valued for societies and help participate fully in economic, social, cultural, political and other activities. Research and development from a plant or a local herbal remedy could be of great benefit to middle-aged and older adults for primary health care and long-term care. It helps to save expenditure of them and also countries.

Agarwood, a fragrant resin-impregnated heartwood of *Aquilaria* species (Thymelaeaceae family), has been used as an incense as well as traditional sedative, analgesic, and digestive medicine in East Asia since centuries. It is the most expensive wood which used to make incense, medicine and aromatherapy. Agarwood has several species as it originates in North Eastern India, Bhutan and parts of Southeast Asia - especially Vietnam and Cambodia - the Philippines, and Indonesia, including Papau New Guinea (Blanchette, 2006; Persoon and van Beek, 2008). Agarwood is well known in Thailand as "Mai Krissana" and at least four species of Aquilaria trees (*Aquilaria malaccencis, Aquilaria subintegra, Aquilaria crassna*, and *Aquilaria Hirta*) are found in several provinces in Thailand such as Trad, Chanthaburi, Nakhon Nayok and Narathiwat.

Several studies have shown the sedative, analgesic and anti-inflammatory activities of the extract from the leaves and the stem. The benzene extract of *A. malaccencis* showed sedative effect on spontaneous locomotor activity, prolong

sleeping time, decreased rectal temperature and reduce effect of acetic acid-writhing (Okugawa, et al., 1996). Single oral administration of *A. sinensis* leaves extract at a dose of 848 mg/kg has shown the increasing analgesic activity on hotplate analgesic test and reducing inflammation activity on xylene or carrageenan-induced edema, carboxymethylcellulose sodium (CMC-Na)-induced leukocyte migration in mice (Zhou, et al., 2008). For pharmacological studies of agarwood oil, inhalation of agarwood oil vapor which contained benzylacetone (agarwood oil from a Hong Kong market) or α-gurjunene and (+)-calarene (agarwood oil made in Vietnam) could sedate mice as indicated by decreasing spontaneous locomotor activity (Takemoto, et al., 2008). An active ingredient of *A. malaccencis* leaves, 4'-hydroxyacetanilide, has been used as acetaminophen for treating analgesic and antipyretic (Afiffudden, Alwi and Hamid, 2015). In addition, ethanol extract of *A. agallocha* leaves was found to decrease serum aspartate aminotransferase (AST), serum alanine transaminase (ALT), alkaline phosphatase (ALP), serum lactate dehydrogenase (LDH), cholesterol, bilirubin and increased albumin (ALB), total protein concentration (TP) against paracetamol induced hepatotoxicity in Sprague Dawley (SD) rats (Alam, et al., 2016).

In Japan, agarwood leaves have been consumed as herbal tea for sedative and laxative effect (Kakino, et al., 2008). The main constituent contributing to the laxative effect of the acetone extract in agarwood leaves from *A. crassana* is genkwanin 5-O-β-primeveroside (Ray, et al., 2014). Laxative effect of ethanol extract of *A. sinensis* leaves was also reported when given to the rat model of low-fiber diet-induced constipation at single doses of 300 and 600 mg/kg for 14 days (Kakino, et al., 2010).

Agarwood leaves extract also have the positive effects on brain function. Spirovetivane-type sesquiterpene, a constituent of agarwood identified from ethanol extract of Vietnamese agarwood leaves, showed significant induction effect on brain-derived neurotrophic factor (BDNF) mRNA expression in rat cultured neuronal cells (Ueda, et al., 2006). Interestingly, the chloroform extracts of leaf and stem *of A. subintegra* contains phenols, flavonoids, terpenoids, and alkaloids compounds. These extracts show acetylcholinesterase (AChE) inhibitory activity and could improve memory impairment when given to the mice with valium-impaired memory (Bahrani, et al., 2014).

Different species of *Aquilaria* have been studied and explored their biological effects such as antioxidant, anti-ischemic, antifungal, and antibacterial effects (Kamonwannasit, et al., 2013; Ray, et al., 2014; Sattayasai, et al., 2012; Zhou, et al., 2008). The specie in particular, *A. subintegra*, is indigenous to the southern part of Thailand and few researches have been done to identify its effects on the CNS function. Recent study by Ray and colleagues (2014) reported the high antioxidant activity from the ethanolic extract from the leaves of *A. subintegra* as compared to vitamin C. Moreover, the leaves extract of this specie showed an AChE inhibitory activity *in vitro* and ability to reverse the cognitive impairment *in vivo* study (Bahrani, et al., 2014). Therefore, the objective of this study is to obtain essential information regarding an ability of AS to improve cognitive impairments in another animal model of amnesia, an ovariectomized rat. In addition, changes in serum estrogen level and brain neurotransmitters was also studied to identify the underlying mechanism(s) of this plant. In the present study, several behavioral tests was also employed and the effects of AS leaves extract on the CNS function were evaluated.

Main objectives

To screen the acute and subchronic effects of the aqueous extract of *A. subintegra* leaves extract (AS) on the CNS function when administered orally to the rats.

Specific objectives

1. To investigate CNS active properties of the aqueous extract of AS by measuring locomotor activity, anxiety, nociception and hypnotic activity in ovariectomized (OVX) female rat.

2. To study the effects of the aqueous extract of AS on learning and memory in ovariectomy- induced memory deficits rat.

3. To determine the effect of the aqueous extract of AS on basal neurotramsmitter levels in ovariectomy-induced memory deficit rats.

The scope of the study

This study was conducted in the adult sham-operated and ovariectomized female Sprague Dawley rats to investigate the effects of the aqueous extract of AS on the rat behavior such as a) locomotor activity by using an open field test, b) anxiety by using an elevated plus maze, c) nociception by using hotplate assay and tail-flick assay, d) hypnotic activity by measuring hexobarbiturate-induced sleeping time, and e) learning and memory abilities by using Morris's water maze and novel object recognition tests. The aqueous extract of AS at dose either 100 or 1000 mg/kg body weight was administered daily for 60 days.

After completion of all behavioral tests, the animal's brain was removed for determining of the basal level of monoamines and amino acid neurotransmitters by employing a high performance liquid chromatography system (HPLC) with an electrochemical detector (ECD) and/or fluorescence detector.

Preliminary Agreement

Since, the present study involved the usage of laboratory animals, the protocol of this study, therefore, must be submitted and approved by Naresuan

University Animal Care and Use Committee (NUACUC) before purchasing the animals and conducting the experiments.

Keywords

agarwood, locomotor activity, anxiety, nociception, hypnotic activity, learning and memory, neurotransmitters

Hypotheses

1. Since, there is evidence indicating the effects of *A. malaccensis* wood extract on the CNS function in mice (Okugawa, et al., 1992), I, therefore, would expect to obtain similar effects when *A. subintegra* leaves extract was administered to the rats. These effects included: a) a decrease in locomotor activity, b) an increase the sleeping time induced by barbiturate, c) a decrease of anxiety, and d) an increase of pain threshold.

2. Since the facts that estrogen is essential for learning and memory (Bean, Ianov and Foster, 2014; Frick, et al., 2015; Talebi, et al., 2010) and *A. subintegra* leaves extract has been shown to have high antioxidant properties (Ray et al., 2014) and claimed to be effective in treating mice with valium-impaired memory by inhibiting acetylcholinesterase (Bahrani, et al., 2014), thus, sub-chronic administration of *A. subintegra* leaves extract in an animal model of dementia should be able to improve the memory impairment caused by age and estrogen depletion.

The anticipated outcomes of this study

Results from this study may support the previous data on CNS action of agarwood wood extract in animals with supplementary data of brain neurotransmitters. In addition, the amelioration of learning and memory impairments following subchronic administration of *A. subintegra* leaves extract, may provide useful scientific information for future studies focusing on its underlying mechanism(s). The findings will also support the potential application of agarwood in treatment of cognitive impairments in postmenopausal women. It will also support the developing of a dietary supplement that enhance memory performance for middle aged and old people.

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Amnesia

Amnesia is the most common cause of progressive decline of cognitive function in aged humans. It refers to the loss of memories that is characterized as dementia and other disorders of brain function. There are 2 types of amnesia. The first type is anterograde amnesia which is an impaired learning or access to new explicit information due to brain damage. Such that, people were unable to remember things for long periods of time. The second type is retrograde amnesia which refers as the loss of explicit memory acquired before the occurrence of brain damage i.e. patient shows an inability to retrieve information that was acquired before brain damage. Both types can occur within one patient at one time (Kopelman and Bright, 2012; Köppen, et al., 2016).

Neuropathology of amnesia

The criteria of neuropathic (i.e. Alzheimer's disease or AD, dementia, amnesia) are the presence of 2 main components; amyloid plaques and neurofibrillary tangles (NFT) (Selkoe, 2001). Amyloid plaques are extracellular accumulated of amyloid- β (Aβ) peptides and NFT are intracellular accumulated of hyperphosphorylate tau protein. These components will lead to taupathy, neuronal injury, and cognitive impairment (Hardy and Selkoe, 2002).

Aβ peptides results from the cleavage of amyloid precursor protein (APP) by β and γ-Secretase leading to release of the ectodomain or hydrophilic amino-terminal extracellular domain, carboxy-terminal cytoplasm domain or cytosolic domain, hydrophobic putative transmembrane domain or spanning domain of APPs (Savonenko, et al., 2015).

NFT is found on cell bodies and dendrites of neurons. In normal cell, tau protein display hydrophilic properties and stabilize microtubules which become microtubule binding protein (Goedert and Spillantini, 2012). The

hyperphosphorylation of tau protein causing accumulation of paired-helical filament (PHF) which has double helix character and insoluble in the pyramidal cell of cerebral cortex and hippocampus (Corbo and Alonso, 2010).

Previous study found that memory impairment in the cholinergic system of the mice occur due to Aβ accumulation following the increase of acetylcholinesterase (AChE) (Terry and Buccafusco, 2003). Acetylcholine (ACh) binds to two families of receptors such as nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). Action of both receptor families can lead to the cognitive processes (Barrantes, Borroni and Vallés, 2010). The activity of enzyme choline acetyltransferase (ChAT) that involved in ACh production is decreased in AD (Auld, et al., 2002).

Animal model of amnesia

Amnesia can be produced genetically and pharmacologically in animals. It is possible to discover novel drugs that slow the progress or alleviate the clinical symptoms of amnesia by using animal models to evaluate anti-amnesia drugs. Nowadays, several animal models of amnesia are generally accepted and widely used. Animals induced by amyloid β-peptide (Aβ) inhibit long-term potentiation (LTP) in the hippocampus (Jensen, et al., 1999; Lambert, et al., 1998). In addition to the direct neurotoxicity, Aβ activates microglia to produce cytokines, free radicals, and nitric oxide (NO) (Li, et al., 2014).

Memory deficits in animal could also be induced by scopolamine for the screening of anti-dementia drugs (Chen, et al., 2013). This drug is a non-selective post-synaptic receptor antagonist. Scopolamine blocks muscarinic receptors at the synapse and inhibits depolarization of acetylcholine action via oxidative stress and neuroinflammation similar to memory impairments as observed in the elderly (Sherbiny, et al., 2003; Kim, et al., 2013). Scopolamine causes changes partially which can mimic the age-associated changes and also influence electroencephalogram as observed in patients of dementia (Lee, Jung and Kim, 2009).

Traumatic brain injury (TBI) can led to the increase in Aβ42 level (Dekosky, et al., 2007), phosphorylated tau protein level (Zanier, et al., 2013) and the presence of neurofibrillary tangles (NFT) (Johnson, Stewart and Smith, 2012). Moreover, TBI is

associated with neuroinflammation and neuronal injury (Johnson, et al., 2013; Shojo, et al., 2010). These could result in memory impairment, dementia, pyramidal and extrapyramidal dysfunction, as well as cerebellar impairment (Smith, Johnson and Stewart, 2013). TBI model has been used in humans which show several define pathological of amnesia and also in mice, rats, rabbits, pigs, and monkeys (Breunig, Guillot-Sestier and Town, 2013).

Normal pressure hydrocephalus (NPH) is one of the cause of dementia which results from abnormal accumulation of the cerebrospinal fluid in the brain due to an imbalance between the rates of CSF production and absorption (Bateman, 2002; Bateman, 2004). These reduced transportation of beta amyloid (β amyloid) in Alzheimer's disease and increased accumulation of CSF in the cord (Weller, et al., 2000). Animal models of NPH could be induced by cisterna kaolin injection (Klinge, et al., 2006; Silverberg, et al., 2015).

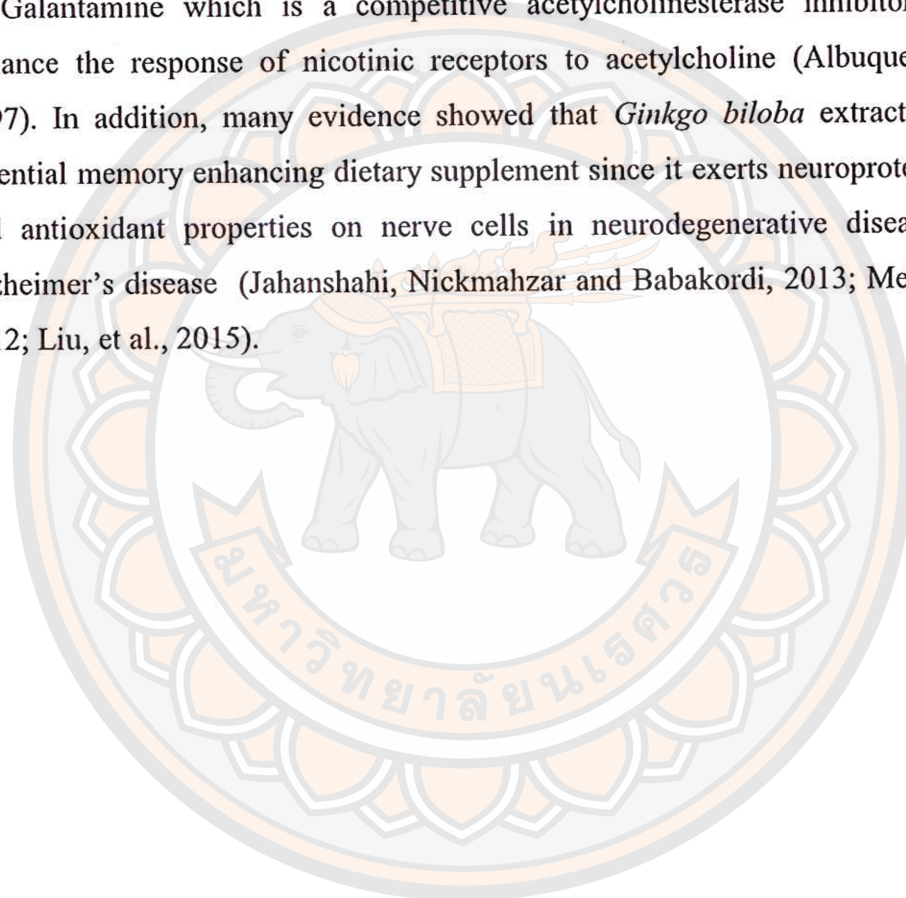
Arterial hypertension has been investigated as the effect of hypertension on cognitive function are risk factors for amnesia (Moss and Jonak, 2007). Animal that has high levels of blood pressure also shows an accumulation of amyloid (Carnevale and Lembo, 2011). Animal with arterial hypertension by angiotensin II (Ag II) injection showed impairments in spatial and recognition memories (Csiszar, et al., 2013).

The ovariectomy has been used in basic research for understanding basic biological mechanism and effects of menopause. The removal of ovaries in rat could reduce effect of hormone such as estradiol (E_2) similarly to menopause in human. There are several reports demonstrating side effects related to estrogen deficiency including decrease in sexual behavior (Zavatti, et al., 2009), diabetes (Appiah, Winters and Hornung, 2014), osteoporosis (Zhang, et al., 2015), cardiovascular disease (Xu, et al., 2008), parkinsonism, cognitive impairment, depression and anxiety (Baeza, et al., 2010; Hogervorst and Bandelow, 2010). The ovariectomized rat model is undoubtedly one of the model used in the study of Alzheimer's disease (Zhang, et al., 2012).

In the present study, OVX rat was chosen to induce memory impairments in adult female rat similarly to the symptom observed in older age.

Treatment of amnesia

At the present time, there are several drugs (Figure 1) prescribed for treatment of amnesia such as 1) donepezil which is an acetylcholinesterase inhibitor used in the treatment of mild to moderate dementia in Alzheimer's disease (Nagasawa, et al., 2016), 2) Rivastigmine which can inhibit both butyrylcholinesterase (BuChE) and acetylcholinesterase (AChE) (Rösler, et al., 1999), 3) Memantine which is N-methyl-D-aspartate (NMDA) receptor antagonist (Marszalek-Grabska, et al., 2016), 4) Galantamine which is a competitive acetylcholinesterase inhibitor that could enhance the response of nicotinic receptors to acetylcholine (Albuquerque, et al., 1997). In addition, many evidence showed that *Ginkgo biloba* extract serves as a potential memory enhancing dietary supplement since it exerts neuroprotective effects and antioxidant properties on nerve cells in neurodegenerative diseases such as Alzheimer's disease (Jahanshahi, Nickmahzar and Babakordi, 2013; Mehrdad, et al., 2012; Liu, et al., 2015).



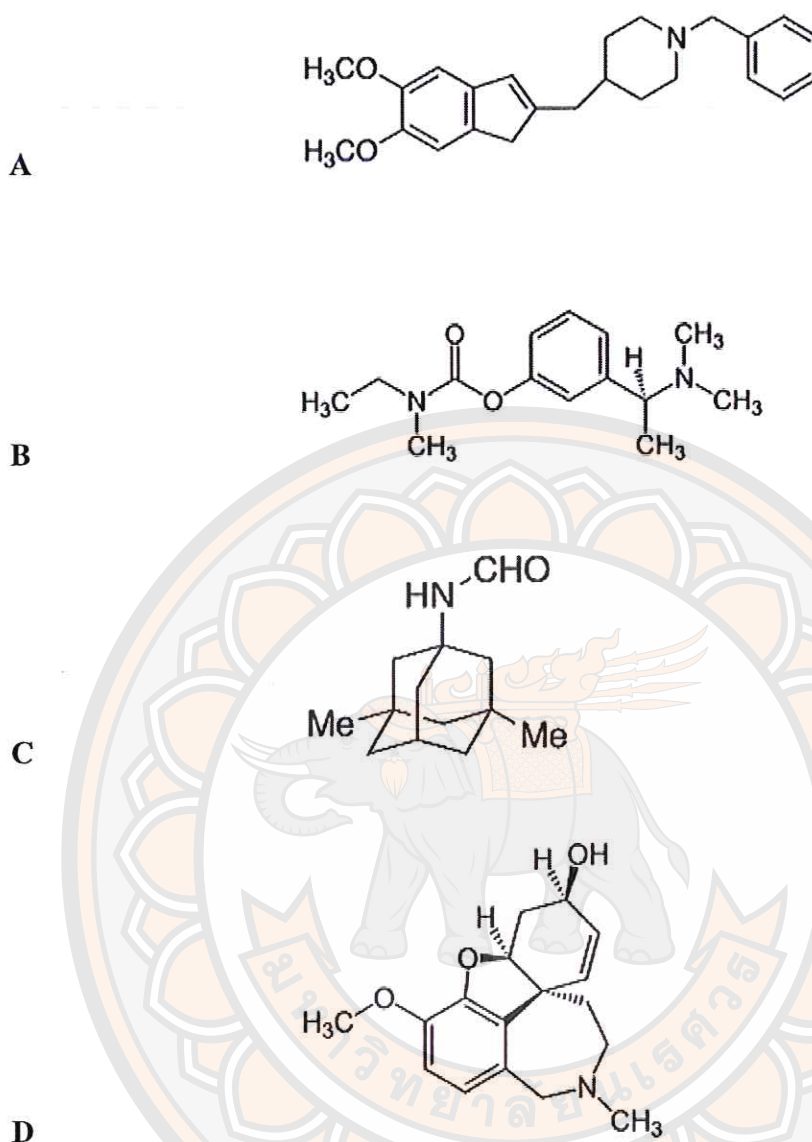


Figure 1 Molecular structure of (A) donepezil, (B) rivastigmine, (C) memantine, (D) galantamine

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https://upload.wikimedia.org/wikipedia/commons/0/0a/Galantamine_Structural_Formulae.png

Estrogen effect on learning and memory

Estrogens (Figure 2) is a steroid gonadal hormone produced mainly by ovarian follicles. Estrogen is syntheses from cholesterol and cholesterol, and catalyzed to pregnenolone by cytochrome P450 side-chain cleavage enzyme (P450scc). Pregnenolone is then converted to progesterone and dehydroepiandrosterone (DHEA) by 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450c17, respectively. Progesterone and DHEA are converted to androstenedione via cytochrome P450c17 and 3β-HSD. Androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD). Then, androstenedione and testosterone are converted to 17β-estradiol or estrogen by aromatase (Figure 3). Previous study, P450scc and aromatase have been detected in hippocampus and some other tissues such as liver, adrenal gland, adipose tissue and the breast (Do Rego, et al., 2009; Fester, et al., 2011).

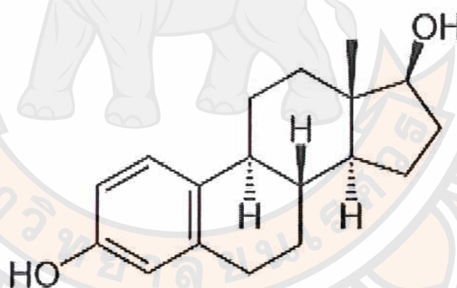


Figure 2 Chemical Structure of estrogen

Source: https://upload.wikimedia.org/wikipedia/commons/thumb/0/00/Estradiol.svg/200px-Estradiol.svg.png

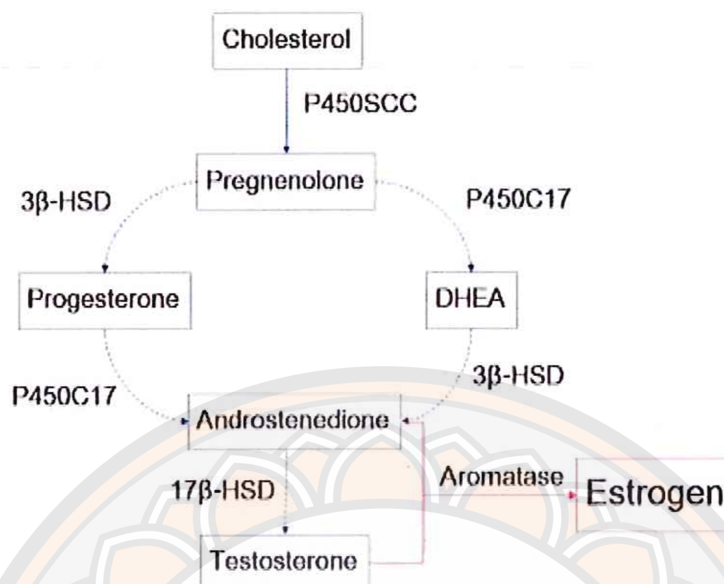


Figure 3 Estrogen synthesis in brain

This hormone plays an important role in several body systems including the nervous system. A number of evidence have shown that estrogen could protect neuron and improve learning and memory by modulate synaptic plasticity while aging or removal of ovaries have led to decrease in estrogen level and subsequently leading to cognitive decline (Chamniansawat and Chongthammakun, 2010; Li, Cui and Shen, 2014; McEwen, et al., 2012; Varea, et al., 2009). There are several evidence reporting that estrogen could affected cognitive functions (Amantea, et al., 2005; Frick, et al., 2015; Luine and Frankfurt, 2015; Luine, 2014). An *in vitro* experiment by McCullough and Hurn has shown that estrogen can act as potent neuroprotectants (McCullough and Hurn, 2003). The evidence showing that estrogen could enhance spatial memory have also been reported (Luine, et al., 1998). Additionally, when female OVX rats were treated with estrogen, rats' ability in object recognition test were improved (Gervais, et al., 2013; Jacome, et al., 2010).

Estrogen receptor (ER) can be divided into 2 classical subtypes; estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Both receptors are found to distribute in the cerebral cortex, prefrontal cortex, and hippocampus, areas associated with learning and memory (Shughrue, Lane and Merchenthaler, 1997). The genomic pathway is proposed for estrogen enhancing cognitive function in which estrogen molecule enters and binds to both subtypes of ER nucleus receptor to form a complex binding to estrogen response element (ERE). This complex then stimulate gene transcription which result in an increase in protein synthesis and subsequently increasing neural transmission and as a result, enhancing cognitive functions (Chamniansawat and Chongthammakun, 2010; Luine, 2014). The second pathway is also known as nongenomic or nonnuclear, in which estrogen bind to membrane receptor which lead to stimulate intracellular signal pathway by trigger the phosphorylation of cAMP response element binding protein (CREB) for rapidly activated target cell of estrogen (Figure 4).

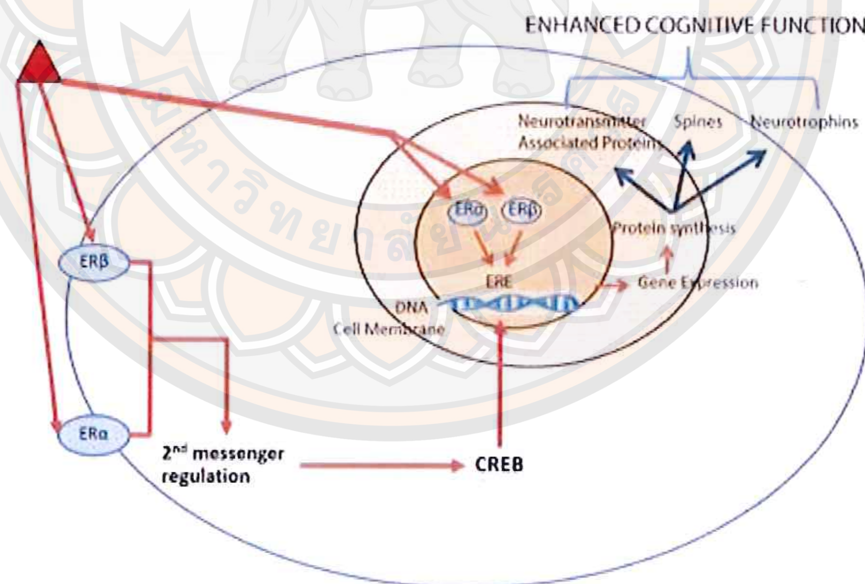


Figure 4 Estrogen enhances memory by genomic and nongenomic pathways

Source: modified from Luine, 2014

Agarwood

Agarwood is well known in Thailand as "Mai Krissana". It is the most expensive wood that can be used to make incense, herbal medicine and aromatherapy (Chen, et al., 2011). In Japan, agarwood leaves has been used to consume as herbal tea for sedative and laxative effect (Kakino, et al., 2010).

Figure 5 Agarwood trees (A) and leaves (B)

Source: www.pharmacy.mahidol.ac.th

http://forestpathology.cfans.umn.edu/photos/aquilaria%20leaves%20with%20moisture.jpg

Agarwood has many species including *Aquilaria agallocha* (*A. agallocha*), *Aquilaria malaccencis (A. malaccencis), Aquilaria sinensis* (*A. sinensis*), *Aquilaria subintegra* (*A. subintegra*), *Aquilaria crassna (A. crassna)*, *Aquilaria Hirta* (*A. Hirta*). Four species are found in Thailand such as 1) *A. malaccencis* which is found in the rainforests and dry evergreen forest in the Northcentral and Northeast regions. It can grow up to 20 meters tall, 2) *A. crassna* which is often found in the Southern part of Thailand where a high humidity is. The tree can normally grow from 15 to 20 meters tall, 3) *A. subintegra* is found mostly in the Eastern part of Thailand and can grow up to 40 meters tall with the diameter is greater than 60 cm. The trunk of this tree is straight, smooth bark, soft whitish and stripping off in long phloem (Figure 5), and 4) *A. Hirta* which it leaves are hairy, pointed and can grow up to 14 meters which can be found mainly in Narathiwat Province (Ray, et al., 2014).

The benefit of the bark and oil from agarwood has shown in several reports. *A. crassna* oil could inhibit metastasis and induction of apoptosis in the pancreatic cell line (MIA PaCa-2) (Dahham, et al., 2015). The ethanol extract of *A. sinensis* when given at single doses of 300 and 600 mg/kg for 14 days has laxative effect on rat model of low-fiber diet-induced constipation (Kakino, et al., 2010). The ethanol extract of Vietnamese agarwood showed an improvement in brain-derived neurotrophic factor (BDNF) mRNA expression in rat cultured neuronal cells which serve as an important role in the survival, differentiation and synaptic plasticity of neurons (Ueda, et al., 2006). Inhalation of agarwood oil vapor obtained benzylacetone (agarwood oil from a Hong Kong market) or α-gurjunene and (+)-calarene (agarwood oil made in Vietnam) could sedate mice by decreasing spontaneous locomotor activity (Takemoto, et al., 2008). The benzene extract of *A. malaccencis* is shown to have sedative effect on spontaneous locomotor activity, prolong sleeping time, decreased rectal temperature and reduce effect of acetic acid-writhing (Okugawa, et al., 1992).

For the benefit of agarwood leaf, ethanol extract of *A. agallocha* leaves was found to decrease serum aspartate aminotransferase (AST), serum alanine transaminase (ALT), alkaline phosphatase (ALP), serum lactate dehydrogenase (LDH), cholesterol, bilirubin and increased albumin (ALB), total protein concentration (TP) against hepatotoxicity in SD rats induced with paracetamol (Alam, et al., 2016). An active ingredient, 4'-hydroxyacetanilide, extracted from *A. malaccencis* leaves has

been widely used as acetaminophen which is used to treat analgesic and antipyretic (Afiffudden, et al., 2015). Study in mice received single oral administration of *A. sinensis* leaves extract at the dose of 848 mg/kg B.W. has shown an increase in analgesic activity and reduction in inflammation activity on xylene or carrageenan-induced edema, carboxymethylcellulose sodium (CMC-Na)-induced leukocyte migration (Zhou, et al., 2008). *In vitro* study have found that the decreasing of iNOS on lipopolysaccharide (LPS)-induced nitric oxide (NO) release from mouse peritoneal macrophages (Zhou, et al., 2008). Moreover, the leaves and stem *of A. subintegra* which have phenols, flavonoids, terpenoids, and alkaloids compounds could inhibit acetylcholinesterase (AChE) resulting in an increase in acetylcholine level and improve memory impairment in mice on radial arm maze test (RAM) (Bahrani, et al., 2014). Previously study of Ingkaninan, K., et al (unpublished data) has shown the antioxidant properties (i.e. mangiferin 8-14%) of AS which could decrease lipid peroxidation on thiobarbituric acid reactive substances (TBARS) assay and the 2,2-diphenylpicrylhydrazyl (DPPH) assay when compared with Trolox, a derivative of vitamin E. Oral administration of ethanol extract of *A. subintegra* at single dose of 2,000 mg/kg and repeated dose of 500 mg/kg for 6 months showed mild laxative effect and no abnormalities in hematology and cytology (Taepavarapruk, N., et al., unpublished data). Thus, *A. subintegra* leaves extract is relatively safe for the long term consumption.

CHAPTER III

RESEARCH METHODOLOGY

Chemicals and reagents

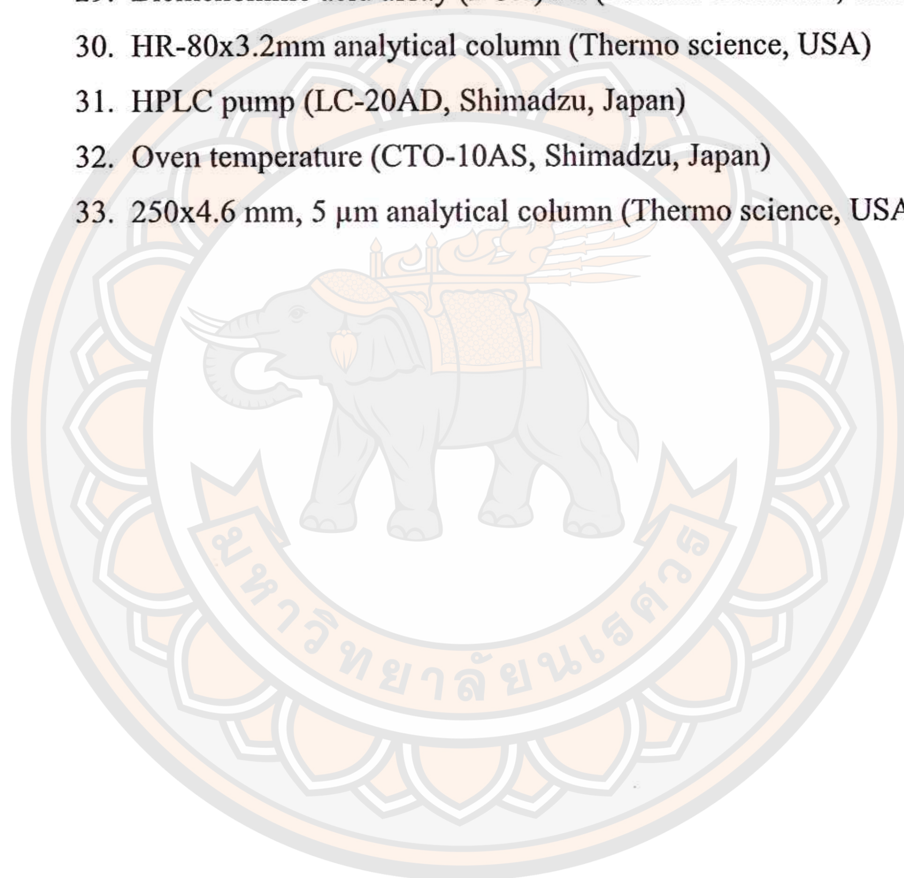
1. 70% alcohol (Leopard Medical Brand Co., Ltd., Thailand)
2. Sodium chloride (Merck, Germany)
3. Sodium pentobarbital (Nembutal) (CEVA sante animale, France, France)
4. Ethylenediaminetetra-acetic acid (EDTA) (Fluka, USA)
5. Diazepam (Valium, Hoffmann-La Roche, Switzerland)
6. Donepezil hydrochloride (Pfizer, UK)
7. Povidone-iodine (Alcon Laboratories, Inc., USA)
8. Sodium thiopental (Jagsonpal, India, India)
9. di-sodium hydrogen orthophosphate anhydrous (Ajax Finechem, Australia)
10. Sodium dihydrogen phosphate monohydrate (Ajax Finechem, Australia)
11. Disodium hydrogen phosphate (Na_2HPO_4) (Riedel-19 de Haën, USA)
12. Perchloric acid (ORëC)
13. Methanol (HPLC grade, Lab-Scan Analytical Sciences, Lab-Scan Asia Co., Ltd., Thailand., Thailand)
14. Acetonitrile (HPLC grade, Lab-Scan Analytical Sciences, Lab-Scan Asia Co., Ltd., Thailand.)
15. Phthaldiadehyde reagent (Sigma–Aldrich, Germany)
16. β-mercaptoethanol (Merck, Germany)
17. Sodium Hydroxide (Ajax Finechem, Australia)
18. Citric acid (Fisher chemical, USA)
19. Isoflurane (Piramal Critical Cares, Inc, USA)
20. Dopamine (Sigma–Aldrich, Germany, Germany)
21. Serotonin (Sigma–Aldrich, Germany)
22. Aspartate (Fluka, USA)
23. Glutamate (Fluka, USA)
24. Serine (Sigma–Aldrich, Germany)

25. Glutamine (Himedia, India)
26. Glycine (Sigma–Aldrich, Germany)
27. Gamma aminobutyric acid (Sigma–Aldrich, Germany)

Materials and research instrument

1. Feeding needles (National Laboratory Animal Center, Mahidol University, Thailand)
2. Stopwatch (SW01-X008, Alba, USA)
3. Morris Water Maze apparatus
4. Elevated Plus Maze apparatus
5. Novel Object Recognition apparatus
6. Surgical equipment
7. Decapitator
8. Tail-Flick Analgesic Meter (model 31, Lafayette Instrument Company, USA)
9. Hotplate Analgesic Meter
10. Analytical Balance (PB403-S, Metter-Toledo Ltd, Thailand)
11. Computer (a1150l, Hp pavilion, USA)
12. VDO camera (LYD-808C, China)
13. Microsyringe 25 μl (gastight syringe, Hamilton co.,reno Nevada, USA.)
14. Vortex (G-560E, Scientific Industries, Uohimai, USA)
15. Homogenizer (Ultra-turexT8, Ika-werke GMBH & CO.KG Staufen, Germany)
16. Centrifugation machine (Labofuge 400R, Heraeus instruments, Germany)
17. Water bath (WB22, Memmert, Germany)
18. Microplate reader (1401, LabSystem, iEMS Reader MF, Finland)
19. Microplate shaker/incubator (Vortemp 56 EVC, National Labnet Co., Inc. Woodbridge, NJ USA.)
20. pH meter (Mettler Toledo 320, Beaumont Leys, England)
21. Autoclave (AMA260S, ASB260, AMA270S, ASTELL EduSystems Ltd, Thailand)

22. Spherisorb column 5 µm ODS2 particle (Waters Corp, USA)
23. Fluorescence detector (model 7725, Rheodyne, Shimadzu, Japan)
24. System controller (model SCL-10 AVP, Shimadzu, Japan)
25. Solvent delivery module (LC-10AD vp, Shimadzu, Japan)
26. Degasser (DGU-14A, Shimadzu, Japan)
27. Column oven (CTO-AS vp, Shimadzu, Japan)
28. Scanning fluorescent detector (model 474, Waters, USA)
29. Bicinchoninic acid assay (BCA) kit (Thermo Scientific, USA)
30. HR-80x3.2mm analytical column (Thermo science, USA)
31. HPLC pump (LC-20AD, Shimadzu, Japan)
32. Oven temperature (CTO-10AS, Shimadzu, Japan)
33. 250x4.6 mm, 5 µm analytical column (Thermo science, USA)



Method

1. Agarwood leaves extract

Preparation of *A. subintegra* leaves extract was done by Eakkaluk Wongwad and Kornkanok Ingkaninanat the Bioscreening Unit, Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand. During the feeding period, the test solutions were prepared by dissolving the extract powder in RO filtered water. Each test solution was then force fed into rat's stomach by using a rodent feeding needle (No. 16, 4 inches length, National Laboratory Animal Center Mahidol University, NLAC).

1.1 Preparation of *A. subintegra* leaves extract

The preparation of *A. subintegra* leaves extract was employed from Dr. Ingkaninan's lab at Bioscreening Unit, Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University. Young leaves of *A. subintegra* were collected from Phitsanulok, Thailand. They were dried in a hot-air oven (60 °C, 48 h.) and ground. The dried powder was macerated in hot water (95-100 °C) at the ratio of 100 g: 1 L for 30 minutes, then sieved through cheesecloth. The filtrate was centrifuged and then the supernatant was lyophilized for 72 h. The crude extract was kept at -20 until use.

1.2 HPLC analysis of *A. subintegra* leaves extract

The HPLC analysis process of *A. subintegra* leaves extract was also employed at Dr. Ingkaninan's lab. Quantitative measurement of mangiferin in *A. subintegra* leaves extract was conducted on an HPLC (Shimadzu, Kyoto, Japan) equipped with a SPD-20A UV/Vis detector, an LC-20AT pump, CTO-10AS VP column oven and an injector with a 20 µL loop. A phenomenex Luna 5µ C-18 column (150 x 4.6 mm) was used as a stationary phase. The flow was isocratic at 1.0 mL/min with acetic acid: acetonitrile: methanol (87:12:1; v/v/v). The detector was set at 257 nm. *A. subintegra* leaves extract (12.5 mg) was dissolved in distilled water and volume adjusted to 25 ml in volumetric flask to produce a solution of 500 µg/ml. The solution was diluted to solution of 400 µg/ml with a mobile phase solution. Then, the sample

solution was filtered through a syringe filter (Nylon 13 mm, 0.45 μm) before submitted to HPLC.

The percent yield of *A. subintegra* leaves extract obtained was 9.48-22.21%. The crude extract contained not less than 7% (w/w) of mangiferin determined using HPLC analysis (Table 1). The chromatogram of HPLC analysis of *A. subintegra* leaves extract is shown in Figure 6.

Table 1 %yields and %mangiferin contents in the different lots of *A. subintegra* leaves extract

Lot	Date of preparation	Raw material (date received)	Weight (g)	%yield	% mangiferin	Notes
3	12/12/2014	12/11/2014	365.00	21.26	13.10±0.19	Brownish powder
4	09/02/2015	12/11/2014	300.00	9.48	8.92±0.10	Brownish powder
5	21/02/2015	12/11/2014	600.00	22.21	10.40±0.24	Brownish powder
6	17/03/2015	12/11/2014	600.00	21.42	8.81±0.03	Brownish powder
7	23/03/2015	12/11/2014	700.00	20.92	12.52±0.25	Brownish powder
8	01/05/2015	12/11/2014	700.00	21.30	9.56±0.56	Brownish powder

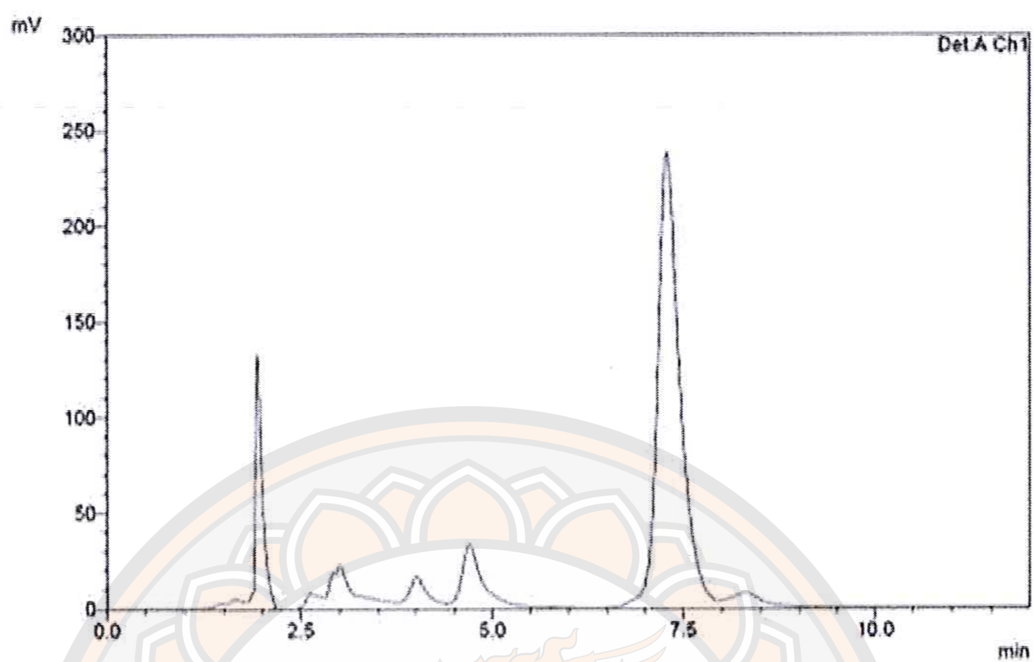


Figure. 6 HPLC chromatogram of 400 µg/ml *A. subintegra* crude extract. The retention time of mangiferin is 7.28 minutes.

2. Experimental animals

All the animal protocol presented herein had been approved by the board of Naresuan University Animal Care and Use Committee (NUACUC) since 08/17/2012. The protocol number is NU-AE550731 and the approval number is 56040030. After the protocol had been approved, 60 retired female Sprague Dawley (SD) rats at the aged of 8 months were ordered from National Laboratory Animal Center (NLAC), Mahidol University. All rats were housed, two-three animals per a shoe-box cage, in an animal room of Naresuan University Center for Animal Research (NUCAR). The animal room was maintained in constant temperature at 22±1.0 C°, relative humidity (RH) at 45-65% , and a reverse dark-light cycle (light on at 11:00 p.m.-11:00 a.m. and light off at 11:00 a.m.-11:00 p.m.). These animals had free access to food and water (food pellet formula G82, Feed Marketing Bureau CPF (Thailand) PCL, Bangkok) in their home cages. In addition, the animals were allowed to acclimatize to the animal room with a reverse dark-light cycle for at least 5 days before conducting an experiment. Rats were kept until the aged of 12 months and were subjected to ovariectomy. The rats were divided into 6 group (ten each):

2.1 OVX group (n=10), the OVX rats were treated with reverse osmosis (RO) water.

2.2 Sham group (n=10), the rats were treated with RO water

2.3 OVX+AS100 group (n=10), the OVX rats were treated with AS 100 mg/kg B.W.

2.4 OVX+AS1000 group (n=10), the OVX rats were treated with AS 1000 mg/kg B.W.

2.5 OVX+ Diazepam (D#1) group (n=10), the OVX rats were treated with diazepam 2.5 mg/kg B.W.

2.6 OVX+ Donepezil (D#2) group (n=10), the OVX rats were treated with Donepezil 5 mg/kg B.W.

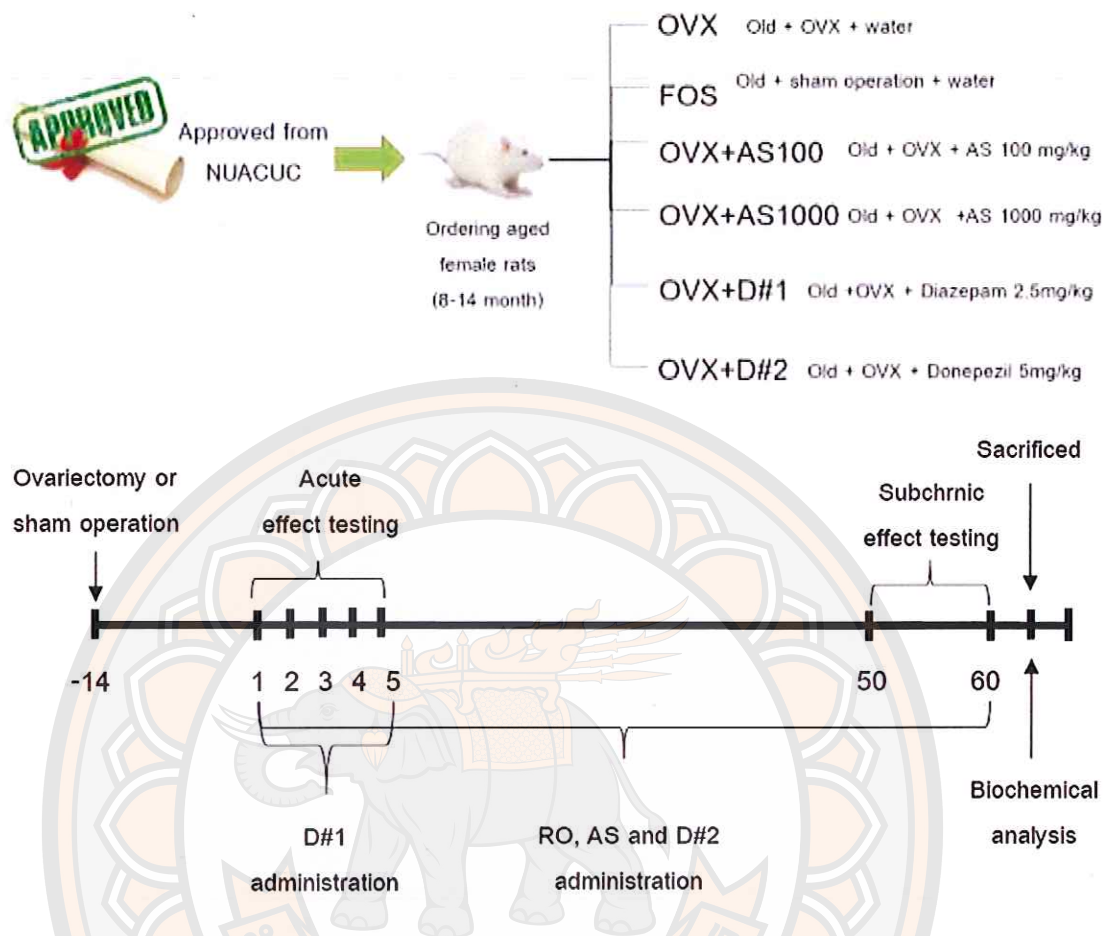


Figure 7 Group of animal and experimental schedule

3. Procedures for bilateral ovariectomy

Before starting all behavioral tests, the animals were subjected to a bilateral ovariectomy surgery in order to create an animal model of memory deficit caused by estradiol (E_2) hormone deficiency. This animal model has been previously employed by several investigators as it could represent a cognitive impairment condition in menopausal women (Baeza, et al., 2010; Hogervorst and Bandelow, 2010).

When the animals' age reached 12 months old, they were subjected to a bilateral ovariectomy surgery. Prior to the surgery, each rat was induced and maintained to deeply anesthesia with 3-4% isoflurane. Then, the lower abdomen region was clean with an alcohol pad, and a midline incision was performed to expose the intestinal organs. After that, the ovaries were identified, ligated at the distal uterine

horns, and cut from the uterine horns. Finally, the incision was sutured, applied with a Betadine solution, and covered with a Fixomull® pad. These ovariectomized rats were allowed to recover from the surgery for at least two weeks. During the 2-week recovery period, estrogen were reported to be decreased from ovariectomy (Baeza, et al., 2010).

Figure 8 Procedures for bilateral ovariectomy, (A) A female rat was anesthetized and performed a midline incision at the lower abdomen. (B) Then, the uterine horns were isolated and ligated at the distal uterine horns. (C) The ovaries were removed and the incision was sutured and covered with a Fixomull® pad.

4. Screening of the CNS action of AS crude extract

The screening of effects of AS crude extract on the CNS were conducted in 2 set of experiments. The first set of experiments was aimed to screen an acute effect of AS crude extract. This set of behavior tests consisted of 1) locomotor activity test, 2) elevated plus maze test, 3) hotplate analgesic test, 4) tail-flick analgesic test, and 5) barbiturate potentiation test, respectively. The second set of experiments was aimed to screen the effect of AS crude extract on learning and memory. This set of experiments consisted of 1) a novel object recognition (NOR) test and 2) Morris water maze (MWM) test.

4.1 Procedures for testing the acute effect of AS

After the 2-week recovery period, the animals were randomly divided into 4 groups (10 rats per each group) as 1) a control group (OVX) which was fed with reverse osmosis (RO) water, 2) a diazepam-treated group (OVX+D#1) which was fed with 2.5 mg/kg diazepam, 3) a low dose AS-treated group treated which was fed with 100 mg/kg AS crude extract (OVX+AS100), and 4) a high dose AS-treated group treated which was fed with 1,000 mg/kg AS crude extract (OVX+AS1000). The series of behavioral tests were:

4.1.1 Locomotor activity test

Spontaneous locomotor activity test is a simple method for screening the drug effecting motor function by observing rat's spontaneous locomotor activity after a drug administration (Nieradko-Iwanicka and Borzecki, 2016). This test was performed by allowing the animal to freely move in an open field in order to test whether the drug could depress or stimulate the CNS function (Lynch, Castagne, et al., 2011). The open field setup consists of a circular tank (150 cm diameter, 45 cm height) and a CCTV video camera connected to a PC computer equipped with a video tracking software (Anymaze, Stoelting Co., USA).

The rat was allowed to adapt to the open field (or circular) tank for 10 minutes. After 10 minutes of adaptation, each group was administered with RO water (control, OVX), 2.5 mg/kg B.W. diazepam (positive control, OVX+D#1), 100 mg/kg B.W. AS (OVX+AS100) or 1000 mg/kg B.W. AS (OVX+AS1000). After administration, each rat was put back in the circular tank and allowed to freely explore the place for another 60 minutes. The number of total distance and immobile time

were recorded as parameters of locomotor activity and the data were analyzed every 10 minutes. After finish each test, rat was removed and the floor of the tank was carefully cleaned with 70% alcohol (Leopard Medical Brand Co., Ltd.).

Figure 9 Locomotor activity apparatus (A) locomotor activity apparatus consists of a circular tank and a digital video camera connected to a desktop computer. (B) Examples of track plots of the animal after the drug administration (10-min period each) analyzed by using Anymaze software.

4.1.2 Elevated plus maze test

Elevated plus maze test was originally designed by Pellow et al. in 1985. This behavioral test is an experimental tool for determining anxiety of the animal after administering anxiolytic substance. In ordinary situation, rat prefers to stay in closed arms rather than open arms. The maze used in this study was made of wood, consisted of 2 open arms which had no wall and 2 close arms which had three side walls. This maze was elevated 50 cm from the floor and had a CCTV camera directly above the maze. The animal's locomotive behaviors were continuously recorded by using the CCTV camera connected to a PC computer which had Anymaze software (Stoelting Co.). Recording the number of entries into the open arms and closed arms, and the total time spent in the open arms and closed arms were analyzed by using Anymaze software. The anxiolytic index was calculated from the ratio of the mean time in open arms/the mean time in all arms.

For the experimental protocol, a rat was allowed to acclimatize to the maze for 5 min by gently placing at the center of the maze. This data was recorded as a baseline. Then the rat was fed with either RO water (control, OVX), 2.5 mg/kg B.W. diazepam (positive control, OVX+D#1) (Gamberini, et al., 2015), 100 mg/kg B.W. AS (OVX+AS100), or 1000 mg/kg B.W. AS (OVX+AS1000). After the administration for 30 min, each rat was returned to the maze and its behaviors were recorded for 5 min (Telonis and Margarity, 2015). After finishing each test, the maze was cleaned with 70% alcohol and let it dry before starting the next test.

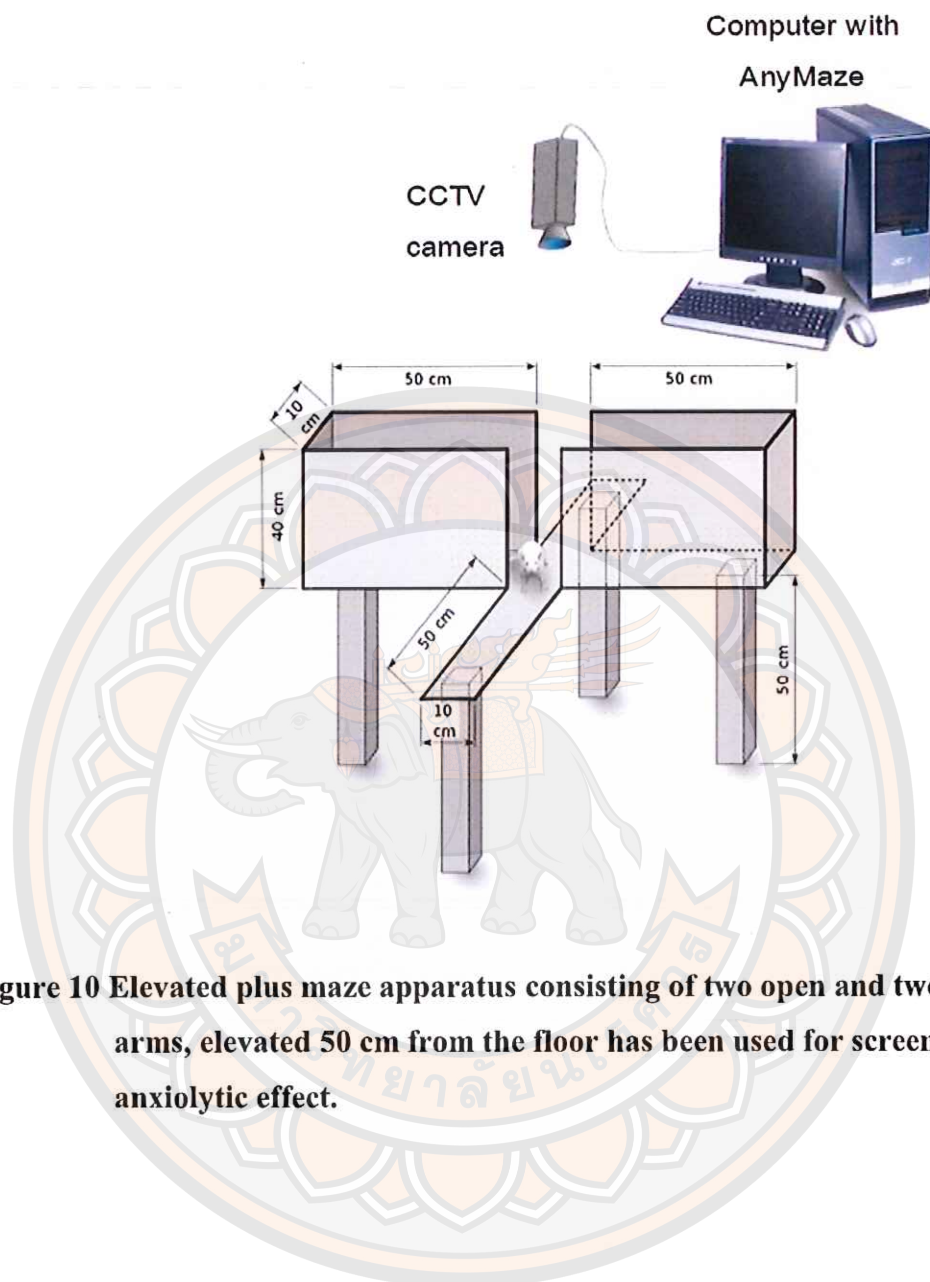
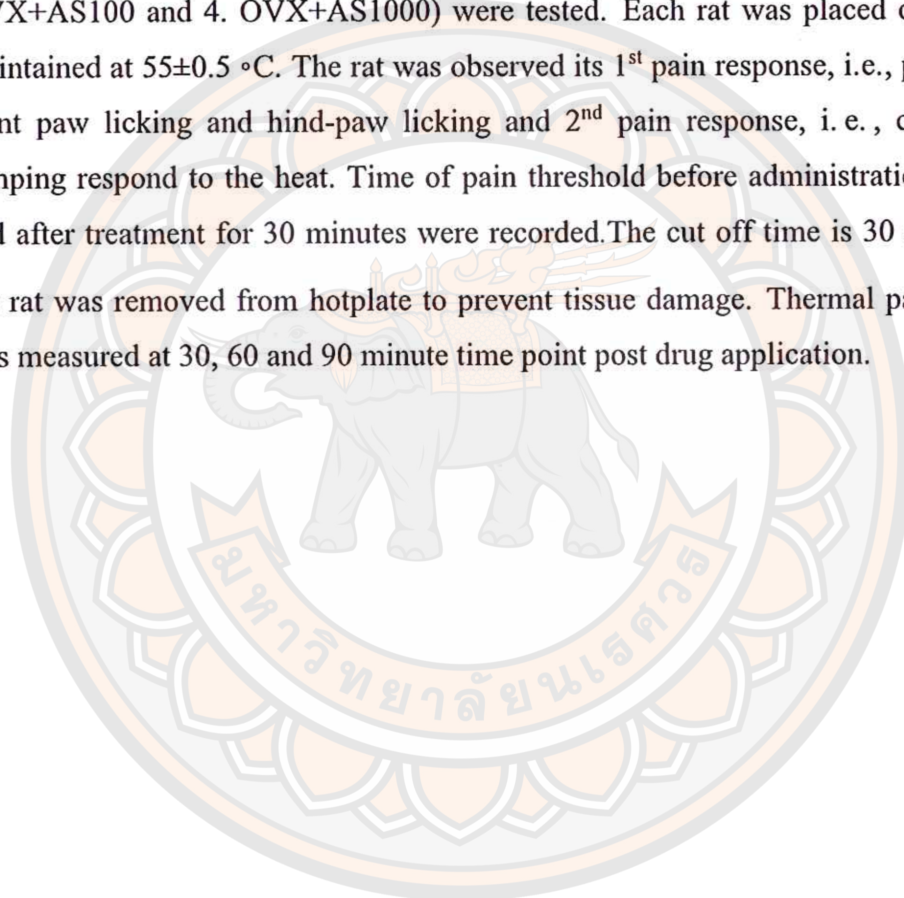


Figure 10 Elevated plus maze apparatus consisting of two open and two closed arms, elevated 50 cm from the floor has been used for screening anxiolytic effect.

4.1.3 Hotplate analgesic test

The screening of antinociceptive effect of a drug using thermal stimulus was firstly introduced by Eddy and Leimbach in 1953 (Eddy and Leimbach, 1953). The equipment used in this study consisted of a thermos-regulated hotplate, a clear plastic cylindrical cage (25 cm height, 30 cm diameter) used to prevent the animal escaping from the hotplate, and a stopwatch.

Four groups of rats (1. OVX control, 2. OVX+D#1, 3. OVX+AS100 and 4. OVX+AS1000) were tested. Each rat was placed on a hotplate maintained at 55 ± 0.5 ∘C. The rat was observed its 1st pain response, i.e., paw shaking, front paw licking and hind-paw licking and 2nd pain response, i. e., climbing and jumping respond to the heat. Time of pain threshold before administration (baseline) and after treatment for 30 minutes were recorded.The cut off time is 30 sec in which the rat was removed from hotplate to prevent tissue damage. Thermal pain threshold was measured at 30, 60 and 90 minute time point post drug application.



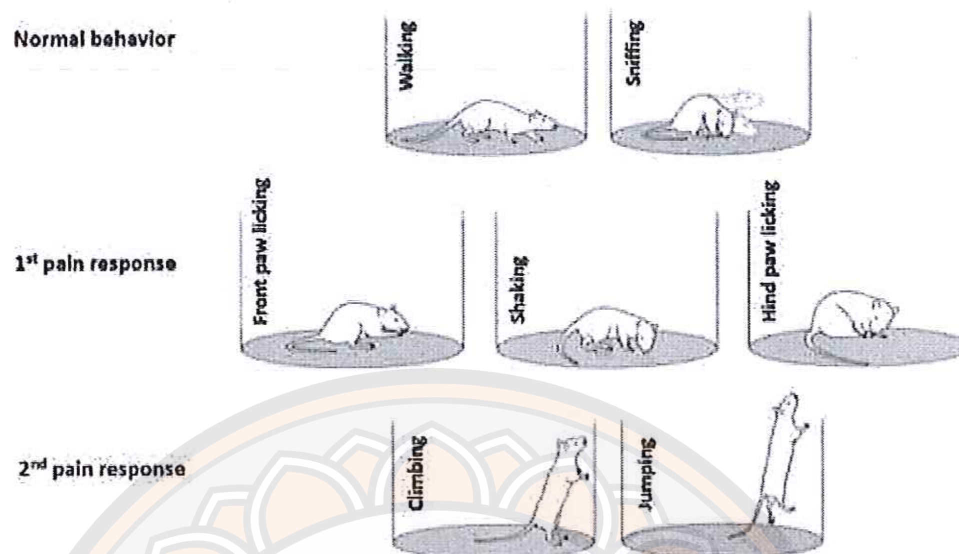


Figure 11 Hotplate analgesic test. Rat behavior in the hotplate test such as normal behavior, i.e., walking and sniffing, 1st pain response, i.e., paw shaking front paw licking and hind-paw licking and 2nd pain response, i.e., climbing and jumping were recorded.

Source: modified from Casarrubea, et al., 2012

4.1.4 Tail-flick analgesic test

Tail flick analgesic test was used for determining tail reflex response or tail withdrawal to noxious stimulus. This technique was first introduced in 1941 by D'Amour and Smith (D'Amour and Smith, 1941). Thermal stimulation of the tail root assay in order to confirm analgesic properties. In the normal rats, the beam of light produces a painful heat causing a reflex of tail. Thermal noxious stimuli was set at 55±0.5°C. Thermal pain thresholds were determined the mean of three stimulations tested before administration for baseline, 30, 60 and 90 min after the administration. Rats were divided 4 groups including 1. OVX control, 2. OVX+D#1 , 3. OVX+AS100 and 4. OVX+AS1000. Cut-off time is 10 sec to prevent tissue damage.

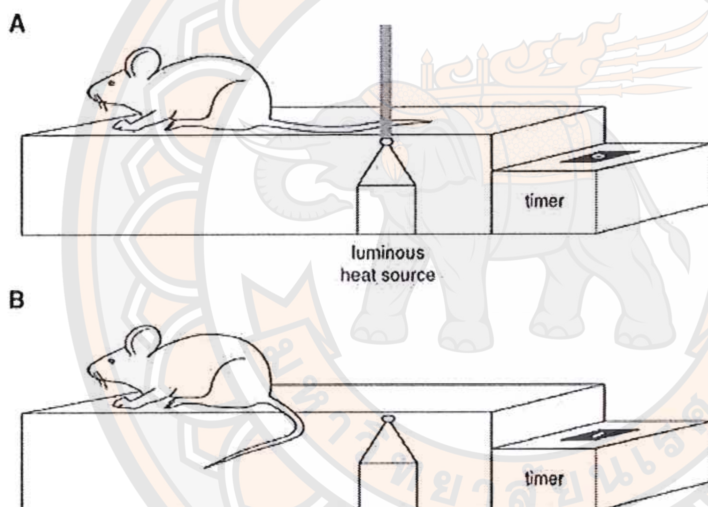


Figure 12 Procedure for tail-flick analgesic test in rat

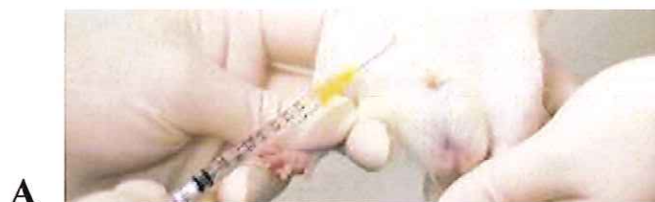
A) Rat's tail exposes an infrared beam

B) The rat's tail withdraws from the radiation when it hurts

Source: http://ja.brc.riken.jp/lab/bpmp/SDOP/en/mc/tail-flick/figure2.jpg

4.1.5 Barbiturate potentiation effect test

Barbiturate is classified as a general anesthesia drug that most widely used as central nervous system depressants. GABA-A receptor consists of barbiturate and benzodiazepine site in CNS. When barbiturate binds to the barbiturate site, it prolong the duration of GABA binding to GABA-A receptor causing a long period of Cl^- channel and hyperpolarization of membrane (Enna and McCarson, 2005; Nobay and Acquisto, 2014). This experiment was aimed to examine the physiological effects of AS on GABA-A receptor. Diazepam binds to the benzodiazepine site on GABA-A receptor causing an increase in frequency of Cl^- channel opening. In this experiment 4 groups of rats (OVX, OVX+D#1, OVX+AS100 and OVX+AS1000) were tested. The number of induction time (loss of righting reaction) and duration of anesthesia were recorded. After treatment for 30 minutes, rats were injected with sodium thiopental (Jagsonpal, India) 25 mg/kg B.W. by i.p injection. Induction time or the time point that rat loss righting reaction (when the rats cannot back to stand on their legs) and duration of anesthesia (when the rats can stand on their legs) were recorded. (Figure 13).

A 

B

C

Figure 13 Procedure for barbiturate potentiation effect test (A) The rat was injected with Thiopental 25 mg/kg, i.p. after the drug administration for 30 min. (B) The rat was anesthetized. (C) The rats can stand on their legs.

4.2 Cognitive function tests

To investigate the subchronic effect of AS, sham and forty ovariectomized rats were used and were divided into 5 groups (ten each) including 1) female old control (OVX) treated with vehicle (reverse osmosis water), 2) female old sham (FOS) which it ovaries were not removed, 3) female old rats treated with donepezil hydrochloride 5 mg/kg B.W. (OVX+D#2), 4) female old rats treated with Agarwood leaves extract (AS) 100 mg/kg B.W. (OVX+AS100) and 5) female old rats treated with AS 1,000 mg/kg B.W. (OVX+AS1000). Rats were subjected to behavior tests include subchronic effect such as the locomotor activity test, the Morris's water maze test and the novel object recognition test.

4.2.1 Morris's water maze (MWM) test

To determine effect of AS on learning and memory in animal model of cognitive deficit by using bilateral OVX. Spatial learning and memory was a retrieval of location data in the environment, assessed using the Morris's water maze test. Hippocampus was part of brain regions associated with spatial memory (Bird and Burgess, 2009). This experiment was developed by Richard Morris in 1981 (Morris, 1981) Morris's water maze setup consists of a circular tank filled with water and a video camera connect to a computer which has Anymaze software installed. There are 4 different symbols, such as star, circle, square and triangle, attached to each wall (curtain) which the rat could use as the visual cues (Figure 14). Before the MWM test, the rats was induced to express memory deficits by using bilateral ovariectomy (OVX) technique. After full recovery, the OVX rats were divided into 4 groups as vehicle, donepezil-treated, and AS-treated groups. In the training phase, each rat was allowed to swim in the circular pool to locate the hidden platform within 90 seconds. The rat was trained 3 times a day for seven days or until the rat could remember where the hidden platform was. On day eighth (day 8th), the hidden platform was removed and the rat was allowed to swim in the circular pool for 90 seconds. The pool was divided into 4 quadrant corresponding to the 4 visual cues (figure 15 (A)). The quadrant triangle was the target zone where the hidden platform used to be located. Escape latency which was a duration used to find a hidden platform and retention memory which was time of spent in target quadrant were recorded and analyze by using video-camera connected with computer had Anymaze software (Stoelting Co.) (Figure 15

(B)). Spatial memory was assessed using a spatial memory index calculated for each animal using the formula:

Spatial memory index = [time of spent in target quadrant / time of spent in non-target quadrant] × 100

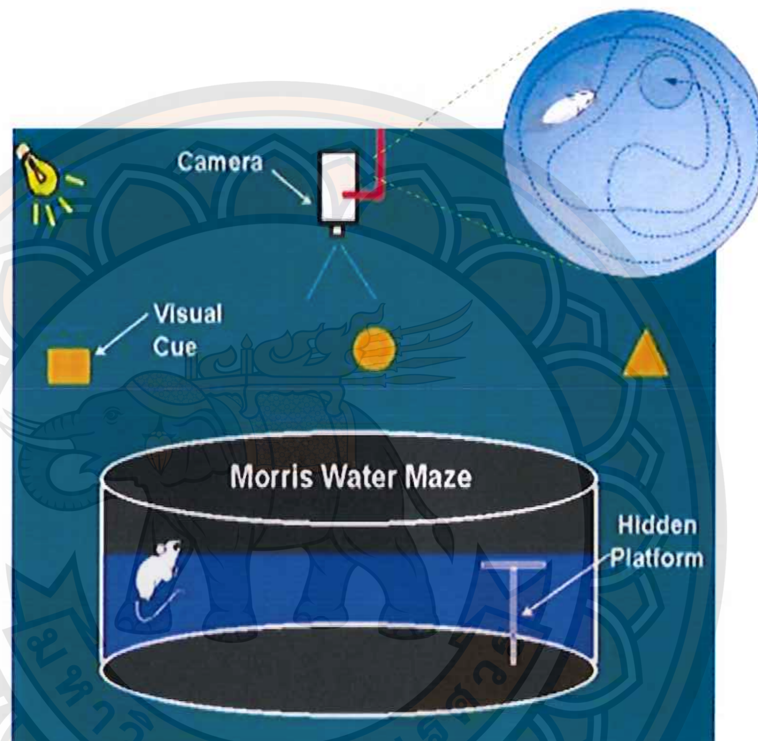


Figure 14 Morris's water maze setup consists of a circular tank filled with tap water, a hidden platform, 4 visual cues, and a video camera.

Reference: http://www.augusta.edu/core/labs/sabc/images/MWM.jpg

Figure 15 Video recording (A) and track plots (B) of the rat's swimming activity from Anymaze software

(A) An example of video recording of rat swimming in a circular pool without a hidden platform.

(B) Examples of track plots of rat swimming cross each quadrant.

4.2.2 Novel object recognition (NOR) test

To determine effect of AS on learning and memory in animal model of cognitive deficit by using bilateral OVX. NOR was employed to evaluate recognition memory, developed by Mumby (D. G. Mumby, 2001) which referred to the ability to remember previously of events, objects, or people. The NOR setup consisted of an empty circular tank and 3 different objects. The rat was placed in the middle of tank bottom to explore 2 different objects. This experiment has two phases. In the training phase, the rat was exposed to 2 different objects such as object A and B for 5 minutes. The rat was removed to its home cage for 5 minutes. Then the rat was put back into the tank again in the testing phase. In testing phase, object B was replaced with object C which was a novel object. Rat was allowed to explore these 2 objects for another 5 minutes (Figure 16). Object exploration was recorded by the following parameters include time(s) of object A exploration during testing phase (TA) and time(s) of object exploration during testing phase (TC). Recognition memory was assessed using a recognition index calculated for each animal using the following formula:

$$\text{Recognition index} = [TC / (TA + TC)] \times 100$$

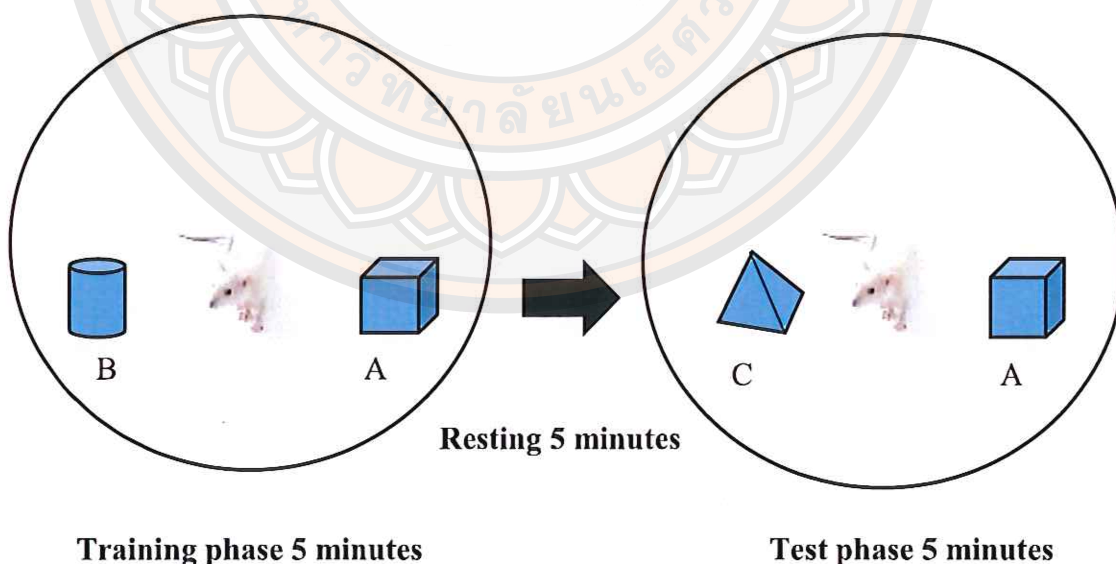


Figure 16 Experimental scheme for novel object recognition test.

5. Tissue preparation

After completing all behavioral studies, the rat was euthanized an overdose of pentobarbital (100 mg/kg B.W., intraperitoneal injection). Then, the brain was removed, weighted and placed on an ice-chilled petri dish. The brain tissue was submerged in nitrogen liquid and stored at a -80°C refrigerator until analyzed. Prior to analysis, the brain was thawed, with normal saline and homogenized in 0.1 M perchloric acid (volume 200 μl). After that, the homogenized tissue was centrifuged at 12,000 rpm at 4 ◦C for 20 minutes. The supernatant was collected and stored at -80 ◦C for future analysis. The concentrations of neurotransmitter were determined by using a high performance liquid chromatography technique (HPLC) and the amount of protein was determined by using a BCA assay kit.

Figure 17 procedures for brain tissue collection

6. Determination of serum estrogen (estradiol or E_2) level

Blood samples from OVX, FOS, OVX+AS100, OVX+AS1000, groups were collected at the end of experiment. Serum was separated from the blood by using centrifugation and then kept on ice until analysis. Serum (80 µl) was filled into cuvette and incubated 4.7 minutes at 37 °C. Serum estradiol level was measured by using electrochemiluminescence immunoassay (ECLA). The serum biochemical assay was employed at Biolab Medical Clinic, Phitsanulok, Thailand.

7. Determination of neurotransmitter levels by biochemical analysis

Determination of monoamine neurotransmitters by using a high performance liquid chromatography technique

To determine the basal monoamine neurotransmitter level in the brain, a reverse phase HPLC protocol was performed by using HR-80x3.2mm (Thermo science, U.S.A.) analytical column protected with a security guard cartridge spherisorb column contained 5 µm ODS2 particle (Waters Corp, USA.). Components were eluted isocratically using a used HPLC pump (LC-20AD, Shimadzu, Japan) flow rate 0.7 ml per minute, Injection volume 20 µl. Oven temperature (CTO-10AS, Shimadzu) was for controlling the oven temperature at 32 °C. Monoamine neurotransmitter were oxidized and detected by using an ECD (ESA Coulochem III, USA). The mobile phase was circulated on the HPLC system and changed every week or as required. LC solution analysis software was used to analyze chromatogram samples.

The mobile phase for HPLC-ECD analysis consisted of sodium dihydrogen phosphate monohydrate (Ajax Finechem, Australia), ethylenediaminetetra-acetic acid (EDTA) (Fluka, USA) and Citric acid (Fisher chemical) dissolved in Methanol (HPLC grade, Lab-Scan Analytical Sciences, Lab-Scan Asia Co., Ltd., Thailand.) and acetonitrile (HPLC grade, Lab-Scan Analytical Sciences, Lab-Scan Asia Co., Ltd.), and diluted with distillated water. The solution pH was adjusted to pH 5.60 as needed with 14 N Sodium hydroxide.

For the preparation of standard solution, stock solutions of dopamine (DA), serotonin (5-HT) 1 mg were prepared by dissolving in 0.1 M perchloric acid volume 1000 µl. The stock solutions were filtered with 0.22 µm nylon membrane filters and kept at 4 ◦C in refrigerator.

For constructing of standard calibration curve, five different concentrations of each standard amine solution were injected into the HPLC-ECD system in order to yield 5 chromatograms, i.e. standard of 5-HT at 5, 10, 15, 20, 25 μg/mL, standard of DA at 10, 20, 40, 60, 80 mg/mL. The peak area of each chromatogram was measured and used for plotting a standard calibration curve (Figure 38 and Figure 39).

Determination of amino acid neurotransmitters by using a high performance liquid chromatography technique

To examine the basal amino acid neurotransmitter levels, a reverse phase HPLC protocol was performed by using 250x4.6 mm, 5 micron (Thermo science, USA) analytical column protected with a security guard cartridge spherisorb column 5 µm ODS2 particle (Waters Corp, USA). Components were eluted isocratically using a Degasser (DGU-14A, Shimadzu, Japan) and solvent delivery module (LC-10AD vp, Shimadzu, Japan) flow rate 1 ml per minute, Injection were 50 µl. Column oven (CTO-AS vp, Shimadzu) was controlled at 32 ºC. A fluorescence detector (Waters. Corp) was used to determining amino acid. The mobile phase was circulated on the HPLC system and changed every week or as required. Shimadzu, Japan 10AVP HPLC System was used to analyze samples.

Stock solutions of aspartate (ASP), glutamate (Glu), serine, glutamine (Gln), Glycine, gamma aminobutyric acid (GABA) were dissolved in 0.1 M perchloric acid volume 1000 µl. Stock solutions filtered with 0.22 µm nylon membrane filters and kept at 4 ◦C.

The derivatization process of amino acid analysis was done by mixing of 100 µl derivatizing solution and 50 µL of standard solution or sample at room temperature 2 min before injecting into the HPLC system for amino acid analysis.

The concentration of amino acid neurotransmitters in the supernatant were obtained from their peak areas of the chromatogram.

Protein Analysis

To determine protein the amount of neurotransmitter in rat's brain, the supernatant of homogenized brain was assayed by using a BCA protein assay kit. The procedure for protein analysis can be described in the following steps.

1. Diluted BSA standard into clean vials, as shown in Table 2

Table 2 Preparation of Diluted Albumin Standards

Vial	Volume of diluent (µL)	Volume and source of BSA (µL)	Final BSA concentration (µg/mL)
A	465	465	1000
B	200	600 of vial A dilution	750
C	200	400 of vial A dilution	500
D	200	200 of vial A dilution	250
E	200	180 of vial A dilution	125
F	200	100 of vial A dilution	25

2. Diluted supernatant and added 90 µl of 0.1M perchloric acid solution.
3. 25 µl of the supernatant, protein standard and blank was pipetted into well plate.
4. Prepared the BCA Working Reagent (WR) by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B)
5. Added 200 µL of the WR to each tube and mix well 30 seconds.
6. Covered and incubated 30 minutes at 37° C
7. Measured the absorbance at 562 nm by microplate reader (1401, LabSystem, iEMS Reader MF, Finland) within 10 minutes.
8. Plotting a standard curve based on absorbance of the protein standards and determined the protein concentration of each unknown sample according to the BCA Protein Assay Kit.

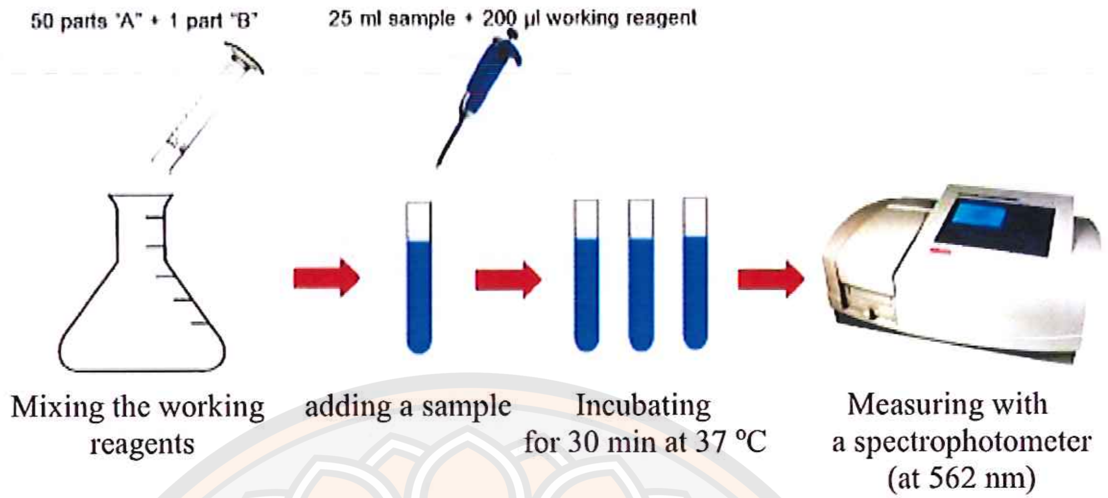
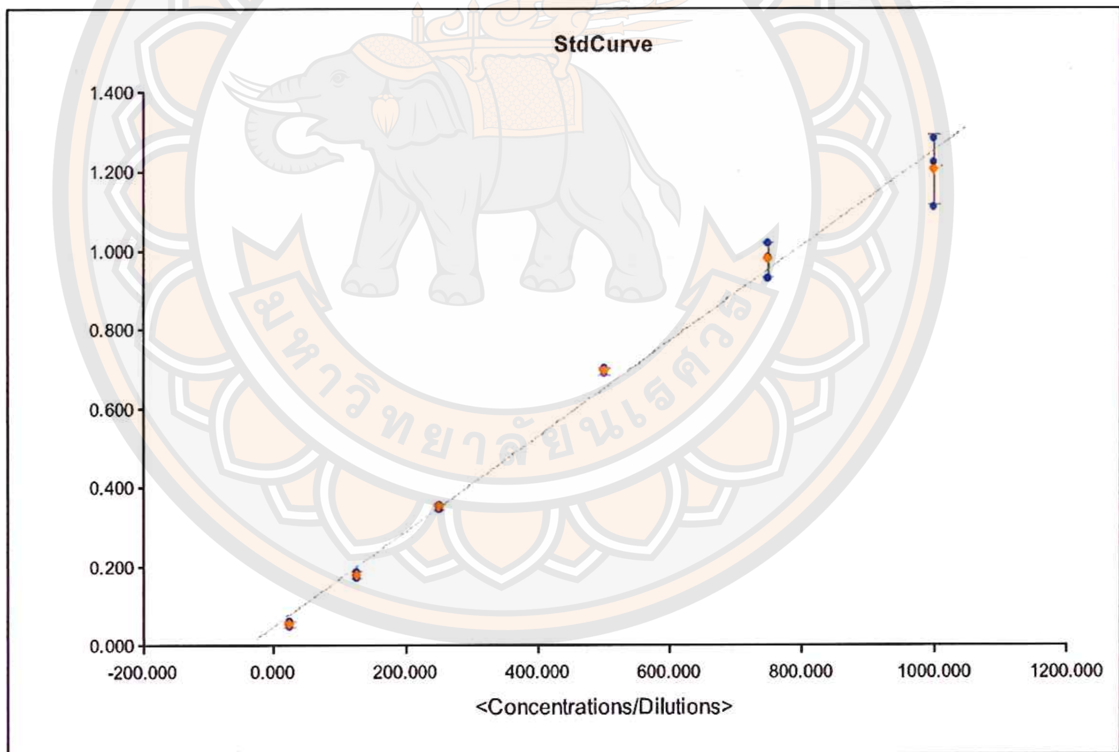


Figure 18 Procedure for protein analysis



Curve Name	Curve Formula	A	B	R^2
StdCurve	Y=A*X+B	0.0012	0.046	0.995

Figure 19 Standard calibration curve of BSA standard

Statistical analysis

The results were analyzed by *t*-test $P < 0.05$. The control was considered statistically significant using Microsoft Office Excel software, version 2013. The experiments were performed for statistical analysis and expressed as mean±SEM.

CHAPTER IV

RESULTS

Screening of the CNS action of agarwood leaves extract

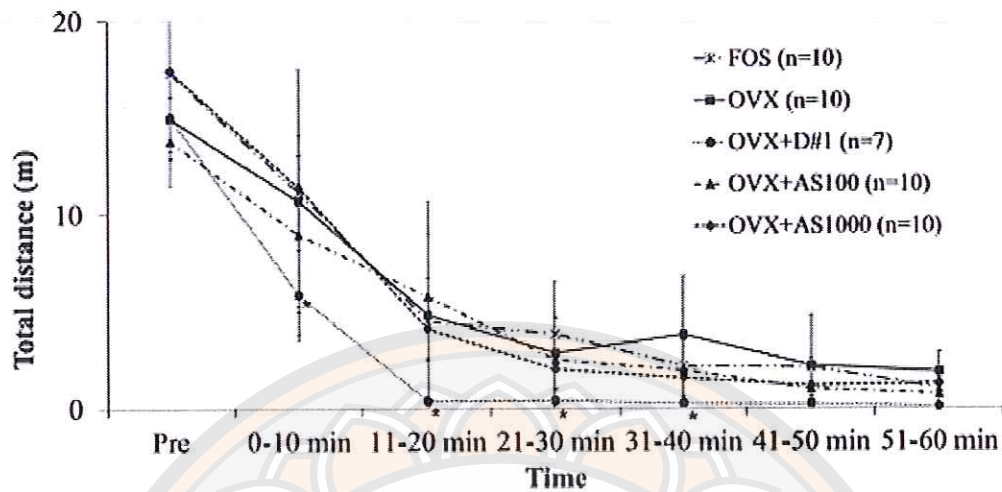
1. Acute effect of agarwood leaves extract on locomotor activity

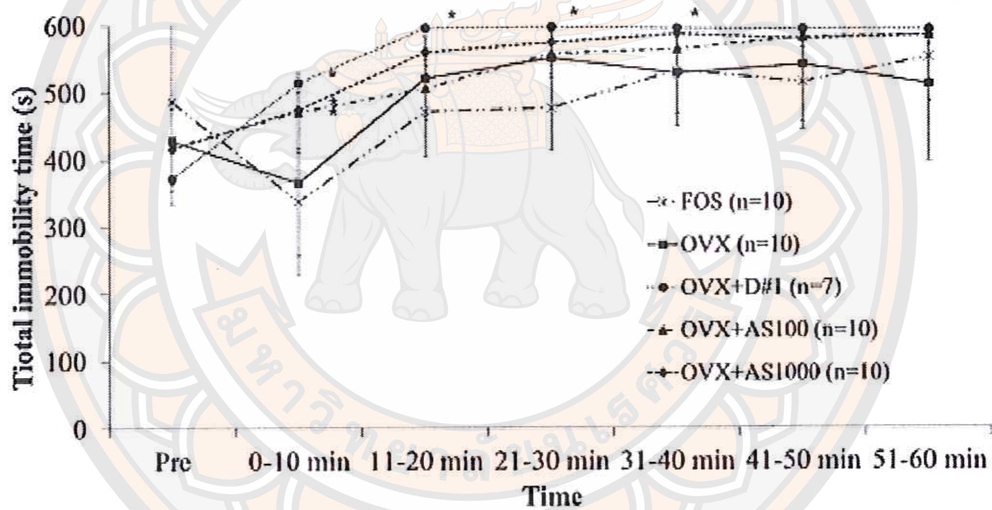
By sampling within a 10-min time point, the profile of rat's locomotion in a circular chamber for each group are shown in Figure 20A. When compared to the control (OVX), the diazepam-treated groups (OVX+D#1) showed significantly lower spontaneous locomotion during 0-10, 11-20, 21-30, 31-40, 41-50, 51-60 min after administration ($P < 0.05$, unpaired t-test). In addition, Figure 20B the AS-treated groups (OVX+AS100 and OVX+AS1000) showed significantly longer immobilization time during 0-10 min post administration of the AS-treated groups when compared to the control ($P < 0.05$, unpaired t-test).

When compared the total data during a 60-min period after administration, the total distance of control (OVX) and positive control (OVX+D#1) groups measured 26.13 ± 10.73 m and 7.02 ± 2.29 m, respectively. The total immobilization time of the control group (OVX) and positive control group (OVX+D#1) measured 3016.39 ± 269.92 s and 3474.70 ± 32.11 s (Figure 21A). On the other hand, the total distance of FOS, OVX+AS100, and OVX+AS1000 groups measured 24.63 ± 13.53 m, 20.65 ± 10.77 m, and 21.37 ± 13.05 m, respectively. The total immobilization time of FOS, OVX+AS100, and OVX+AS1000 groups measured 2884.21 ± 296.43 s, 3262.82 ± 120.07 s, and 3336.57 ± 96.63 s (Figure 21B).

All together, the results show that AS leaves extract has a mild CNS depressant action.

A





B

Figure 20 Profile of rat's locomotor activity before (Pre) and 10 min epochs (i.e. 0-10, 10-20, 20-30, 30-40, 40-50, 50-60 min post administration). Each bar represents mean ± SD.

(A) Distance of rat's locomotor activity obtained every 10 min before and after administration.

(B) Immobilization time obtained every 10 min before and after administration. * = $p < 0.05$ when compare to the control.

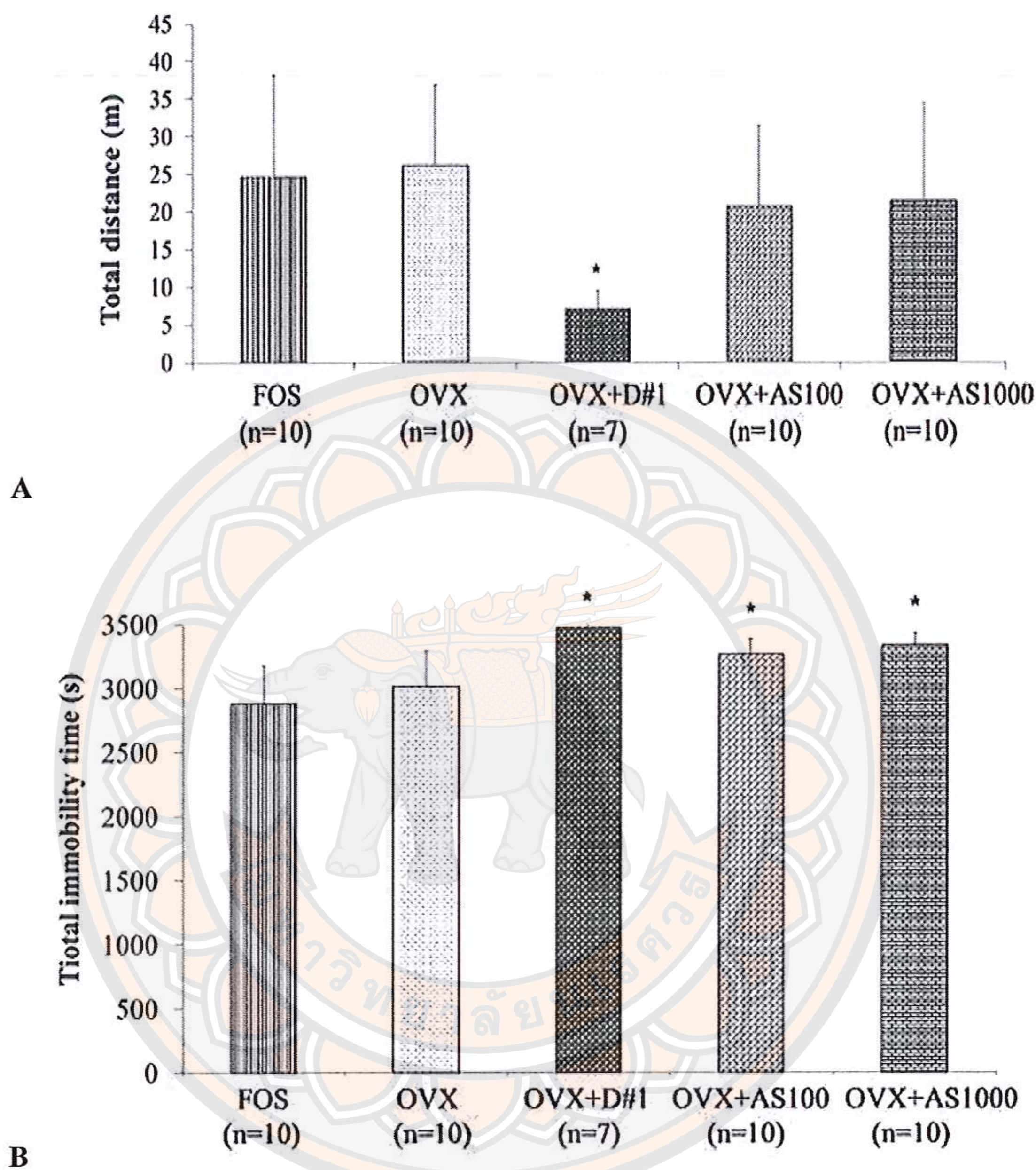


Figure 21 (A) Total distance post administration. (B) Total immobilization time post administration. Each bar represents mean ± SD. * = $p < 0.05$ when compare to the control.

2. Screening of an acute anxiolytic effect of agarwood leaves extract

Figure 22 shows the anxiolytic indices from each group during pre and post drug administrations. The indices of control group show no significant difference between Pre (0.45 ± 0.12) and post administration (0.49 ± 0.16) (P > 0.05, paired t-test). Interestingly, the indices of OVX+D#1 group during pre treatment period (0.36 ± 0.05) show significantly lower than that of during post administration (0.52 ± 0.04) (P < 0.05, paired t-test). However, the index of FOS group is slightly higher but does not significantly different during post treatment (0.41 ± 0.11) when compared to during pre treatment (0.37 ± 0.11) (P > 0.05, paired t-test). Similarly, the indices of OVX+AS100 and OVX+AS1000 groups during pre treatment are 0.35 ± 0.14 and 0.36 ± 0.12 which do not significantly different when compared to those from post treatments which are 0.36 ± 0.07 and 0.41 ± 0.12, respectively (P > 0.05, paired t-test). These results indicate that AS leaves extract does not have anxiolytic action.

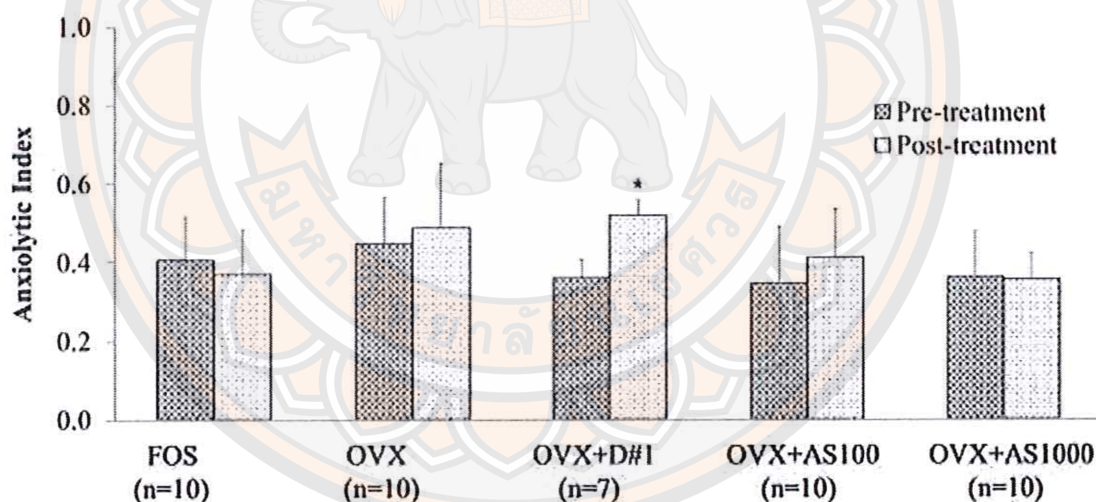


Figure 22 Screening of the acute anxiolytic action of AS leaves extract using an elevated plus maze test. Each bar represents mean ± SD of anxiolytic index, * = $p < 0.05$ when compare to the control (paired t-test).

3. Screening of acute anti-nociceptive effect of agarwood leaves extract

3.1 Hotplate analgesic test

By using a constant (55±0.5 C°) temperature hotplate as a pain stimulus, the mean latencies of 1st and 2nd pain responses calculated from each group are shown in Figure 23. For the results of control (OVX) and sham (FOS) groups, 1st and 2nd pain response latencies measured 6.02 ± 5.38 s and 20.07 ± 8.38 s, and 9.81 ± 5.06 s and 23.41 ± 6.19 s, respectively.

For the results of AS-treated groups, OVX+AS100 group measured 13.08 ± 5.14 s and 27.24 ± 5.99 s, whereas those of OVX+AS1000 group measured 27.27 ± 9.01 s and 30 ± 1.63 s. When compared to the control, the 1st and 2nd pain response latencies of OVX+AS100 and OVX+AS1000 groups were significantly longer ($P < 0.05$, unpaired t-test).

3.2 Tail-flick analgesic test

By using a tail-flick analgesiometer, the latencies of pain response obtained from each group are shown in Figure 24. The pain response latencies of control (OVX) and sham (FOS) groups during post administration for 30, 60 and 90 min measured 1.3 ± 0.5, 1.2 ± 0.3, and 1.2 ± 0.3 s, and 1.06 ± 0.3 s, 1.90 ± 0.3 and 1.4 ± 0.7 s, respectively. The pain response latencies of OVX+AS100 group during post administration for 30, 60 and 90 min show no significant difference when compared to the control ($P > 0.05$, unpaired t-test). However, the pain response latencies during post administration for 30, 60 and 90 min of OVX+AS1000 measured 2.0 ± 1.0 s, 1.9 ± 0.8 and 2.0 ± 0.9 s and positive control groups (OVX+D#1) measured 2.2 ± 0.5 s, 2.2 ± 0.9 and 2.1 ± 1.0 s. are significantly longer than the control ($P < 0.05$, unpaired t-test).

All together, the results from these 2 analgesic tests show that AS leaves extract has an acute effect on pain.

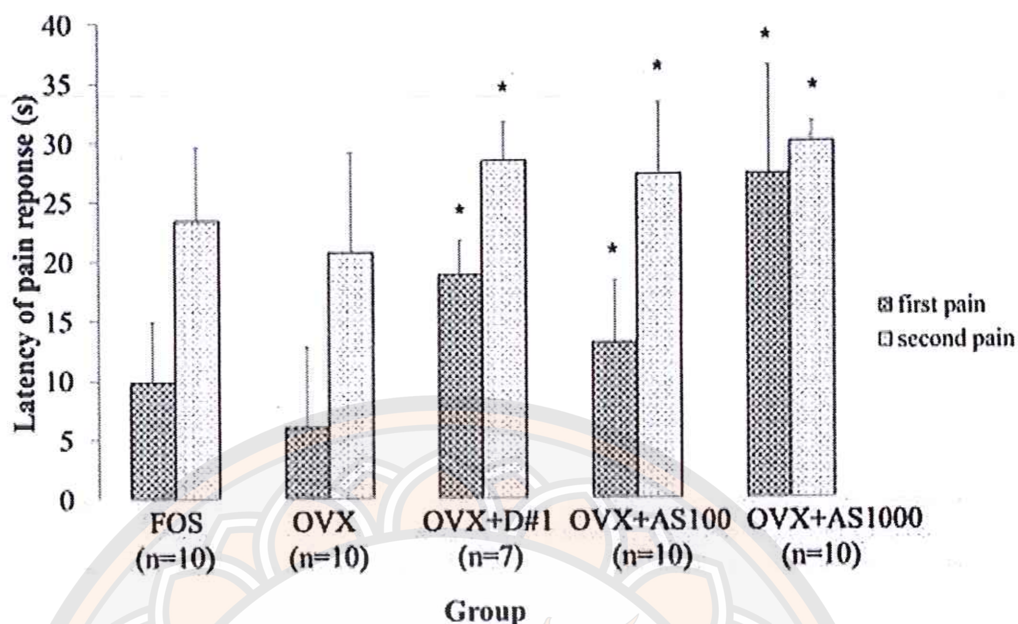


Figure 23 Screening of the acute antinociceptive action of AS leaves extract using a hotplate analgesic test. After administration for 30 min, the pain response latencies were quantified as 1st and 2nd pain responses to a constant heat (55.0 ± 0.5 °C). Each bar are mean ± SD, * = $p < 0.05$ when compared to the control (unpaired t-test).

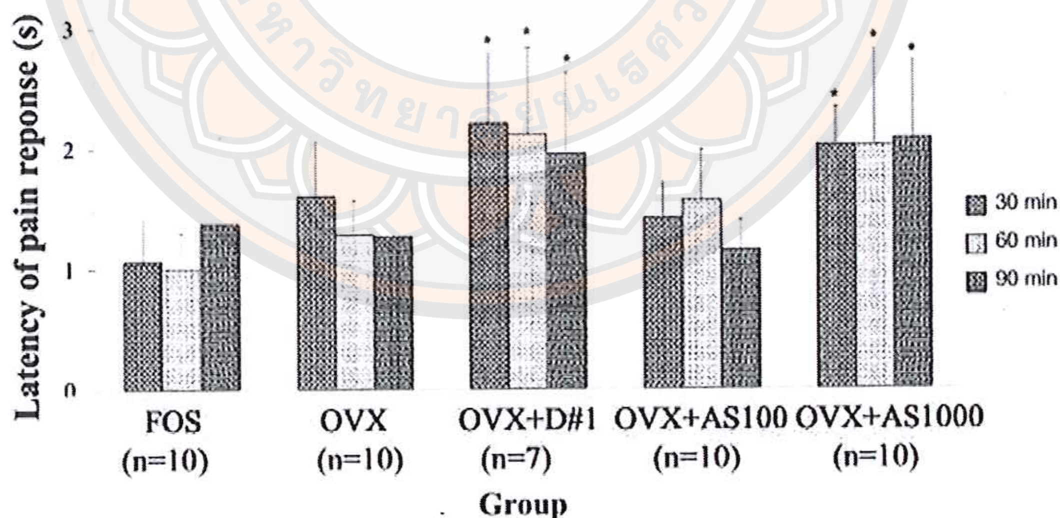


Figure 24 Screening of the acute antinociceptive action of AS leaves extract using a tail-flick analgesic test. The pain response latencies were quantified at 30, 60, and 90 min after administration. Each bar represents mean ± SD, * $p < 0.05$ when compare to the control (unpaired t-test).

4. **Screening of barbiturate potentiation efficacy of agarwood leaves extract**

After RO water administration for 30 min, the onset time (induction time) and duration of thiopental induced anesthesia for control (OVX) and sham (FOS) groups measured 2.97 ± 0.72 min and 15.10 ± 3.46 min, 1.94 ± 0.31 min and 17.26 ± 2.34 min, as shown in Figure 38. For the test groups, the onset times of OVX+AS100 and OVX+AS1000 groups (measured 2.82 ± 0.62 min and 3.37 ± 0.68 min, respectively) did not differ from the control (P > 0.05, unpaired t-test). However, the duration of anesthesia of OVX+AS100 and OVX+AS1000 groups (measured 26.88 ± 6.70 min and 20.98 ± 6.24 min, respectively) were significantly longer than the control (P < 0.05, unpaired t-test). The result indicates that AS leaves extract has mild efficacy on potentiating barbiturate effect.

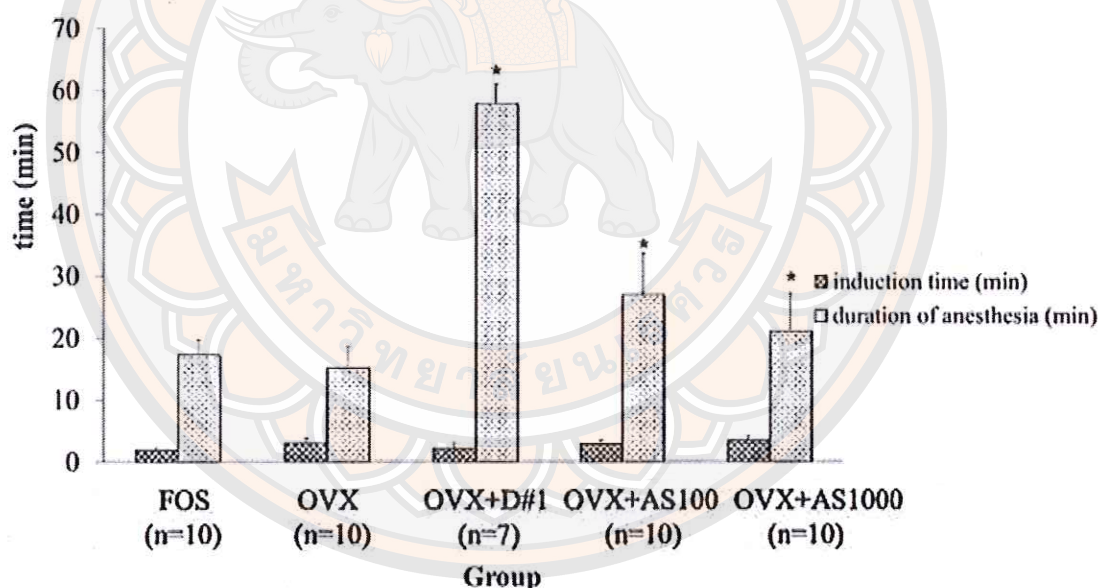


Figure 25 Screening of barbiturate potentiation on induction time and duration of anesthesia after administration for 30 minutes. The diazepam group, OVX+AS100 and OVX+AS1000 showed significantly longer duration of anesthesia than the control group. Each bar represents mean ± SD, * = p < 0.05 when compare to the control (Student's unpaired t-test).

5. Screening of cognitive effect of agarwood leaves extract

5.1 Morris's water maze test (MWM)

After a 2 month period of administration, the learning profile of the animal to locate a hidden platform in a circular pool within 7 days during the training phase is shown in Figure 26. Although the escape latencies (time to locate the hidden platform) from each group of animals were vary in Day 1 (ranging from 12.3 – 25.5 s), these values were relatively no difference during Day 4 to Day 7 (ranging from 3.3 – 6.8 s). On the test phase (testing without the hidden platform), the spatial memory indices of control (OVX) and sham (FOS) groups were 17.2 ± 5.1 and 22.3 ± 3.10, respectively (Figure 26). However, the spatial memory index of OVX+AS100 and OVX+AS1000 were 25.99 ± 5.59 and 25.53 ± 3.76, which were significantly higher compared to the control ($P < 0.05$, unpaired t-test). The values of these tested group are close to that of the positive control group (OVX+D#2,) which received 5 mg/kg donepezil for 2 months and the index is 26.79 ± 4.43. This indicates that AS leaves extract could help to improve spatial memory in ovariectomized rats.

5.2 Novel object recognition test (NOR)

The recognition memory of each group was determined by using a novel object recognition test after a 2 month period of administration. As shown in Figure 28, the recognition index of control (OVX) and sham (FOS) groups were 42.63 ± 8.41 and 64.13 ± 11.32, respectively. When compared to the control group, the indices of both OVX+AS100 and positive control group (OVX+D#2) are significantly higher (69.27 ± 12.07 and 77.61 ± 16.44, respectively) ($P > 0.05$, unpaired t-test). However, the index of OVX+AS1000 group (52.86 ± 19.42) does not significantly differ when compared to the control. This result may imply that AS leaves extract at the dose of 100 mg/kg can help to improve recognition memory in ovariectomized rats as well.

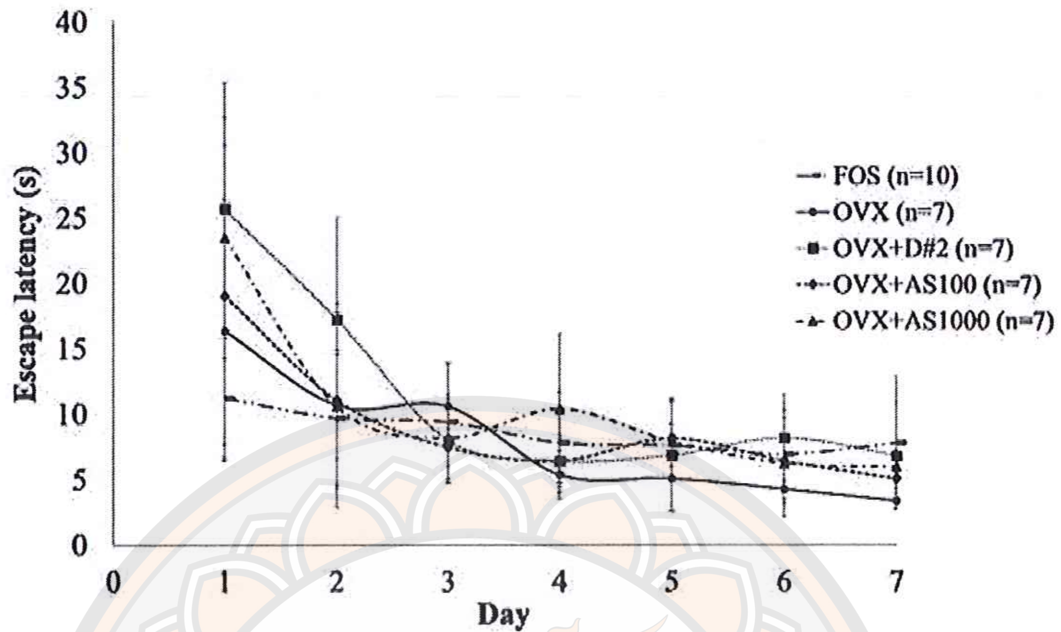


Figure 26 Learning profile of each group of the rats within a 7-day training phase of a Morris water maze test. Each data point represents mean ± SD of escape latency.

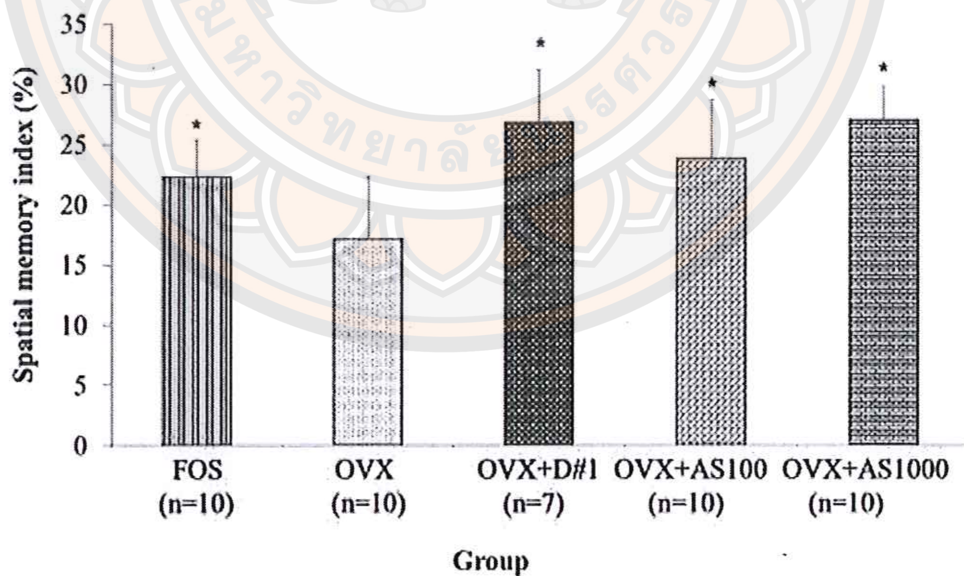


Figure 27 Comparison of spatial memory index of each group evaluated by using a probe trial test (on Day 8). Each bar represent the mean ± SD, * = $p < 0.05$ when compare to the control (OVX) using Student's unpaired t-test.

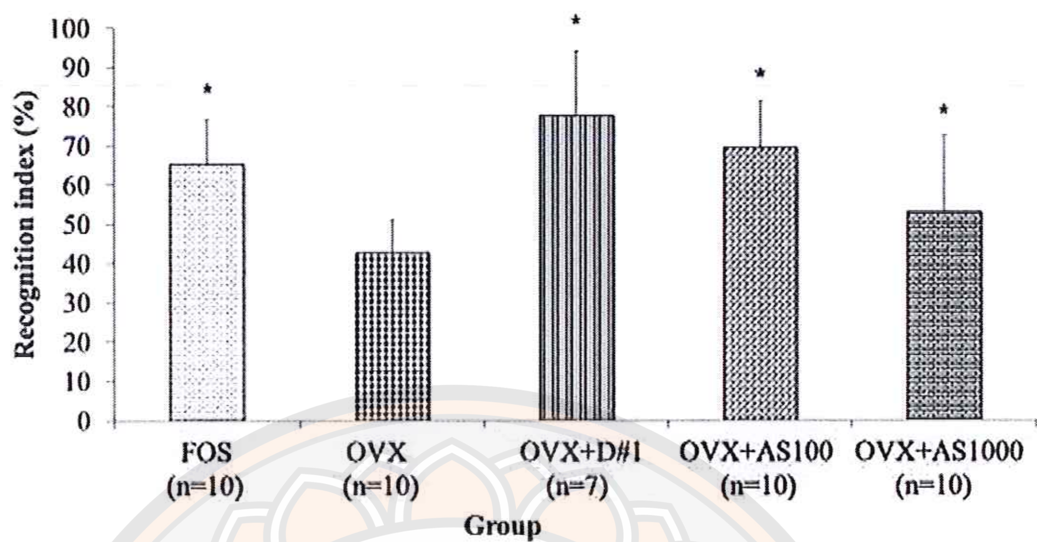


Figure 28 Comparison of recognition index of each group evaluated by using a novel object recognition test. Each bar represent the mean ± SD, * = p < 0.05 when compare to the control (OVX) using Student's unpaired t-test.

6. Serum estrogen analysis

By using electrochemiluminescence immunoassay (ECLA), serum estrogen level from each group of animals was determined and summarized as shown in Figure 29. In ovariectomized control rats (OVX), the serum estrogen level measured 20.90 ± 10.55 which was significantly lower than those of sham group (FOS, 39.87 ± 15.11) ($P < 0.05$, unpaired t-test). In contrast, the serum estrogen level of both OVX+AS100 and OVX+AS1000 groups were 37.38 ± 4.98 and 44.13 ± 8.65, which were significantly higher than that of the control (OVX) ($P < 0.05$, unpaired t-test).

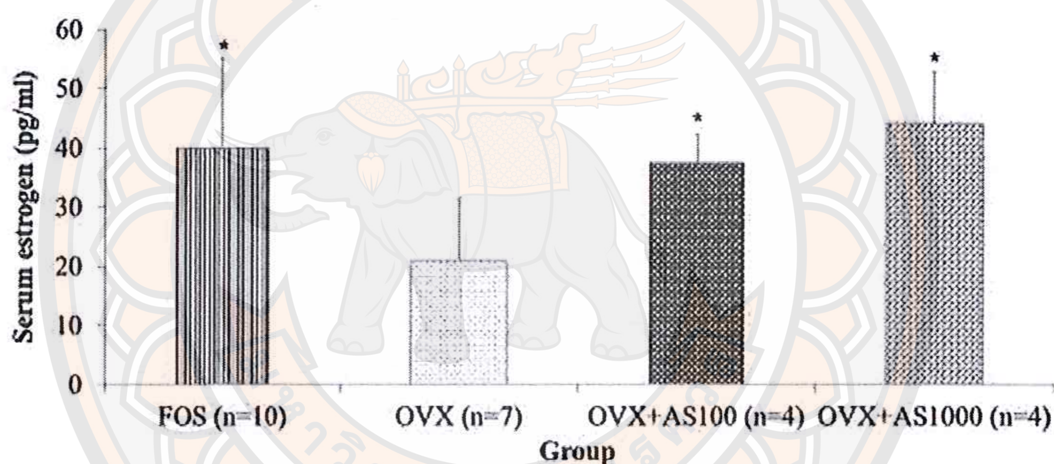


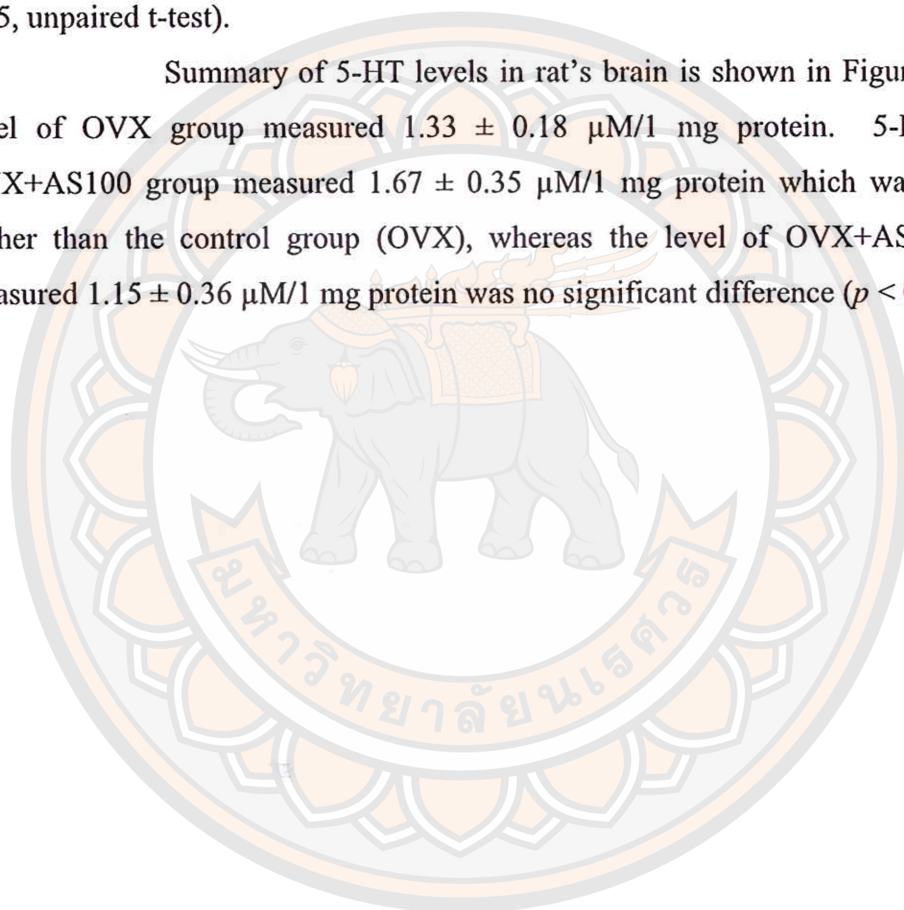
Figure 29 Comparison of serum estrogen levels of control (OVX), sham (FOS), and AS-treated (OVX+AS100, OVX+AS1000) groups. Each bar represents the mean ± SD, * = $p < 0.05$ when compare to the control (OVX) using Student's unpaired t-test.

7. Assay for brain neurotransmitters

7.1 Levels of brain amine neurotransmitters

Summary of DA levels in rat's brain is shown in Figure 30. DA level of control group (OVX) measured 9.25 ± 0.99 μM/1 mg protein. Likewise, DA level of OVX+AS100 measured 8.84 ± 1.31 μM/1 mg protein, which was no significant difference when compare to the control ($P > 0.05$, unpaired t-test). However, DA level of OVX+AS1000 measured 6.36 ± 2.03 which was significantly than the control ($P < 0.05$, unpaired t-test).

Summary of 5-HT levels in rat's brain is shown in Figure 31. 5-HT level of OVX group measured 1.33 ± 0.18 μM/1 mg protein. 5-HT level of OVX+AS100 group measured 1.67 ± 0.35 μM/1 mg protein which was significant higher than the control group (OVX), whereas the level of OVX+AS1000 group measured 1.15 ± 0.36 μM/1 mg protein was no significant difference ($p < 0.05$).



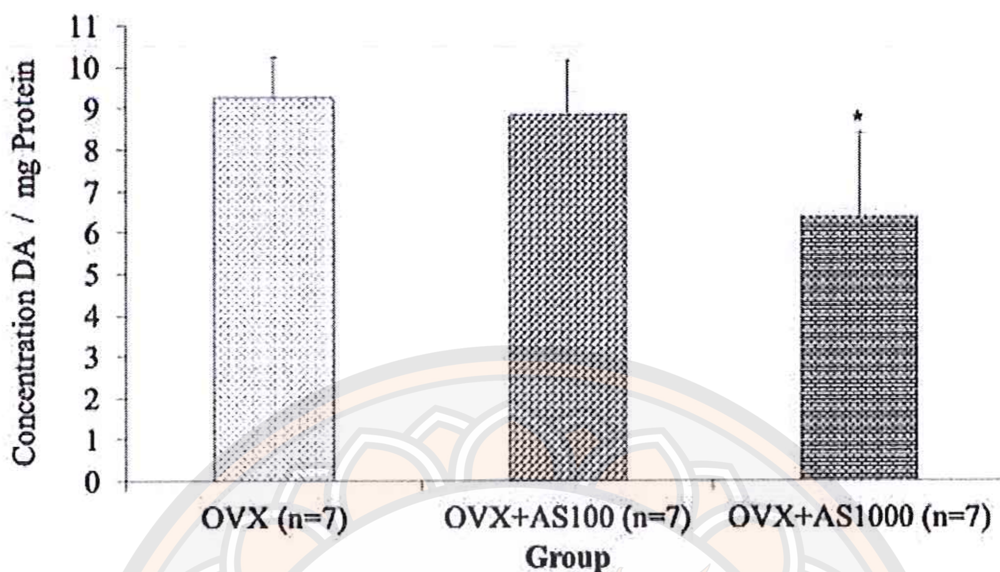


Figure 30 DA levels in rat's brains analyzed by using a HPLC-ECD. Each bar represents mean ± SEM, * = $p < 0.05$ when compare the control (Student's unpaired t-test).

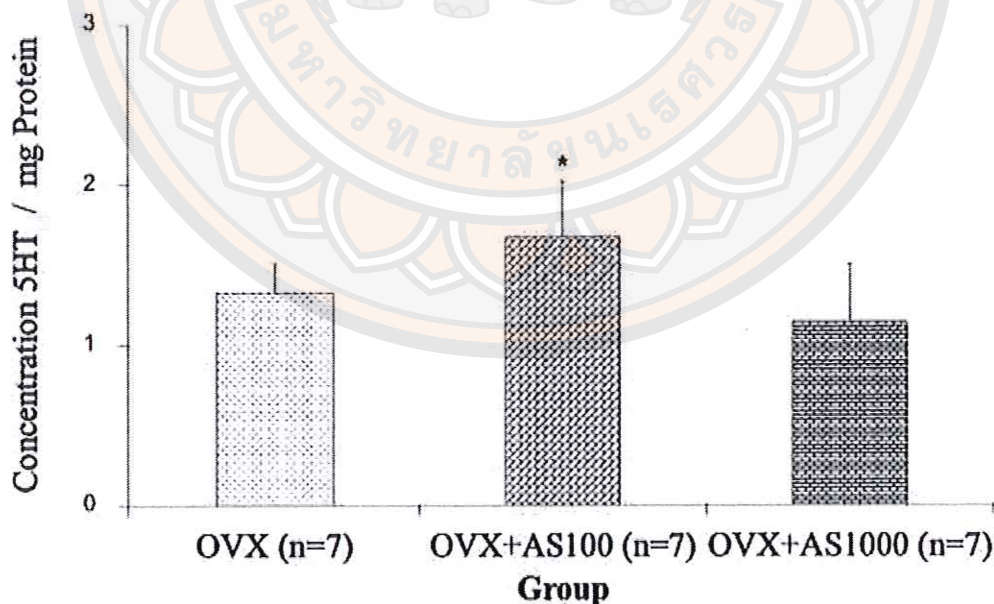


Figure 31 5-HT levels in rat's brains analyzed by using a HPLC-ECD. Each bar represents mean ± SEM, * = $p < 0.05$ when compare the control (Student's unpaired t-test).

7.2 Levels of brain amino acid neurotransmitters

Summary of hippocampal aspartate (ASP) levels of each group is shown in Figure 32. The ASP levels of the rat OVX, OVX+AS100, and OVX+AS1000 measured 0.89 ± 0.38, 0.96 ± 0.40, and 0.76 ± 0.29 µM/1 mg protein, respectively. There was no significant difference of the ASP levels in AS-treated group when compared to the control group ($P > 0.05$, unpaired t-test).

Summary of hippocampal glutamate (GLU) levels of each group is shown in Figure 33. The GLU levels of the rat OVX, OVX+AS100, and OVX+AS1000 measured 2.39 ± 0.48, 2.15 ± 0.62, and 1.50 ± 0.38 µM/1 mg protein, respectively. There was a significant decrease of the GLU level in AS1000-treated (OVX+AS1000) group when compared to the control group ($P < 0.05$, unpaired t-test).

Summary of hippocampal serine (SER) levels of each group is shown in Figure 34. The SER levels of the rat OVX, OVX+AS100, and OVX+AS1000 measured 0.12 ± 0.02, 0.12 ± 0.03 and 0.10 ± 0.05 µM/1 mg protein, respectively. There was no significant difference of the SER levels in AS-treated group when compared to the control group ($P > 0.05$, unpaired t-test).

Summary of hippocampal glutamine (GLN) levels of each group is shown in Figure 35. The GLN levels of the rat OVX, OVX+AS100, and OVX+AS1000 measured 0.27 ± 0.08, 0.27 ± 0.06, and 0.17 ± 0.06 µM/1 mg protein, respectively. There was a significant decrease of the GLN level in AS1000-treated (OVX+AS1000) group when compared to the control group ($P < 0.05$, unpaired t-test).

Summary of hippocampal glycine (GLY) levels of each group is shown in Figure 36. The GLY levels of the rat OVX, OVX+AS100, and OVX+AS1000 measured 0.18 ± 0.02, 0.20 ± 0.07, and 0.13 ± 0.05 µM/1 mg protein, respectively. There was no significant difference of the GLY levels in AS-treated group when compared to the control group ($P > 0.05$, unpaired t-test).

Summary of hippocampal GABA levels of each group is shown in Figure 37. The GABA levels of the rat OVX, OVX+AS100, and OVX+AS1000 measured 0.52 ± 0.14, 0.63 ± 0.16 and 0.44 ± 0.14 μM/1 mg protein, respectively. There was no significant difference of the GABA levels in AS-treated group when compared to the control group ($P > 0.05$, Student's unpaired t-test).

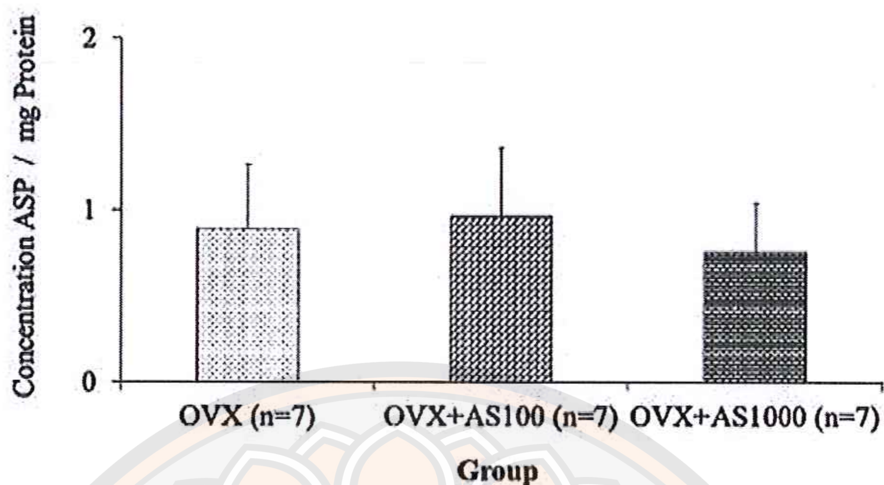


Figure 32 Hippocampal aspartate (ASP) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector. Each bar represents mean ± SEM. There was no significant difference of the ASP level of AS-treated groups when compare the control (P > 0.05, Student's unpaired t-test).

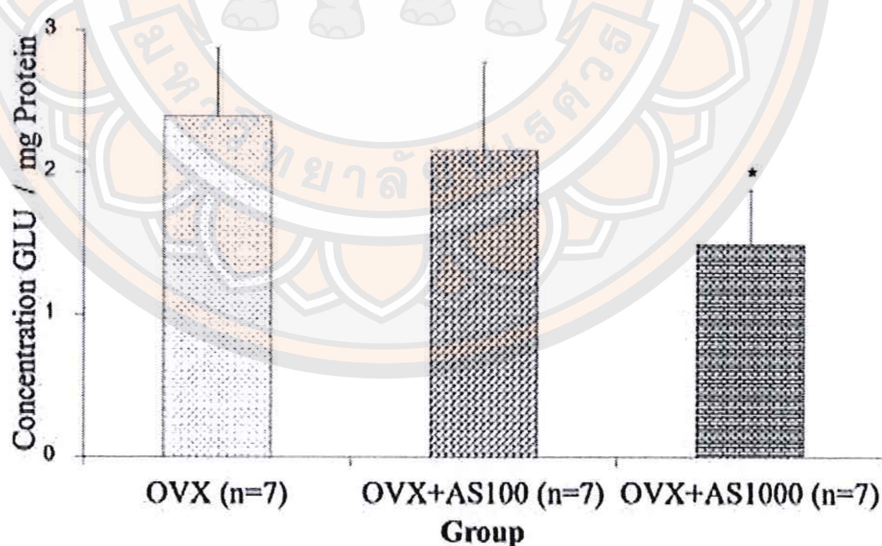


Figure 33 Hippocampal glutamate (GLU) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector. Each bar represents mean ± SEM, * = $p < 0.05$ when compare the control (Student's unpaired t-test).

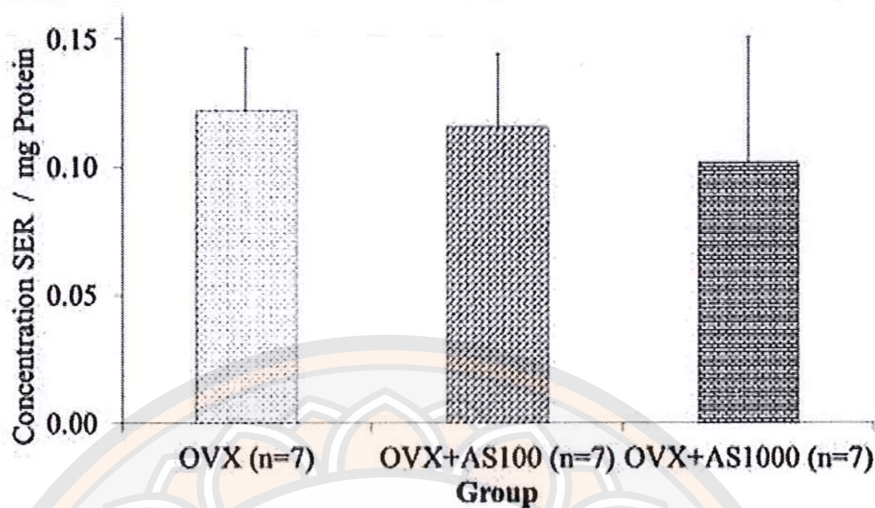


Figure 34 Hippocampal serine (SER) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector. Each bar represents mean ± SEM. There was no significant difference of the SER level of AS-treated groups when compare the control (P > 0.05, Student's unpaired t-test).

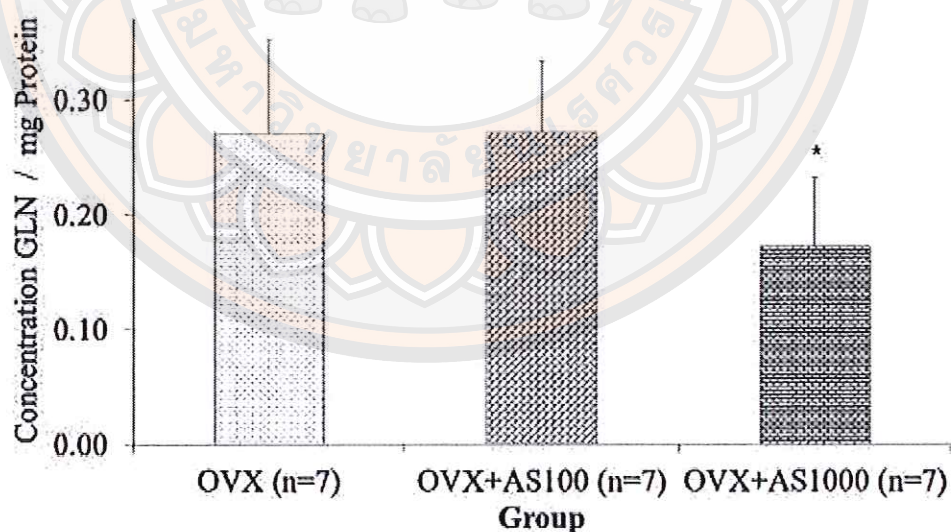


Figure 35 Hippocampal glutamine (GLN) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector. Each bar represents mean ± SEM, * = $p < 0.05$ when compare the control (Student's unpaired t-test).

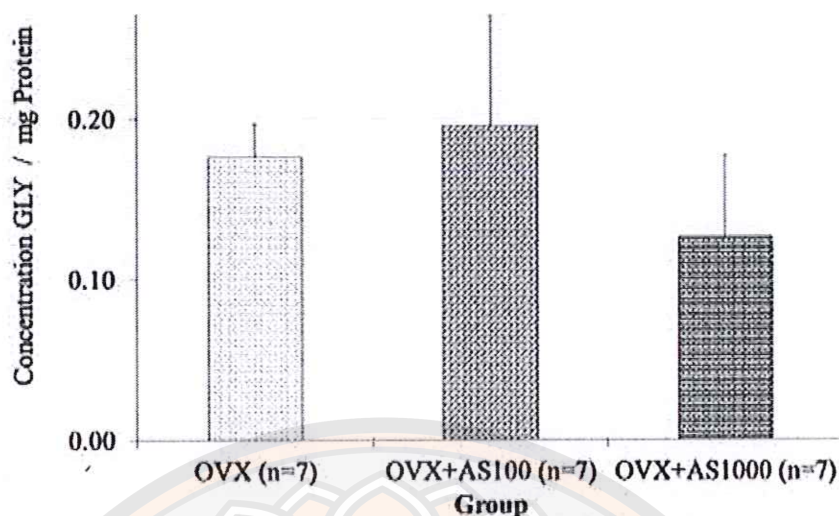


Figure 36 Hippocampal glycine (GLY) levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector. Each bar represents mean ± SEM. There was no significant difference of the GLY level of AS-treated groups when compare the control ($P > 0.05$, Student's unpaired t-test).

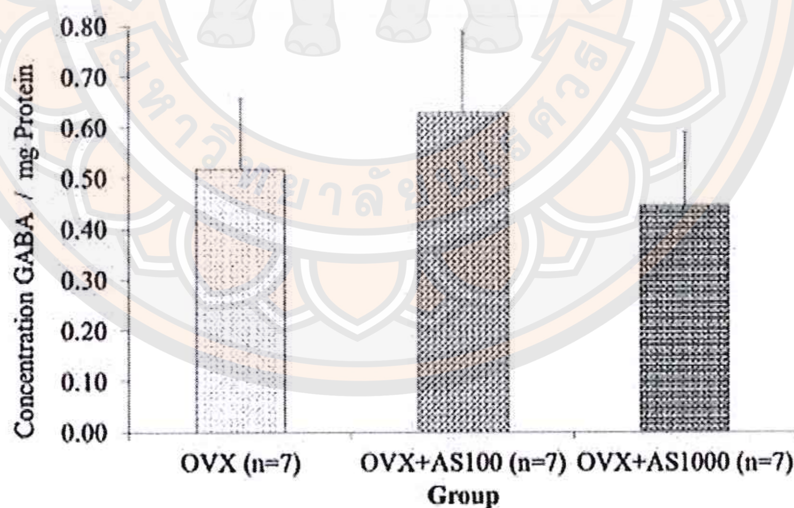


Figure 37 Hippocampal GABA levels of control (OVX) and AS-treated (OVX+AS100, OVX+AS1000) groups analyzed by using a HPLC-fluorescent detector. Each bar represents mean ± SEM. There was no significant difference of the GABA level of AS-treated groups when compare the control ($P > 0.05$, Student's unpaired t-test).

CHAPTER V

DISCUSSION AND CONCLUSIONS

In the present study, the effects of agarwood leaves extract on the CNS were evaluated in ovariectomized aging rats. From the results of behavioral tests, it indicates that *A. subintegra* leaves extract has mild sedative, anxiolytic, mild antinociceptive, and memory enhancing properties. Previously, the behavioral studies by Okugawa and his colleagues have shown that agarospirol and jinkoh-eramol in agarwood oil have antinociceptive on reduced spontaneous locomotor activity, prolong hexobarbital induced sleeping time, hypothermic, and suppressive action on acetic acid-induced writhing in mice (Okugawa, et al., 1996). Moreover, Takemoto group has found that agarwood oil contains benzylacetone, calarene, and α-gurjunene which have a sedative effect on locomotor activity when tested in mice (Takemoto, et al., 2008). Though, the sedative effect of *A. subintegra* leaves extract does not likely due to estrogen depletion in ovariectomized rats (Zhang, et al.1999; Chaves, et al. 2009). It is possible that *A. subintegra* leaves extract may contain some bioactive compounds similar to those of agarwood oil that can produce mild depressant on animal's spontaneous locomotor activity. Another possibility is that the reduction of locomotor activity in AS leaves extract treated group may be caused by the effect on lower dopamine levels in the brain (Carlsson, 1974, Dolphin, et al., 1975).

In agarwood leaves and oil extracts, several bioactive compounds have been recently found. Some of these compounds have been demonstrated the ability to cross the blood–brain barrier. In 1994, Vidya, et al. reported that co-administration of agarospiorl and jinkoh-eramol extracted from *Nardostachys jatamansi* could induce increases of GABA and 5-HT levels in rat's brain after the treatment for 15 days (Vidya, et al., 1994). Subsequent studies have shown that *A. subintegra* leaves contains flavonoids, terpenoids, and alkaloids compounds (Vidya, et al., 1994, Bahrani, et al., 2014). These bioactive compounds are able to bind to benzodiazepine site of GABA-A receptors producing membrane hyperpolarization (Vidya, et al., 1994,

Verma, et al., 2010, Hanrahan, et al., 2011). Since, carvacrol, one of monoterpenes in subclass of terpenoids found in agarwood leaves, can bind to GABA-A receptors and produce an anxiolytic-like property (Pires, et al., 2013, Guimarães, et al., 2014, Manayi, et al., 2016). Our result from elevated plus maze test indicated that the rats treated with AS leaves extract at the dose 100 mg/kg B.W. could significantly explore the open arms more than the closed arms. The anxiolytic property of AS leaves extract may be resulting from its bioactive compounds that act through benzodiazepine binding sites of GABA-A receptors (Guimarães, et al., 2014). Thus, both sedative and anxiolytic effects of *A. subintegra* leaves extract may be produced by mild depressive action of the bioactive compounds.

In the present study, the results from tail-flick and hotplate analgesic tests in rats similar to that of Zhou, et al. who reported the analgesic action of *A. sinensis* leaves extract in mice by using a hotplate analgesic test (Zhou, et al., 2008). In addition, Zhou, et al. (2008) also reported that a single oral administration of *A. sinensis* leaves extract at the dose of 848 mg/kg could reduce the inflammation induced by xylene- or carrageenan-injection to a mouse's paw. One of the possible mechanisms is that *A. subintegra* extract may act directly at peripheral nociceptors to become desensitizing to pain stimuli. Another possibility is that the crude extract may activate the descending pain control pathway in the brainstem such as periaqueductal gray (PAG) and nucleus raphe magnus (NRM) causing a reduction of pain signal transmission to the higher center (Heinricher, et al., 2009).

For the results of tail-flick and hotplate analgesic tests, researchers found that *A. sinesis* leaves extract also has antinociceptive action in rats. Zhou and his group used an *in vitro* study to demonstrate the decreases of inducible nitric oxide synthase (iNOS) and lipopolysaccharide (LPS)-induced nitric oxide (NO) release from mouse peritoneal macrophages after the exposure of AS leaves extract (Zhou, et al., 2008). In addition, Afiffudden and his co-workers have recently reported that *A. malaccencis* leaves contain 4'-hydroxyacetanilide which is an acetaminophen compound generally used in the synthesis of non-steroid anti-inflammatory drugs (NSAIDs) (Afiffudden, et al., 2015). Since the results from control and sham groups showed no difference in 1st and 2nd pain respond latencies, thus the bioactive compounds in AS leaves extract

might exert its action at peripheral nociceptors or through iNOS mechanism, and/or at the central (pain descending pathway) through GABA-A receptors but did not cause by estrogen depletion (Karami, et al. 2011; Li, et al. 2014).

For barbiturate potentiation test, the result shows that rats treated with AS leaves extract could potentiate anesthetic action of thiopental as indicated by significantly longer durations of anesthesia than that of the control group. Previously, Okugawa and his group have reported that two active compounds extracted from AS leaves, such as jinkoh-eremol and agarospirol, show potentiating action on hexobarbital-induced sleeping time in mice (Okugawa, et al., 1996). Subsequently, several *in vivo* studies have demonstrated that some bioactive compounds in agarwood leaves such as flavonoids, terpenoids, and alkaloids also have barbiturate potentiating action (Kang, et al., 2000, Sousa, et al., 2007, Bahrani, et al., 2014). From this study, *A. subintegra* leaves extract potentiated barbiturate action did not caused by the effect of ovariectomy (Selye, 1971) or aging (Stevenson and Bull, 1974) as the result showed that there was no different in duration of anesthesia between the control and sham groups.

Finally, *A. subintegra* leaves extract at the doses of 100 and 1000 mg/kg, has been demonstrated to improve learning and memory in ovariectomized rats by using MWM and NOR tests. However, the mechanism(s) of this herbal extract is (are) still unknown. It is possible that this cognitive enhancing property of *A. subintegra* leaves extract may act through these mechanisms: 1) antioxidant activity, 2) increasing some nerve growth factors (NGF), 3) inhibition of acetylcholinesterase (AChE) activity, and/or 4) increasing serum estrogen level.

Previously, Ingkaninan and her colleagues conducted an *in vitro* screening of an antioxidant property of mangiferin extracted from AS leaves using thiobarbituric acid reactive substances (TBAR) and 2,2-diphenylpicrylhydrazyl (DPPH) assays. They found that extract mangiferin could decrease lipid peroxidation in both TBAR and DPPH assays when compared to Trolox which was a derivative of vitamin E (Ingkaninan, et al., unpublished data). Recently, Ray and colleagues using thin-layer chromatography (TLC) and DPPH assay to determine bioactive compounds and antioxidant activity from the ethanolic extract from *A. crassna* leaves. They found 2

main compounds which were mangiferin and genkwanin in the crude extract and these 2 compounds had IC_{50} values on DPPH free radical scavenging activity approximately 15 and 70 %, respectively (Ray, et al., 2014). Thus, it is likely that the cognitive enhancing effect of *A. subintegra* leaves extract may cause by antioxidant property of its bioactive compounds such as mangiferin and genkwanin.

There is evidence showing that agarwood extract can induce a production of brain derived nerve growth factor (BDNF) which is a putative nerve growth factor (NGF). In 2006, Ueda, et al. reported that ethanol extract of Vietnamese agarwood could significantly induce BDNF mRNA expression in rat cultured neurons (Ueda, et al., 2006). BDNF is known to effect the growth of neurons in the hippocampus, cortex, and basal forebrain areas which are essential for learning, memory, and higher thinking process (Benraiss, et al., 2001,Yamada and Nabeshima, 2003, Bekinschtein, et al., 2008). In 2014, Bahrani and his co-researchers used a TLC technique and could indicate 2 main compounds such as kaempferol and dimethoxyflavone from chloroform extracts of the stem and leaves of *A. subintegra*. Then, they employed an *in vitro* screening of AChE activity and found that *A. subintegra* leaves extract and AS stem extract had IC50 values of 80% and 93%, respectively. Subsequently, they used a RAM test to demonstrate that adult male and female mice treated with these chloroform extracts could improve their working memory deficits caused by an administration of valium (Bahrani, et al., 2014). These results imply that *A. subintegra* leaves extract is a natural AChE inhibitor for the treatment of some chronic memory impairments such as Alzheimer's disease.

In conclusion, the administration of agarwood leaves extract in aged OVX rats produces mild sedative effect, reduces anxiety, relieves pain, potentiates barbiturate sleeping time, and restores learning and memory. Altogether, the results from the present study can be summarized that *A. subintegra* leaves extract has mild CNS depressive and cognitive restoration effects in ovariectomized aging rats. This herbal extract has a potential to be developed as the food supplement such as herbal tea that has a mild CNS depressant and help to restore the memory impairment. However, the underlying mechanism(s) of action of this herbal extract is still

inconclusive. Therefore, further investigations are needed to elucidate this (these) underlying mechanism(s).

Summary results of the present study

1. Behavior tests

Table 3 Summary results of behavior testing in positive control and AS-treated groups

No	Effects	Positive control	OVX +AS100	OVX +AS1000
1	Acute CNS depressant	Y	mild	mild
2	Anxiolytic effect	Y	Y	N
3	Nociceptive effect (hotplate analgesic test)	Y	Y	Y
4	Nociceptive effect (tail-flick analgesic test)	Y	Y	Y
5	Barbiturate potentiation	Y	Y	Y
6	Improving the spatial memory	Y	Y	Y
7	Improving the recognition memory	Y	Y	N

Note: All tested data were compared to the control group, Y=yes, N=no effect

2. Biochemical tests

Table 4 Summary results of change(s) of serum estrogen and brain's neurotransmitter levels in AS-treated groups

No	Test	OVX+AS100	OVX+AS1000
1	Serum estrogen level	↑	↑
2	Dopamine level on whole brain	NS	↓
3	Serotonin level on whole brain	↑	NS
4	Aspartate level on hippocampus	NS	NS
5	Glutamate level on hippocampus	NS	↓
6	Serine level on hippocampus	NS	NS
7	Glutamine level on hippocampus	NS	↓
8	Glycine level on hippocampus	NS	NS
9	GABA level on hippocampus	NS	NS

Note: All test data were compared to the control group, ↑ = increase, ↓= decrease, NS = no significant change when compare to the control group

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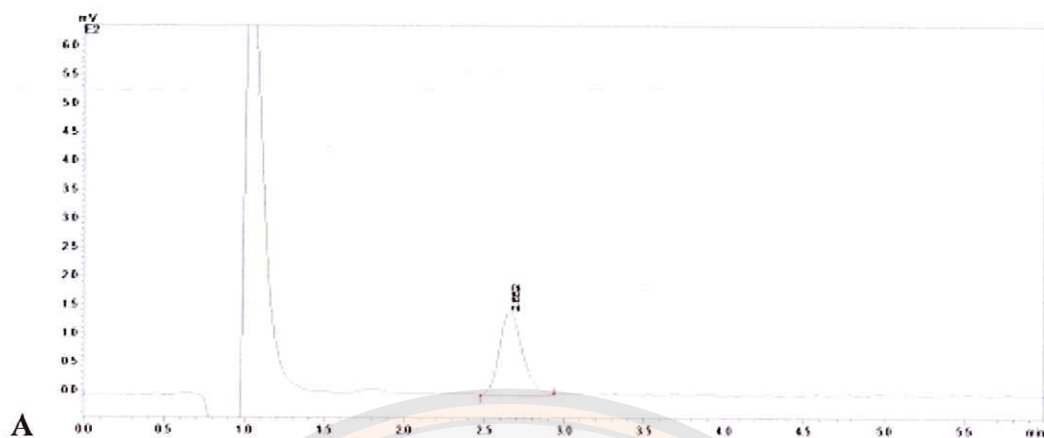
APPENDIX



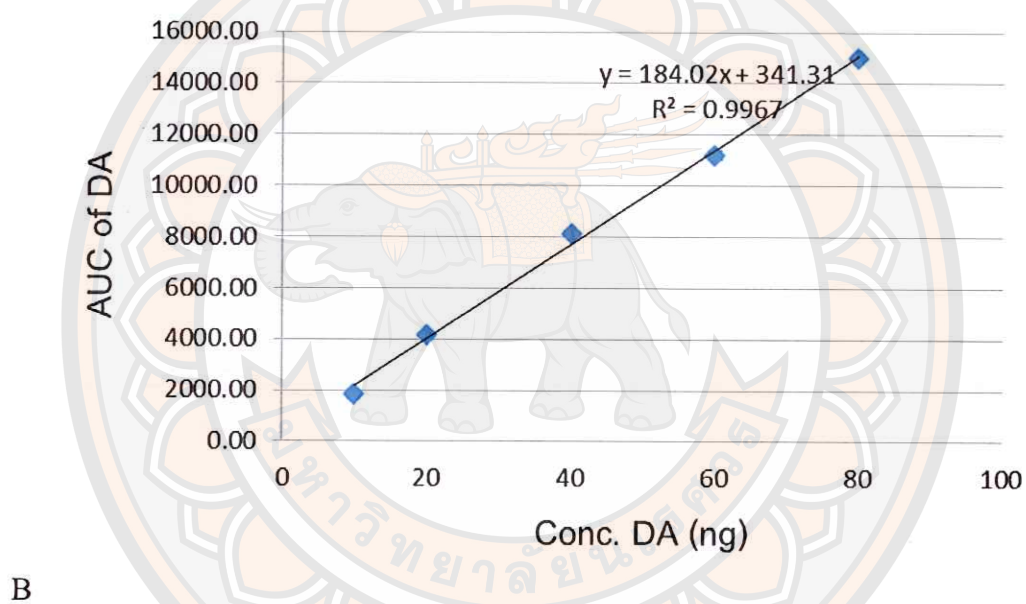
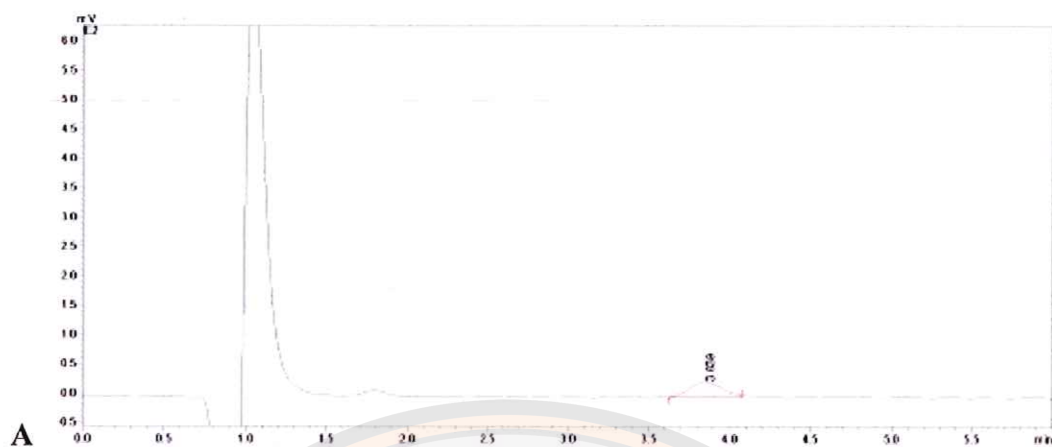


Figure 38 HPLC chromatogram of DA standard(A) and Standard calibrationcurve of DA (B)



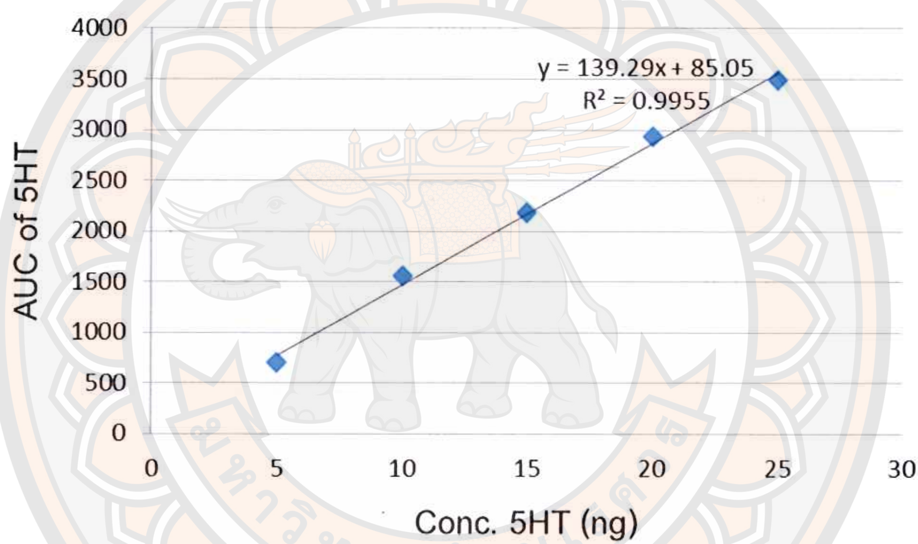


Figure 39 HPLC chromatogram of 5HT standard (A)
Standard calibration curve of 5HT (B)

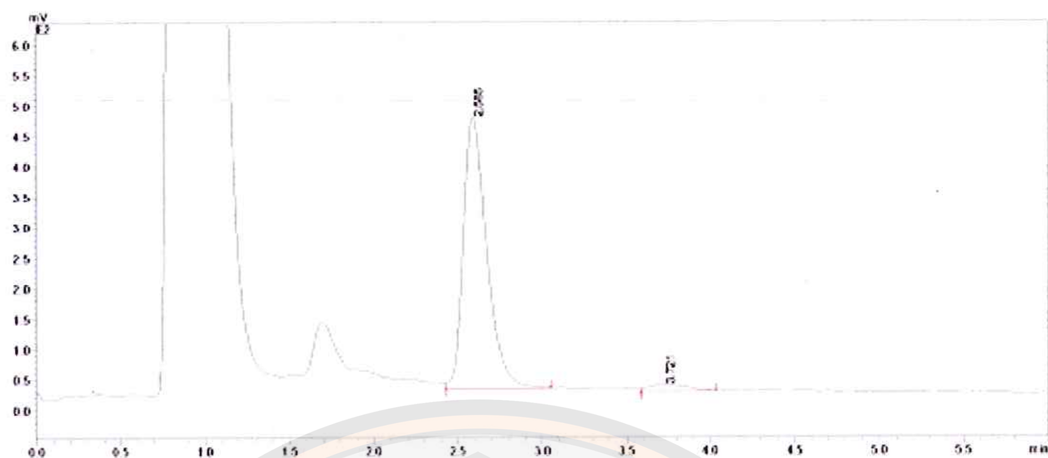
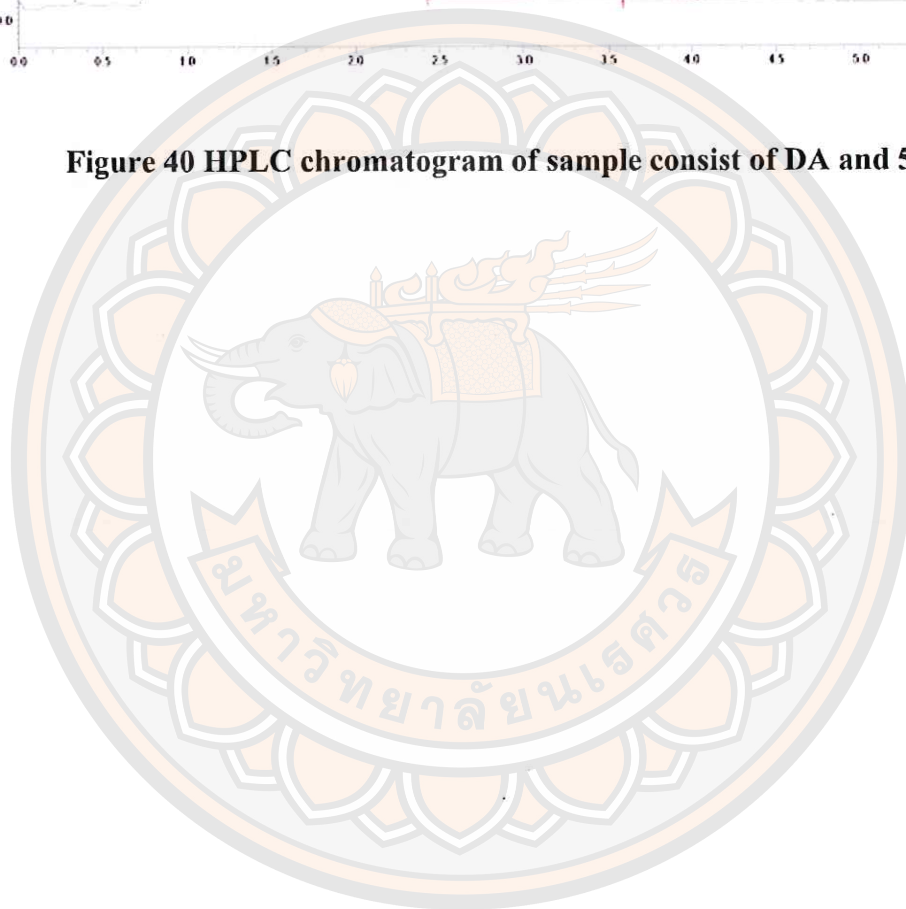


Figure 40 HPLC chromatogram of sample consist of DA and 5HT



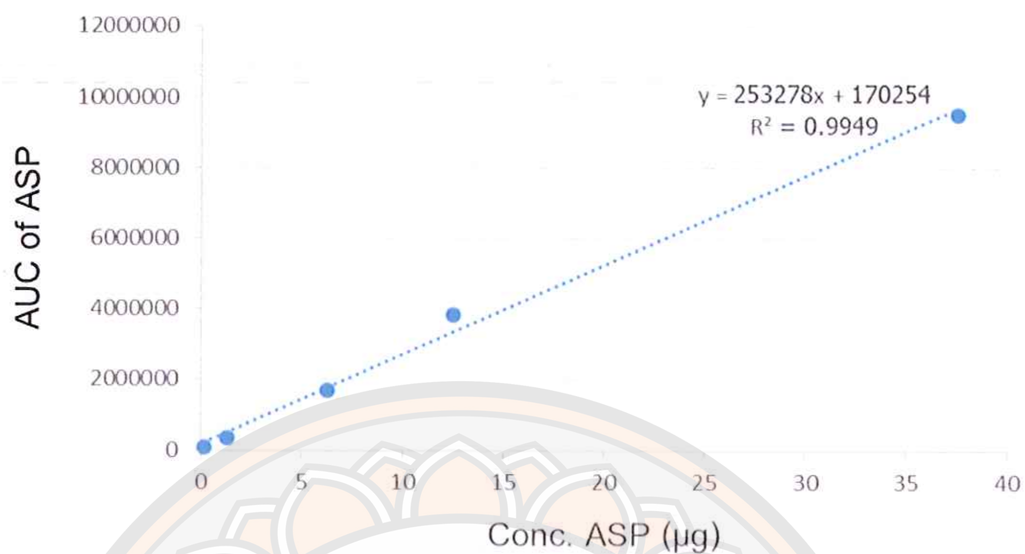


Figure 41 Standard calibration curve of ASP

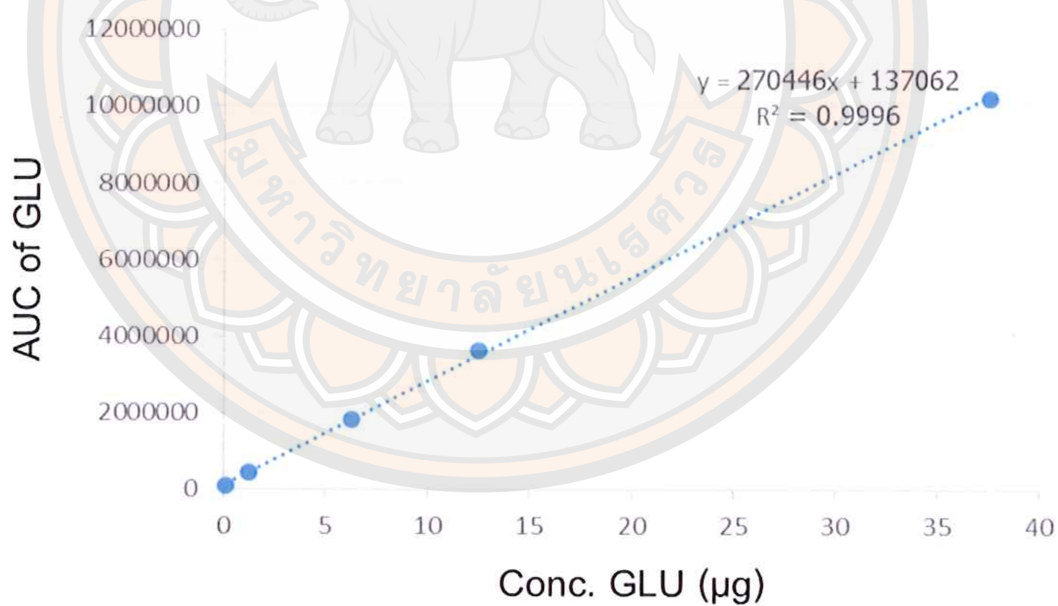


Figure 42 Standard calibration curve of GLU

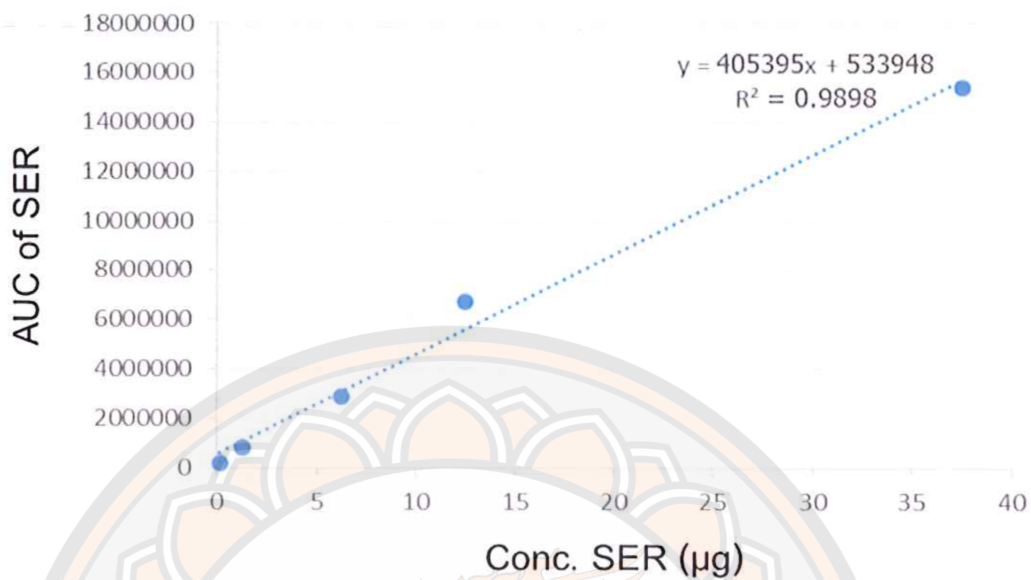


Figure 43 Standard calibration curve of SER

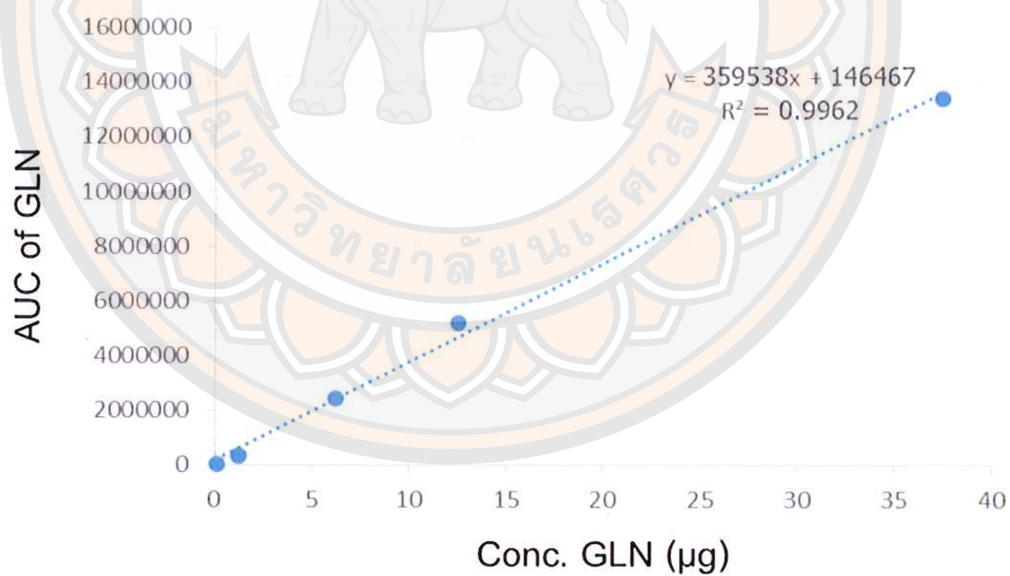
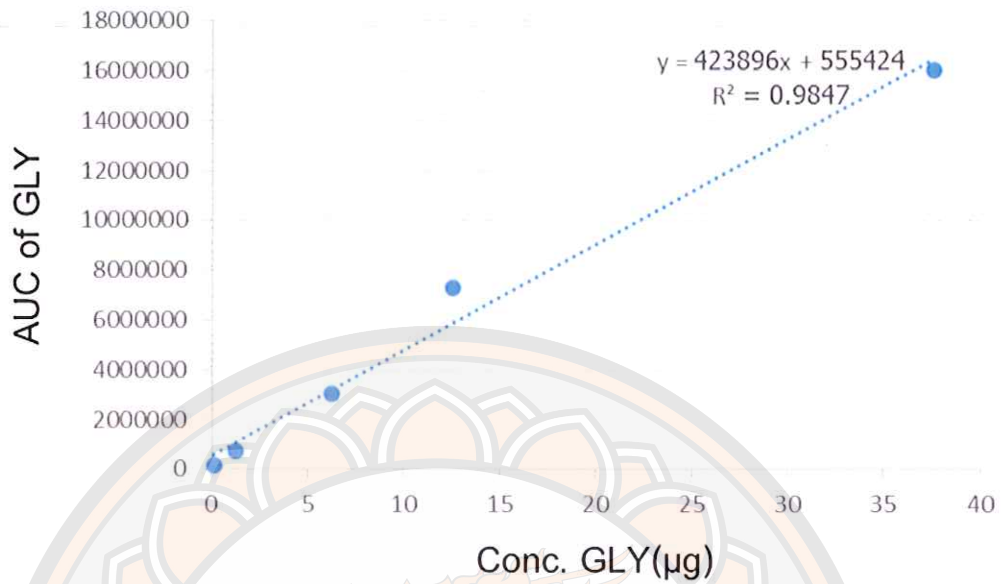
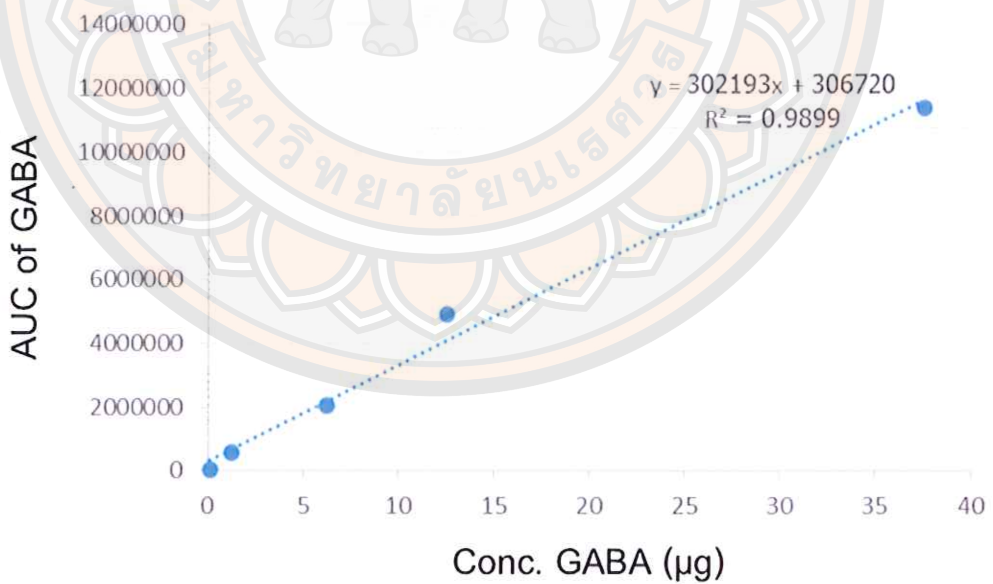


Figure 44 Standard calibration curve of GLN



B

Figure 45 Standard calibration curve of GLY



B

Figure 46 Standard calibration curve of GABA

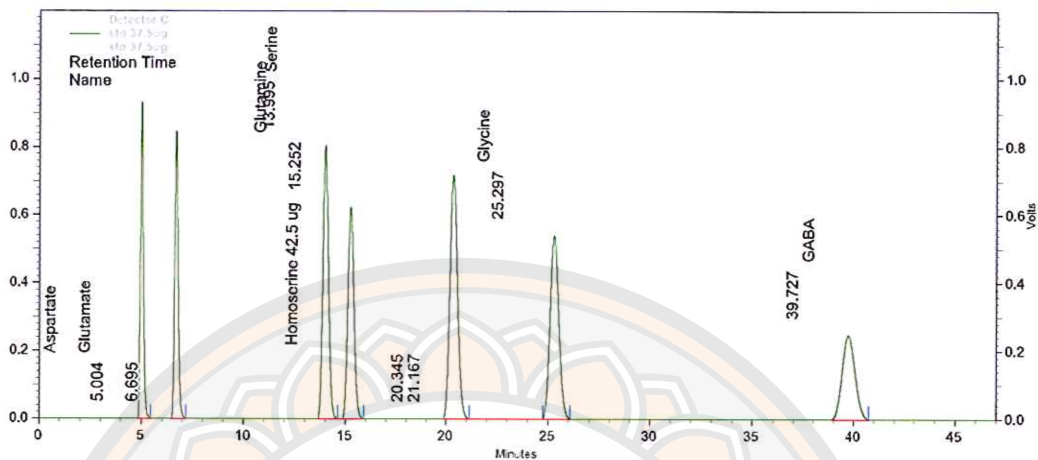


Figure 47 HPLC chromatogram of standard consists of ASP, GLU, SER, GLN, GLY and GABA

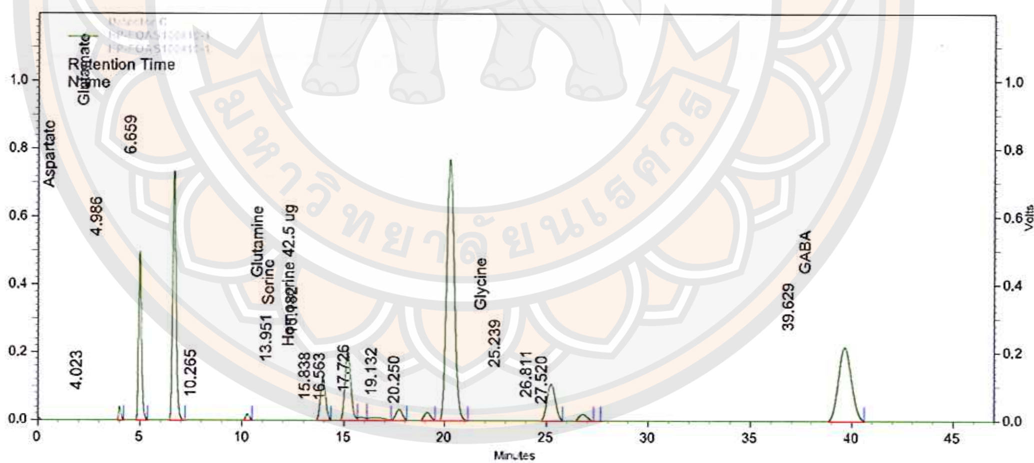


Figure 48 HPLC chromatogram of brain sample consists of ASP, GLU, SER, GLN, GLY and GABA

Table 5 Summary data from HPLC-ECD analyses of standard monoamine

Name	Concentration (μg)	Area 1	Area 2	Area 3	Mean	±SEM
DA	10.000	1687	1754	1721	1721	34
	20.000	4166	4063	4138	4122	53
	40.000	8067	8086	8112	8088	23
	60.000	11464	11265	11036	11255	214
	80.000	14911	15287	15018	15072	194
5HT	5.000	925	798	873	865	64
	10.000	1376	1326	1395	1366	36
	15.000	2009	2119	2076	2068	55
	20.000	2966	2938	2931	2945	19
	25.000	3364	3407	3316	3362	46

Table 6 Summary data from HPLC-fluorescence analyses of standard amino acids

Name	Concentration (μg)	Area 1	Area 2	Area 3	Mean	±SEM
ASP	0.125	75889	61356	72037	69761	7529
	1.250	306980	334299	371948	337742	32621
	6.250	1685737	1658450	1726269	1690152	34124
	12.500	3911460	3794047	3781116	3828874	71813
	37.500	9767897	9880313	8911499	9519903	529883
GLU	0.125	94432	82532	86520	87828	6057
	1.250	454885	451341	463846	456691	6445
	6.250	1841975	1785537	1911400	1846304	63043
	12.500	3704476	3661378	3568572	3644809	69451
	37.500	10545544	10575996	9580839	10234126	565968
SER	0.125	247326	163627	199180	203378	42007
	1.250	720912	792662	966779	826784	126435
	6.250	2872982	2819465	2940155	2877534	60474
	12.500	6751736	6694152	6711652	6719180	29521
	37.500	15919884	15905556	14385836	15403759	881576
GLN	0.125	23557	26351	26119	25342	1550
	1.250	498286	468267	26940	331164	263893
	6.250	2464519	2362287	2495491	2440766	69706
	12.500	5300474	5226254	5114771	5213833	93473
	37.500	13904350	13835585	12578894	13439610	746194

Table 6 (cont.)

Name	Concentration (µg)	Area 1	Area 2	Area 3	Mean	±SEM
GLY	0.125	187596	92267	121923	133929	48785
	1.250	729703	709566	801040	746770	48066
	6.250	3008789	2852496	3190137	3017141	168975
	12.500	7259238	7396931	7210459	7288876	96704
	37.500	17388165	16008006	14656049	16017407	1366082
GABA	0.125	43952	23219	21640	29604	12451
	1.250	577906	559929	566827	568221	9069
	6.250	2051892	1929312	2160808	2047337	115815
	12.500	4948300	4994809	4782527	4908545	111585
	37.500	12001606	11684750	10494873	11393743	794402

เอกสารรับรองโครงการ

คณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

ชื่อโครงการ	การศึกษาฤทธิ์ของสารสกัดจากใบกฤษณาต่อระบบประสาทส่วนกลางในหนูแก่ Screening of the central nervous system action of agarwood leave extract in aged rats
เลขที่โครงการ	NU-AE550731
เลขที่เอกสารรับรอง	55 04 0050
ประเภทการรับรอง	เต็มรูปแบบ
ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ	ผศ.ดร.นิวัติ เทพาวราพฤกษ์
สังกัดหน่วยงาน /คณะ	วิทยาศาสตร์การแพทย์
วันที่รับรอง	วันที่ 17 สิงหาคม 2555

ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

(รองศาสตราจารย์ ดร.รัตติมา จีนาพงษา)

ประธานคณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร