ผลของสารสกัดว่านชักมดลูกต่อเอนไซม์โคลีนอะซิทิลทรานสเฟอเรส
และอะซิทิลโคลีนเอสเตอเรส ในสมองหนูขาว

นางสาว ศศิธร หลูปรีชาเศรษฐ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECTS OF *CURCUMA COMOSA* EXTRACTS ON CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE IN RAT BRAIN

Miss Sasithorn Lupreechaset

สถาบันวิทยบริการ
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ศศิธร หลูปรีชาเศรษฐ: ผลของสารสกัดว่านชักมดลูกต่อเอนไซม์โคลีนอะซิทิลทรานสเฟอเรส และอะซิทิลโคลีนเอสเตอเรส ในสมองหนูขาว (EFFECTS OF *CURCUMA COMOSA* EXTRACTS ON CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTER^SE IN RAT BRAIN) อ.ที่ปรึกษา: รศ. ดร.พ.ต.ท. หญิง สมทรง ลาวัณย์ประเสริฐ, อ.ที่ปรึกษาร่วม: รศ. นวลศรี นิวัติศัยวงศ์, 75 หน้า.

ว่านชักมดลูก (*Curcuma comosa* Roxb.) เป็นพืชสมุนไพรไทยที่รู้จักกันดีในวงศ์ Zingiberacea ตำราแพทย์แผนไทยมีการนำเหง้ามาใช้ในการรักษาอาการผิดปกติของมดลูก ซึ่งจากการศึกษาต่างๆ พบว่า ว่านชักมดลูกมีฤทธิ์คล้ายเอสโตรเจน วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาผลของสารสกัดจากเหง้าของว่านชักมดลูกด้วยเฮกเซนและเอทานอลต่อสมรรถนะของเอนไซม์โคลีนอะซิทิลทรานสเฟอเรส (ChAT) และอะซิทิลโคลีนเอสเตอเรส (AChE) ในสมองของหนูขาว การศึกษานี้ใช้หนูขาวเพศผู้ พันธุ์วิสตาร์ จำนวน 45 ตัว แบ่งหนูขาวแบบสุ่มออกเป็น 6 กลุ่ม กลุ่มที่ 1- 5 กลุ่มละ 8 ตัว ได้รับน้ำมันข้าวโพด ขนาด 1 มิลลิกรัม/กิโลกรัม/วัน (กลุ่มที่ 1) หรือสารสกัดว่านชักมดลูกด้วยเฮกเซน (กลุ่ม 2,3) และเอทานอล (กลุ่ม 4,5) ขนาด 250, 500 มิลลิกรัม/กิโลกรัม/วัน ติดต่อกันทางปากเป็นเวลา 30 วัน และใช้หนูขาว 5 ตัว เป็นกลุ่มเอสโตรเจนอ้างอิง ที่ได้รับ estradiol valerate ขนาด 10 ไมโครกรัม/กิโลกรัม/วัน โดยการฉีดเข้ากล้ามเนื้อเป็นเวลา 7 วัน เมื่อครบระยะเวลา ทำให้หนูขาวหมดความรู้สึก ดึงคอ เก็บตัวอย่างซีรัมเพื่อตรวจค่าความเข้มข้นของเอสตราไดออล เก็บสมอง แยกส่วนของสมอง 3 ส่วนคือ cerebral cortex, basal forebrain และ hippocampus นำส่วนสมองแต่ละส่วนที่ได้มาเตรียม brain homogenate เพื่อวัดสมรรถนะของเอนไซม์ ผลการทดลองพบว่าสารสกัดว่านชักมดลูกด้วยเอทานอลทั้งสองขนาดมีผลเพิ่มสมรรถนะของเอนไซม์โคลีนอะซิทิลทรานสเฟอเรสในสมองส่วน cerebral cortex และ hippocampus แต่ไม่มีผลต่อสมองส่วน basal forebrain สารสกัดว่านชักมดลูกด้วยเฮกเซนและเอทานอลไม่มีผลต่อสมรรถนะของเอนไซม์อะซิทิลโคลีนเอสเตอเรสในสมองของหนูขาวทั้ง 3 ส่วน estradiol ในขนาดที่ใช้ในการทดลองนี้ไม่มีผลต่อสมรรถนะของเอนไซม์โคลีนอะซิทิลทรานสเฟอเรสและอะซิทิลโคลีนเอสเตอเรสในสมองของหนูขาว สารสกัดว่านชักมดลูกด้วยเฮกเซนและเอทานอลทั้ง 2 ขนาด มีผลเพิ่มระดับเอสตราไดออลในเลือดของหนูขาวได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุม อย่างไรก็ตามระดับเอสตราไดออลในเลือดมีค่าต่ำกว่ากลุ่มที่ได้รับ estradiol ผลจากการศึกษานี้ชี้บ่งแนวโน้มว่าสารสกัดว่านชักมดลูกด้วยเอทานอลอาจมีประโยชน์ต่อระบบประสาทโคลิเนอจิกโดยการเพิ่มสมรรถนะเอนไซม์โคลีนอะซิทิลทรานสเฟอเรสซึ่งเป็นเอนไซม์ที่สำคัญในการสร้างอะซิทิลโคลีนในสมอง

ภาควิชา เภสัชวิทยา ....ลายมือชื่อนิสิต......ศศิธร หลูปรีชาเศรษฐ......

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SASITHORN LUPREECHASET: EFFECTS OF *CURCUMA COMOSA* EXTRACTS ON CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE IN RAT BRAIN. THESIS ADVISOR: ASSOC. PROF. POL. LT. COL. SOMSONG LAWANPRASERT, Ph.D., THESIS COADVISOR: ASSOC. PROF. NUANSRI NIWATTISAIWONG, 75 pp.

Curcuma comosa Roxb., a plant in family Zingiberazeae, is one of the well-known medicinal plants in Thailand. Rhizome of this plant has been traditionally used for the treatment of various abnormal symptoms of uterus. Those medicinal purposes of *C. comosa* may be associated with its estrogenic-like effects reported by many studies. The objective of this study was to investigate effects of *C. comosa* hexane and ethanolic extracts on the activities of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in rat brain. Forty five rats were randomly divided into 6 treatment groups. Rats were administered orally with 1 ml/kg/day of corn oil (control group); 250 and 500 mg/kg/day of *C. comosa* hexane extract (group 2, 3); 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (group 4, 5) for 30 days. Each group comprised 8 rats. Estradiol valerate was given intramuscularly to 5 rats at a dose of 10 μg/kg/day for 7 days, serving as an estrogen reference group. At the end of the treatment, rats were anesthesized then cervical dislocated. Serum samples were determined for estradiol concentration. Brain was collected and dissected for cerebral cortex, basal forebrain and hippocampus. Each brain region homogenates was prepared for enzyme activity assay. The results showed that both dosages of *C. comosa* ethanolic extract caused an increase of ChAT activity in cerebral cortex and hippocampus but not in basal forebrain. *C. comosa* hexane and ethanolic extracts did not affect AChE activities in all three brain regions. Estradiol given at the dosage regimen in this study did not affect both ChAT and AChE activities. Even though an increase of serum estradiol level from *C. comosa* extracts was less than the estradiol group, both extracts caused a significant increase of serum estradiol level as compared to the control group. These findings demonstrated that *C. comosa* ethanolic extract potentially possessed a beneficial effect on cholinergic nervous system showing by an enhancement effect on the activity of ChAT, an enzyme responsible for brain acetylcholine synthesis.

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Field of study Pharmacology .... Advisor's signature Pol. Lt. Col. Somsong Lawanprasert

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LIST OF ABBREVIATIONS

β	= beta
μg	= microgram
μl	= microlitre
μmol	= micromole
μM	= micromolar
α	= alpha
ACh	= acetylcholine
AChE	= acetylcholinesterase
AF	= activation function
AD	= Alzheimer's disease
ANOVA	= analysis of variance
APOE	= apolipoprotein
BDNF	= brain-derived neurotrophic factor
BFR	= bile flow rate
BSA	= bovine serum albumin
BuChE	= butyrylcholinesterase
ChAT	= choline acetyltransferase
ChAT-IR	= choline acetyltransferase immunoreactive
cm	= centimeter
CoA	= coenzyme A
%CV	= percent coefficient of variation
DBD	= deoxynucleic acid (DNA) binding domain
DMSO	= dimethylsulfoxide
DTNB	= 5, 5'-dithiobisnitrobenzoic acid
EC_{95}	= 95% of a maximal effective concentration

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EDTA	= ethylenediamine tetraacetic acid
e.g.	= example gratia
ERE	= estrogen response element
ER_s	= estrogen receptors
$ER\alpha$	= estrogen receptor alpha
$ER\beta$	= estrogen receptor beta
FDA	= Food and Drug Administration
g	= gram
g	= gravity
HAPI	= highly aggressive proliferating immortalized
HDL	= high density lipoprotein
5-HT	= 5-hydroxytryptamine
i.g.	= intragastric
IL	= interleukin
kD	= kilodalton
kg	= kilogram
K_m	= Michaelis constant
L	= litre
LBD	= ligand binding domain
LD_{50}	= median lethal dose
LDL	= low density lipoprotein
LPS	= lipopolysaccharide
M	= molar
MCP	= monocyte chemoattractant protein
min	= minute
mg	= miligram
mg/kg	= milligram per kilogram body weight
ml	= milliliter

mM	= millimolar
mmole	= millimole
MS	= medial septum
NBM	= nucleus basalis magnocellularis
NGF	= nerve growth factor
nmol	= nanomole
NMDA	= N-methyl-D-aspartate
NO	= nitric oxide
iNOS	= inducible nitric oxide synthase
pmol	= picromole
OVX	= ovariectomized
4PDS	= 4, 4'-dithiodipyridine
pH	= potential of hydrogen
r^2	= Coefficient of determination
SEM	= standard error of the mean
THA	= 2, 4, 6-trihydroxyacetophenone
4-TP	= 4-thiopyridone
U	= unit
VLDL	= very low density lipoprotein
v/v	= volume by volume
w/v	= weight by volume

CHAPTER I

INTRODUCTION

Curcuma comosa Roxb., a plant in family Zingiberazeae, is one of the well-known medicinal plant in Thailand (Smitinand, 2001). Rhizome of this plant has been traditionally used for treatment of female postpartum uterine inflammation, uterine pain, amenorrhea, peri-menopausal bleeding, lower abdominal pain in male as well as for the stomachic and choleretic effects (Association of the School of Thai Traditional Medicine, 1973).

C. comosa consists of two groups of chemical constituents, diarylheptanoids and acetophenones. Diarylheptanoids include trans-1,7-diphenyl-5-hydroxy-1-heptene, trans-1,7-diphenyl-6-hepten-3-one-5-ol, trans-1,7-diphenyl-3-acetoxy-6-heptene, trans-1,7-diphenyl-6-heptene-3-one, trans,trans-1,7-diphenyl-1,3-heptadien-5-ol, 1,7-diphenyl-5-hydroxy-(1*E*)-1-heptene, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene. Acetophenone found in *C. comosa* is mainly phloracetophenone glucoside (4,6-dihydroxy-2-O-(β-D-glucopyranosyl) acetophenone) (Jurgens *et al*., 1994; Suksamrarn *et al*., 1994, 1997).

Several studies have documented that *C. comosa* extracts exhibit many pharmacological effects in animal models such as *C. comosa* hexane extract possessed uterotrophic effect (Piyachaturawat, *et al.,*1995a,1995b) and estrogenic activity (Piyachaturawat, *et al.,*1998a,1999a). *C. comosa* ethanolic extract reduced uterine contraction induced by oxytocin, acetylcholine, serotonin and potassium chloride (Sawasdipanich, 1994). Methanolic extract *of C. comosa* showed nematocidal activity againsts the free-living nematode *Caenorhabditis elegans* (Jurgens *et al*., 1994). The phloracetophenone glucoside which isolated *C. comosa* by ethyl acetate exhibited a dose-dependent choleretic activity (Suksamran *et al*., 1997). The stimulation of bile secretion and the enhancement of biliary excretion of bile salt and cholesterol consequently led to a decrease in plasma cholesterol in normal rats (Piyachaturawat *et*

al., 1996). Moreover, ethyl acetate extract of *C. comosa* also effectively reduced plasma triglyceride and cholesterol in diet-induced hypercholesterolaemic hamsters (Piyachaturawat, *et al*., 1999b).

Recently, the synthetic phloracetophenone (2,4,6-trihydroxyacetophenone, THA), the aglycone part of the glucoside has been shown to enhance biliary excretion of bile acids in rats, and decrease both plasma cholesterol and triglyceride levels in hypercholesterolemic male hamsters (Piyachaturawat *et al*.,1998b, 2002a). Subchronic toxicity study showed that *C. comosa* ethanolic extract increased alkaline phosphatase enzyme level and caused hyperplasia and hypertrophy of gastric epithelium (Chivapat *et al*., 2003). Subacute toxicity study of THA was assessed in rat, 150 and 300 mg/kg/day of THA induced periportal hepatocyte degeneration and slightly increased concentration of alanine aminotransferase, aspartate aminotransferase, bilirubin, blood urea nitrogen and hepatic triglyceride content (Piyachaturawat *et al*., 2002b).

Alzheimer's disease (AD) is found more common and has an earlier onset and progress more rapidly in women than in men. Recent clinical studies found that deficiency of circulating estrogen in woman after menopause increases risk of cognitive decline associated with AD (Miller *et al*., 1999; Garcia-Segura *et al*., 2000; Shughrue *et al*., 2000). Many epidemiological studies reported the protective potential role of estrogen in AD patients. Estrogen replacement in postmenopausal women has been reported to improve cognitive function and decrease the risk of developing AD, of which the mechanism responsible for these effects are still unclear (Gibbs and Aggarwal, 1998; Wu *et al*., 1999; Garcia-Segura *et al*., 2000; Yaffe *et al*., 2005). In AD patients, acetylcholine (ACh) accompanied with choline acetyltransferase (ChAT), an enzyme catalyzes the biosynthesis of ACh, are reduced in cerebral cortex, basal forebrain and hippocampus. Thus, reduction of cholinergic activity is contributed from the degeneration of neurons in these areas (Alzheimer's Association, 2006). Current studies in animal model have revealed that estrogen replacement can reverse impairment of ACh and ChAT. Luine (1985) and Singh et al. (1994) found that 17β-estradiol caused a restoration of ChAT activity leading to increased ACh levels in ovariectomized (OVX) rats. Estradiol-

17β increased choline acetyltranferase immunoreactive (ChAT-IR) cells in medial septum (MS) and nucleus basalis magnocellularis (NBM) in OVX rats (Gibbs, 1997). Gibbs (1998, 2000) found that short term treatment with physiological levels of estrogen caused a restoration of ChAT mRNA in MS and NBM in OVX rats. In 1999, Wu and collaborated found that raloxifene and estradiol benzoate caused a restoration of hippocampal ChAT activity in OVX rats. While many scientific data indicate that estrogen may increase cognitive function by enhancing ChAT protein or ChAT activity, acetylcholinesterase (AChE) which is an important enzyme for cholinergic neurons was also interested by a number of scientists. Inhibition of AChE is the basic mechanism of most drugs used clinically for symptomatic relief of the early stages of AD. Das and collaborate reported that estradiol exhibited various effects on AChE activity in different brain regions, including a significant decreased AChE activity in thalamus, medulla and hippocampus by the effect of estradiol.

Phytoestogens are defined as plant-derived nonsteroidal substances with a diphenolic ring, of which the structure and function are similar to those of 17β-estradiol (Lee *et al*., 2004). Phytoestrogens have been studied for their potential beneficial effects for the prevention of hormone–dependent cancers (e.g., breast cancer, prostate cancer), cardiovascular disease, osteoporosis, alleviate the symptoms of menopause (e.g., hot flushes) as well as the effect in brain (Lephart *et al*., 2002). Dietary soy phytoestrogens have been shown to improve memory in male and female students, post menopausal women and OVX rats (Pan *et al*., 2000; File *et al*., 2001, 2002; Duffy *et al*., 2003). In 2006, Casini and collaborates demonstrated that soy isoflavone possessed positive effects on post menopausal women, improving cognitive performance and mood. In addition, several animal model studies revealed that phytoestrogen–treated OVX rats were shown to have an increase of brain derive neurotrophic factor (BDNF) in frontal cortex as well as nerve growth factor (NGF) in frontal cortex and hippocampus (Pan *et al*., 1999a, 1999b). Furthermore, Lee et al. (2004) found that soy isoflavone caused an increase ChAT in cerebral cortex basal forebrain and a decrease AChE activity in cerebral cortex, basal forebrain and hippocampus in male rats.

According to the reports mentioned above, estrogen and phytoestrogen possess a potential beneficial effect in cholinergic neurons which play an important role in learning and memory process. Induction of ChAT activity and reduction of AChE activity may be one of the mechanism with a positive effect leading to a restoration of ACh in brain. Taken together with the recent studies which have reported that *C. comosa* possesses estrogenic activity and the effect of *C. comosa* on cholinergic nervous system has never been reported. Therefore, the purpose of this study was to investigate effect of *C. comosa* extracts on ChAT and AChE activity in various region of rat brain.

Hypothesis

C. comosa extracts caused an increase of ChAT activity and/or a decrease of AChE activity in rat brain.

Study design and process

1. Animal treatment: an ex *vivo* study
2. Blood and brain collecting
3. Determination of serum estradiol concentration
4. Preparation of brain homogenates
5. Verification of the analytical methods for determination of ChAT and AChE activities
6. Determination of ChAT and AChE activities in various rat brain regions
7. An *in vitro* study regarding the effect of *C. comosa* on AChE activity
8. Data analysis

Anticipated benefits from the study

A preliminary data of whether *C. comosa* extracts possessed beneficial effects on cholinergic nervous system possibly associated with a restoration of ACh level via either an enhancement of ChAT activity or a reduction of AChE activity in brain.

CHAPTER II

LITERATURE REVIEWS

Curcuma comosa Roxb.

Curcuma comosa Roxb. (Figure 1), a plant belonging to Zingiberazeae family. It is a perennial herb of height up to 1 metre with big tuber, up to 10 centimetre diameter. In Thailand, *C. comosa* is commonly known as Waan chak mod look (Smitinand, 2001). The rhizome of this plant has been wildly used for treatment of female postpartum uterine inflammation, uterine pain, amenorrhea, peri-menopausal bleeding, lower abdominal pain in male and also as an aromatic stomachic and choleretic (Association of the School of Thai Traditional Medicine, 1973). Several studies reported that *C. comosa* extracts have been demonstrated to possess nematocidal (Jurgens *et al*., 1994), uterotropic (Piyachaturawat *et al*., 1995a, 1995b), suppression of male reproductive organ (Piyachaturawat *et al*., 1998a, 1999a), choleretic (Piyachaturawat *et al*., 1996,1998b, 2002a; Suksamrarn *et al*., 1997), hypolipidemic (Piyachaturawat *et al*., 1999b) as well as anti-inflammatory effect (Jantaratnotai *et al*., 2006).

Natural compounds found in rhizome of *C. comosa* can be classified on the basic of their chemical structures into two groups as following: (Jurgens *et al*., 1994; Suksamrarn *et a*l., 1994, 1997)

1. Diarylheptanoids: trans-1,7-diphenyl-5-hydroxy-1-heptene, trans-1,7-diphenyl-6-hepten-3-one-5-ol, trans-1,7-diphenyl-3-acetoxy-6-heptene, trans-1,7-diphenyl-6-heptene-3-one, trans,trans-1,7-diphenyl-1,3-heptadien-5-ol, 1,7-diphenyl-5-hydroxy-(1*E*)-1-heptene, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene)

2. Acetophenones: 4,6-dihydroxy-2-O-(β-D-glucopyranosyl) acetophenone.

Figure 1 *Curcuma comosa* Roxb.

Pharmacological effects

1. Uterotrophic effects

In 1995, Piyachaturawat and collaborate demonstrated that all four crude extracts of *C. comosa* including hexane, ethyl acetate, butanol and aqueous extracts possessed uterotropic effects in female rats. It was found that the hexane extract was most effective in increasing of uterine weight, glycogen content and the cornification of vaginal epithelium as well as the keratinization of mucosal surface of vagina. Butanol and ethyl acetate extracts produced slight increases, whereas the aqueous extracts had no effects on the same parameters mentioned above. In the same year, Piyachaturawat et al. (1995b) performed a study by administration of *C. comosa* extract at a dose of 480 mg/kg/day for two consecutive days to mature OVX rats. The result showed that there was a significant increase of vaginal mucosa thickness and uterine wet weight. Histology of the vaginal mucosa in the *C. comosa* treated animals showed a marked proliferation and keratinization similar to those in the estradiol treated animals. It was

found that most effect of this extract corresponded to the specific action. The extract effectively induced an increase of specific estradiol binding site in the uterine nuclei.

In 1994, Sawasdipanich found that 95% ethanolic extracts of *C. comosa* at doses of 5 and 10 μg/ml reduced the contraction induced by oxytocin, ACh, serotonin (5-HT) and potassium chloride (KCl) in de Jalon solution and 10 μg/ml of this extract also reduced contraction induced by oxytocin, vanadate and $PGF_{2\alpha}$ in Ca -free Lock Ringer solution with ethylene glycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). In anesthetized rat, intraperitoneal injection of 0.5 g/kg of this extract also markedly reduced uterine contraction stimulated by oxytocin.

2. Male reproductive organ

In 1998, Piyachaturawat and collaborate investigated effect of *C. comosa* on male reproductive organs. Intragastric administion of the hexane extract at dose of 500 mg/kg body for 7 consecutive days to immature male rats. The results showed significantly suppressed weights of testes, epididymis, ventral prostrate, seminal vesicle and levator ani muscle whereas the same dose of ethyl acetate extract did not affect the organ weights. Histological examination revealed regression of the spermatogonium in seminiferous tubules and necrosis of epithelial cells in the epididymis. Also, in next year, Piyachaturawat and collaborate found that *C. comosa* hexane extract was able to decrease testicular weight corresponded with a marked regression of spermatogonia and spematids in the seminiferous tubules in adult rats (Piyachaturawat *et al*., 1999a).

3. Choleretic effect

Choleretic effect of hexane, ethyl acetate, butanol and aqueous extracts of *C. comosa* were investigated in rats. An intraduodenal injection of the extract at 1.0 g/kg body weight to rats caused an increase of bile flow rate. The butanol extract was the most potent choleresis and followed by ethyl acetate, hexane and aqueous extracts, respectively. The choleresis of ethyl acetate extract was found to be dose-dependent. For other biliary constituents, both butanol and ethyl acetate extracts (1 g/kg) as well as ethyl acetate fraction markedly increased the concentrations and outputs of bilirubin,

cholesterol and calcium. An increase of cholesterol in biliary constituents possibly caused a decrease in plasma cholesterol level (Piyachaturawat *et al*., 1996).

In 1997, Suksamrarn and collaborates found that three diarylheptanids compound (i.e. 1,7-diphenyl-5-hydroxy-(1E)-1-heptene, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-heptene and 7-(3,4-dihydroxyphenyl)–5-hydroxy-1-phenyl-(1E)-1-heptene) were isolated from the ethyl acetate extract of *C. comosa* rhizomes. A phloracetophenone glucoside, 4,6-dihydroxy-2-o-(β-D-glucopyranosyl) acetophenone, was isolated from the ethyl acetate and n-butanol extracts exhibited choleretic activity in rats. Especially phloracetophenone glucoside exhibited effectively choleretic activity in a dose dependent manner.

The effects of synthetic phloracetophenone (THA) on bile flow and biliary lipid secretion were investigated in male rats. The results showed that a single intraduodenal administration of THA at the doses of 10-150 mg/kg induced a dose-dependent increase of bile flow rate. The increase of bile flow was associated with an increased biliary secretion of bile acid, decreased secretion of cholesterol, phospholipid, and lowered bile lithogenic index. THA at a dose of 100 mg/kg body weight induced a maximal increase of bile flow rate and bile acid output. The stimulation of bile secretion by THA was due to an increase in both bile acid-dependent and bile acid-independent flow (Piyachaturawat *et al*., 1998b).

In 2002, Piyachaturawat and collaborates investigated the choleretic effect of THA in hypercholesterolemic male hamsters. Intragastric administration of THA (300-600 μmol/kg) twice daily for 7 days to these animals caused a dose and time-dependent decreases in both plasma cholesterol and triglyceride levels. THA at a dose of 400 μmol/kg reduced cholesterol and triglyceride levels in plasma with decreases in both plasma very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol but not of high density lipoprotein (HDL) cholesterol.

4. Hypolipidemic effect

In 1999, Piyachaturawat and collaborates investigated the hypolipidemia effect of *C. comosa* ethyl acetate extract in hypercholesterolaemic hamsters. Intragastric administration of *C. comosa* ethyl acetate extract (0-500 mg/kg/day) to

hypercholesterolaemic hamsters for 7 days decreased both plasma triglyceride and cholesterol level in a dose-dependent manner. The *C. comosa* ethyl acetate extract also increased plasma HDL-cholesterol and decreased plasma LDL-cholesterol (Piyachaturawat *et al*., 1999b).

5. Nematocidal effect

Hexane-soluble fraction of methanol extract of *C. comosa* had nematocidal activity. It was found that trans-1,7-diphenyl-5-hydroxy-1-heptene was the most potent inhibitor of nematode mortality with an EC_{95} of 0.7 μg/ml. Trans-1,7-Diphenyl-6-hepten-3-one-5-ol was slightly less active (1μg/ml), followed by trans-1,7-diphenyl-3-acetoxy-6-heptene (9 μg/ml) and trans-1,7-diphenyl-6-hepten-3-on (9 μg/ml), which were less potent, and finally trans,trans-1,7-diphenyl-1,3-heptadien-5-ol, which was inactive (>100 μg/ml) (Jurgens *et al*., 1994).

6. Anti-inflammatory effect

In 2006, Jantaratnotai and collaborate investigated anti-inflammatory effect of *C. comosa* extract on the responses in microglia stimulated with lipopolysaccharide (LPS). Pretreatment of highly aggressively proliferating immortalized (HAPI) cells, a rat microglial cell line, with the *C. comosa* hexane extract at concentrations of 10^{-9} to 10^{-5} g/ml significantly suppressed the levels of nitric oxide (NO) released from these cells. The attenuation in inducible nitric oxide synthase (iNOS) protein and mRNA expression was also observed suggesting an interference at a transcriptional level. In addition, *C. comosa* extract inhibited interferon regulatory factor-1 expression which is an essential transcription factor governing the iNOS expression. Moreover, the levels of mRNA expressions of MCP-1 (monocyte chemoattractant protein) and IL-6 (interleukin-6) induced by LPS were also prominently decreased in the presence of *C. comosa* extract.

Toxicological effects

1. Subacute toxicity

Toxicity of the choleretic compound, THA was investigated in mice, rats and hamsters. Acute toxicity of THA was observed to be dependent on species and route of administration, but not sex and age. Median lethal dose (LD_{50}) of THA given intraperitoneally to male hamsters and mice were 338 and 365 mg/kg body weight, respectively. It was significantly increased to 489 mg/kg body weight in adult male rats. Subacute toxicity was investigated in adult male rats by giving THA at doses of 37-300 mg/kg body weight/day, i.g. for 30 consecutive days. High doses of THA induced periportal hepatocyte degeneration whereas plasma concentrations of alanine aminotransferases, aspartate aminotransferase were slightly increased. (Piyachaturawat *et al*., 2002b).

2. Subchronic toxicity

C. comosa ethanolic extract was investigated in a subchronic toxicity study. The extract was intragastric administered to six groups of Wister rats for 90 consecutive days. The experimental groups were given the extract at doses of 100, 200, 400 and 800 mg/kg/day while two control groups received distilled water and solvent vehicles. The extract did not affect growth and food consumption of rats. Decreasing of hematocrit and hemoglobin in male rats receiving the highest dose of the extract were still within the normal range. Male rats treated with 800 mg/kg/day and female rats receiving 400 and 800 mg/kg/day of the extract had a significant increase of alkaline phosphatase level. Increases in stomach weight and size were observed in male rats receiving the extract at a dose of 100 mg/kg/day or more and in females at a dose of 200 mg/kg/d and more. Histopathological examination of visceral organs revealed no abnormality related to the extract except hyperplasia and hyperkeratosis of the gastric epithelium, the effects of which were dose-dependent (Chivapat *et al*., 2003).

Brain estrogen receptors

Estrogen receptors (ERs) exist as 2 subtypes ERα and ERβ. Although encoded from separate genes, ERα (gene on chromosome 6) and ERβ (gene on chromosome 14) share modular structure features common in the nuclear receptor superfamily. These features include an N-terminal domain that harbons activation function-1 (AF-1, A/B domain), a highly conserved DNA binding domain comprised of two Cys 4 zinc fingers (DBD, C domain), a hinge region (D domain), a less well conserved C-terminal ligand binding domain that harbors AF-2 (LBD, E domain) and a caudal C-terminal F domain (Figure 2) (Zhao *et al*., 2005). The tissue distributions and relative ligand binding affinities of ERα and ERβ in the body are different; this finding may help to explain the selective action of estrogens in different tissues. ERα is moderately to highly expressed in pituitary, kidney, epididymis, placenta, liver, adrenal, bone, and cardiovascular system, ERβ is moderately to highly expressed in brain, prostate, lung, bladder, ovary, testis, and uterus, where ERα is either low or not measurable (Mcewen and Alves,1999; Lee *et al*., 2003; Zhao *et al*., 2005). In the brain, expression of ERs is species and region specific. For instance, in rat brain, ERα and ERβ coexist in select brain regions, including the preoptic area, the bed nucleus of the striaterminalis, the medial and cortical amygdaloid nuclei, ERα is only expressed in the ventromedial hypothalamic nucleus and subfornical organ, ERβ is expressed in the cerebral cortex and hippocampus. In mouse brain, ERα is predominantly detected in the hippocampus. In human brain, although both ERs are predominantly expressed in limbic related areas, the highest ERα mRNA is restricted expression in areas such as the amygdala and hypothalamus, whereas ERβ mRNA is expressed in the hippocampal formation, cerebral cortex and thalamus (Zhao *et al*., 2005).

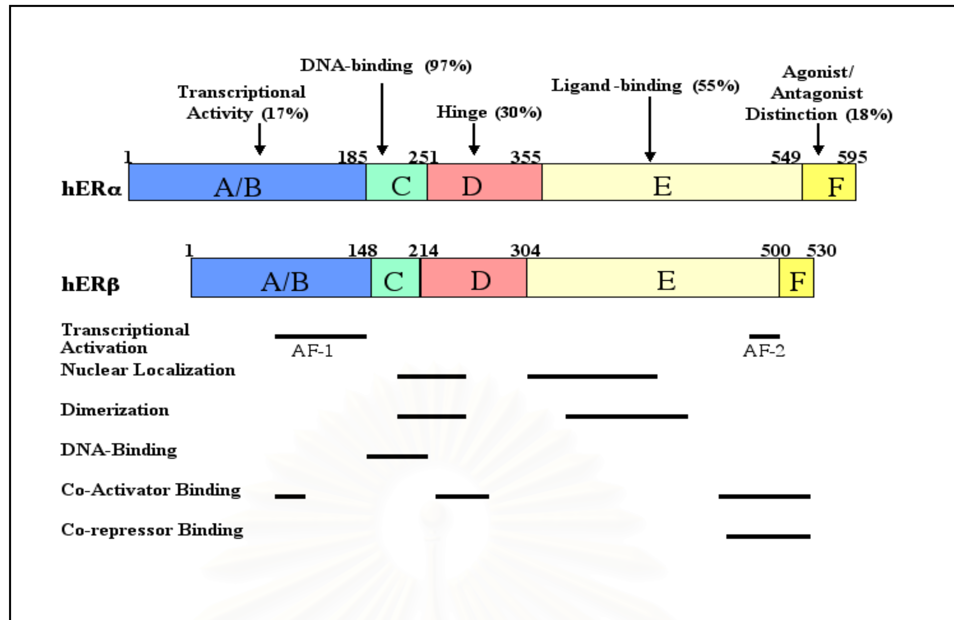

Figure 2 Estrogen receptors (http://nrr.georgetown.edu/Estrogen%20Receptor/ER-PAGE/Structure/Structure%202.html)

Mechanism of estrogen action in brain

ERs localized in the nucleus and form dimers when bound to estrogen. The dimers then interact with the estrogen response element (ERE), which regulates transcription of estrogen response genes. Antagonistic effects can occur when a compound is able to bind to the receptor but dimer formation either dose not occur or the correct configuration to activate the ERE is not attained. A small percentage (2-3%) of ERs are located on the plasma membrane and contribute to non-genomic effects of estrogen (Cornwell *et al*., 2004). In brain, recent studies of neurons indicate that both ERα and ERβ are expressed in nuclear and non-nuclear regions. Several studies on mechanism of neuroprotection by estrogen reported that estrogen may bind to putative ERs in plasma membrane and activate membrane associated signaling cascades or estrogen may also act on conventional nuclear receptors and regulate transcription of growth factor receptors, cytoskeletal proteins and antiapoptotic molecules (Figure 3A) or estrogen may act on unidentified membrane sites and activate membrane associated signaling cascades. Estrogen may act as an antioxidant, or may affect neurotransmitter

receptors, all of them resulting in neuroprotection (Figure 3B) (Mcewen and Alves, 1999; Garcia-Segura *et al*., 2001; Zhao *et al*., 2005).

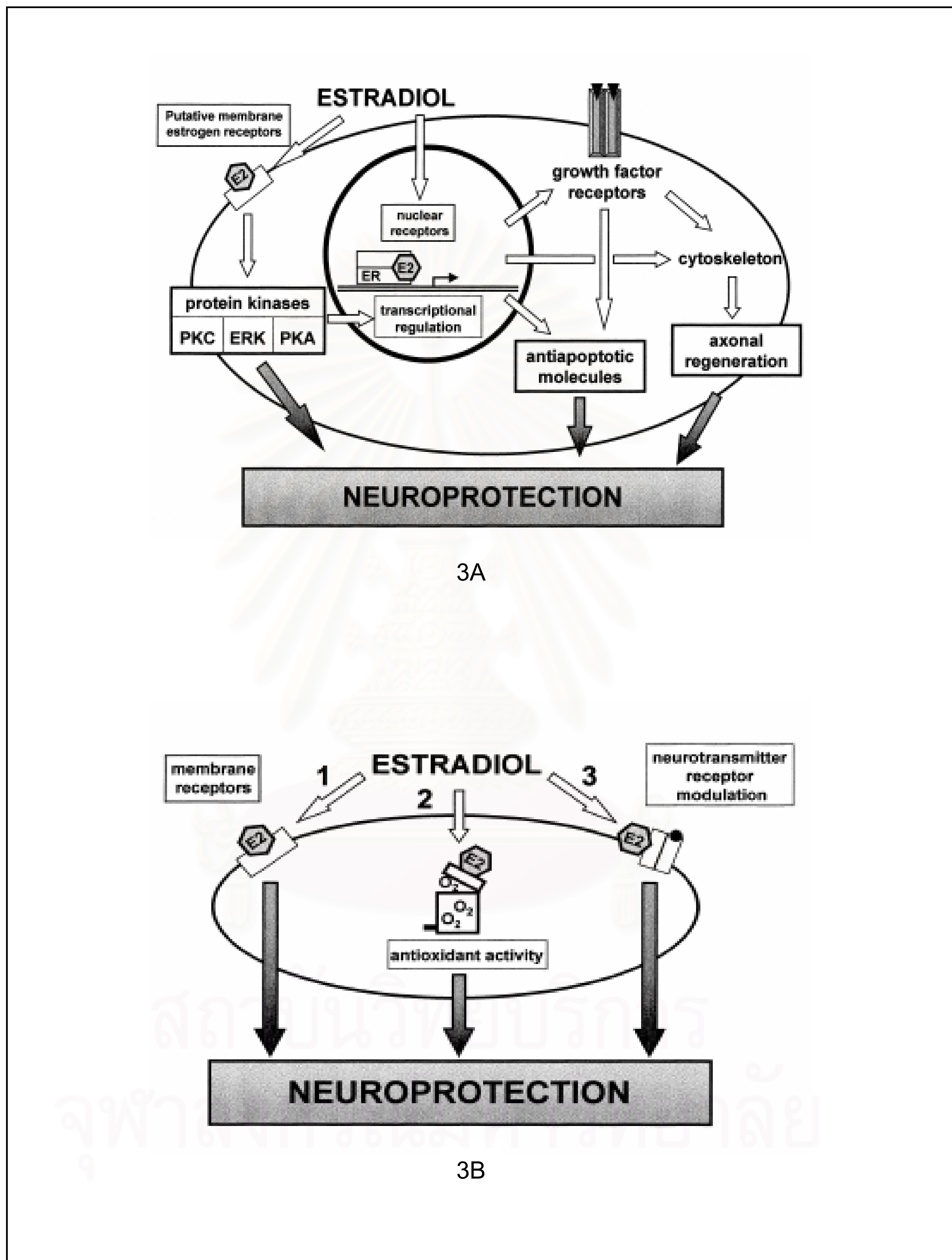

Figure 3 Neuroprotective mechanism of estrogen (Garcia-Segura *et al*., 2001)

Acetylcholine

ACh is the only accepted low-molecular weight amine transmitter substance that is not an amino acid. Biosynthetic pathway of ACh has only one enzymatic reaction, that catalyzed by ChAT and degraded by AChE (Figure 4). ACh is the transmitter used by the motor neurons of the spinal cord and therefore is released at all vertebrate neuromuscular junctions. In the autonomic nervous system, it is the transmitter for all preganglionic neurons and parasympathetic postganglionic neurons as well. It is used at many synapses throughout the brain. In particular, cell bodies synthesizing ACh are in the cholinergic neurons (Kandel *et al*., 2000; Bear *et al*., 2007).

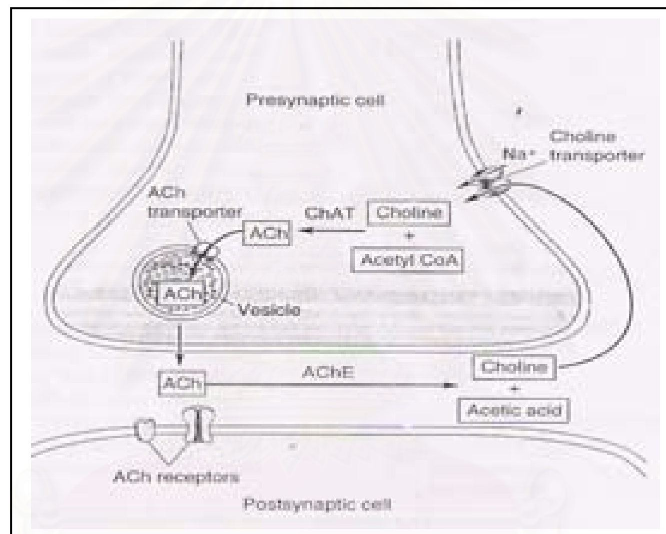

Figure 4 The life cycle of ACh: synthesis of ACh from acetyl CoA and choline, storage of ACh in synaptic vesicles, release of ACh (fusion of synaptic vesicle with presynaptic membrane and release of ACh into the synapse), action of ACh by binding to and activating receptors (nicotinic in autonomic ganglia and neuromuscular junction and muscarinic in many sites), and ACh is degraded in the synaptic cleft by AChE to choline and acetate (Bear *et al*., 2007).

Choline acetyltransferase

ChAT is an enzyme which is synthesized within the body of a neuron. It is found in the nervous system specially at sites where ACh synthesis take place. ChAT transfers an acetyl group from acetylCoA to choline, resulting in the formation of the neurotransmitter, ACh. Only cholinergic neurons contain ChAT, so this enzyme is a good marker for cells that use ACh as a neurotransmitter. With in cholinergic neurons, ChAT is concentrated in nerve terminals, although it is also present in axon, where it is transported from site of synthesis. When highly purified from rat brain, ChAT has a molar weight of 67-75 kD. It has an apparent Michaelis constant (K_m) for choline of 7.5×10^{-4} M and for acetylCoA of 1.0×10^{-5} M. The enzyme is activated by chloride and inhibited by sulfhydryl reagents. Specific substrate of this enzyme are various acyl derivitves of CoA and of ethanolamine. (Cooper *et al*., 1996; Siegel, 1999).

Acetylcholinesterase

Cholinesterase is an enzyme which catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation. There are two types as (1.) AChE also known as RBC cholinesterase or erythrocyte cholinesterase (2.) Pseudocholinesterase, also known as plasma cholinesterase, butyrylcholinesterase (BuChE). In general, neuron tissue contains AChE, while glial cells and non-neuron tissue usually contain BuChE. However, this is a generalization, and some neuron tissue (e.g., autonomic ganglia) contains both esterase, as do some extra-neuron organ (e.g., liver, lung). In blood, erythrocyte contain only AChE, while plasma contains BuChE. Both cholinesterases occur in several molecular forms that are classified as either globular or asymmetric. The globular forms G1, G2, G4 exist as monomer, dimers, and tetramers. Elongated forms that contain as many as 12 subunits and are attached to a collagen tail are classified as asymmetric. Regardless of the form, both cholinesterase occur in both a water-soluble and a membrane bound state. Although their molecular forms are similar, but two enzymes are distinct entities, encoded by specific gene. With its turnover time of 150 μs, equivalent to hydrolyzing 5000 molecules of ACh per molecule of enzyme per second, AChE ranks as one of the most efficient enzyme extant. AChE

hydrolyze ACh faster than BuChE. AChE is inhibited by high concentrations of ACh but, BuChE is not. (Cooper *et al*., 1996)

Cholinergic neurons

Major group of cholinergic neurons is in basal forebrain. The cholinergic cells project to the hippocampus, cerebellum, and cerebral cortex (Bear *et al*., 2007).

Basal forebrain

Basal forebrain is a term for a group of structure that lie near the bottom of the front of the brain, including the nucleus basalis of Meynert, nuclei of the vertical and horizontal limbs of the diagonal band, medial septum and substantia innominata. These structures are important in the production of ACh. Thus, damage to basal forebrain can reduce the amount of ACh in the brain and impair learning (Kandel *et al*., 2000; Bear *et al*., 2007).

Hippocampus

Hippocampus is a one of a group of structures forming the limbic system and is a part of the hippocampus formation, which also includes the dentate gyrus, subiculum, and entorhinal cortex. Different components of the limbic system have been shown to play a critical role in all aspects of emotion, fear, learning and memory (Hains, 2006).

Cerebral cortex

Cerebral cortex is dense aggregation of neuron cell bodies that ranges from 2-4 mm in thickness and forms the surface of each cerebral hemisphere. The total area of the cerebral cortex is about 2500 cm^2, a little larger than a single page of a newspaper. Neurons in the cerebral cortex receive input from many subcortical structures by way of the thalamus and also from other regions of the cortex via association fiber. Cortical neurons, in turn, project to a wide range of neuron structures, including other areas of the cerebral cortex, the thalamus, the basal nuclei, the cerebellum via the pontine nuclei, many of the brainstem nuclei, and the spinal cord. Cerebral cortex is divided into distinct functional areas, some of which are devoted to the processing of incoming sensory information, others to the organization of motor activity, and still others primarily to what are considered “higher intellectual functions”. These functions include memory, judgment, planning of complex activities, processing of language, mathematical

calculations, and the constriction of an internal image of an individual's surrounding (Hains, 2006).

Estrogen and cholinergic system

Basal forebrain contains cholinergic neurons that project to cerebral cortex and hippocampus, where they play an important role in cognitive function. In addition, these neurons are lost and/or impaired in association with aging and Alzheimer's disease. Studies of estrogen effects on the expression of cholinergic enzymes were among the first that pointed to nonreproductive actions of gonadal steroids. Experiments with ovariectomy and estrogen replacement therapy revealed an induction of choline acetyltransferase (Mcewen and Alves, 1999; Gibb, 2000). In 1985, Luine found that administration of 17β-estradiol to OVX rats, is associated with restored activity of ChAT in the medial aspect of the horizontal diagonal band nucleus, the frontal cortex, and CA1 of the dorsal hippocampus. Singh et al (1994) found that ChAT activity was decreased in the hippocampus of 5-week OVX animals but 17β-estradiol replacement resulted in a reversal of this effect. Gibbs (1997) found that 40 and 100 mg/kg of 17β-estradiol–treated OVX rats every other day for 1 week shown an increase of ChAT-IR cells in MS, 17β-estradiol at dose of 40 mg/kg increased ChAT-IR cells in NBM and 8 mg/kg of 17β-estradiol–treated OVX rats every other day for 2 week caused an increase of ChAT-IR cells in NBM. Gibbs (1998, 2000) found that short term treatment with physiological levels of estrogen caused a restoration of ChAT mRNA in MS and NBM in OVX rats. In 1999, Wu and collaborates found that 3 mg/kg of raloxifene and 0.03 and 0.3 mg/kg of estradiol benzoate treated-OVX rats once daily for 3 or 10 days caused a restoration of hippocampal ChAT activity in OVX rats. Das and collaborate (2002) have reported that estradiol showed various effects on AChE activity in different brain regions, including a significant decreased AChE activity in thalamus, medulla and hippocampus by the effect of estradiol.

Estrogen and Alzheimer's disease

Estrogen has important action in central nervous system as well as in peripheral tissues such as bone, breast, uterus, and cardiovascular system (Stahl, 2001). Estrogen has been associated with a decreased risk, delayed onset and progression, or enhanced recovery from numerous traumatic or chronic neurological and mental diseases such as depression, stroke, epilepsy, head injury, as well as the AD. Many epidemiological studies have proposed the protective role of estrogen in AD. The possible of estrogen in AD may be divided into three actions (Figure 5). Firstly, estrogen may affect beta-amyloid depositon by the regulation of beta-amyloid metabolism and ApoE expression. Secondly, estrogen may influence affective disorders associated with AD by acting on serotonin receptor signaling. Finally estrogen may increase cognitive function by enhancing cholinergic neurotransmission (Garcia-Segura *et al*., 2001).

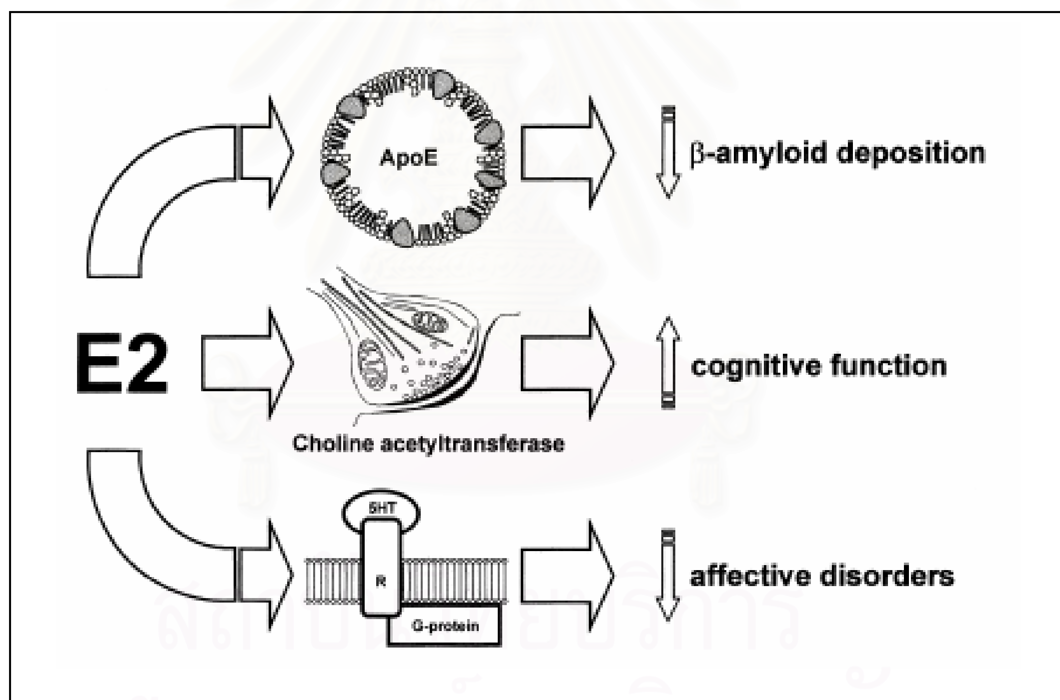

Figure 5 The possible role of estrogen in AD (Garcia-Segura *et al*., 2001)

Alzheimer's disease (AD)

AD is a progressive brain disorder that gradually destroys a person's memory and ability to learn, reason, make judgments, communicate and carry out daily activities. As AD progresses, individuals may also experience changes in personality and behavior, such as anxiety, suspiciousness or agitation, as well as delusion or hallucination. AD advances at widely different rates. People with AD die an average of four to six years after diagnosis, but the duration of the disease can vary from 3 to 20 years. One of characteristic changes in AD is the degeneration of neurons in brain. Scientists are not absolutely sure what causes cell death and tissue loss in the Alzheimer brain, but plaques and tangles are prime suspects. Plaques form when protein pieces called beta-amyloid clump together. Beta-amyloid comes from a larger protein found in the fatty membrane surrounding nerve cells. Beta-amyloid is chemically "sticky" and gradually builds up into plaques. The most damaging form of beta-amyloid may be groups of a few pieces rather than the plaques themselves. The small clumps may block cell-to-cell signaling at synapses. They may also activate immune system cells that trigger inflammation and devour disabled cells. Tangles destroy a vital cell transport system made of proteins. In healthy area: The transport system is organized in orderly parallel strands somewhat like railroad tracks. Food molecules, cell parts and other key materials travel along the "tracks." A protein called tau helps the tracks stay straight. In areas where tangles are forming: Tau collapses into twisted strands called tangles. The tracks can no longer stay straight. They fall apart and disintegrate. Nutrients and other essential supplies can no longer move through the cells, which eventually die.

Risk factors of AD

1. Age: The greatest known risk factor for Alzheimer's is the increasing age. Most individuals with the disease are 65 or older. The likelihood of developing Alzheimer's doubles about every five years after age 65. After age 85, the risk reaches nearly 50 percent.

2. Family history: Research studies have shown that those who have a parent, brother or sister, or child with AD are more likely to develop AD. The risk increases if

more than one family member has the illness. When diseases tend to run in families, either heredity (genetics) or environmental factors or both may play a role.

3. Genetics (heredity): Scientists believe that genes are involved in Alzheimer's. There are two categories of genes that can play a role in determining whether a person will develops a disease. Alzheimer genes have been found in both categories: 1) Risk genes increase the likelihood of developing a disease, but do not guarantee it will happen. Scientists have so far identified one Alzheimer risk gene called apolipoprotein E-e4 (APOE-e4). APOE-e4 is one of the three common forms of the APOE genes; the others are APOE-e2 and APOE-e3. APOE provides the blueprint for one of the proteins that carries cholesterol in the bloodstream. Scientists do not yet know how APOE-e4 raises risk. In addition to raising risk, APOE-e4 may tend to make symptoms appear at a younger age than usual. 2) Deterministic genes directly cause a disease, guaranteeing that anyone who inherits them will develop the disorder. These cases have been identified in a relatively small number of families with many people in multiple generations affected. True familial Alzheimer's accounts for less than 5 percent of cases.

Other risk factors include head injury, heart-head connection which the risk of developing AD or vascular dementia appears to be increased by many conditions that damage the heart or blood vessels. General healthy aging may help keep the brain healthy and may even offer some protection against developing AD or related diseases.

Treatment of AD

Currently, there is no cure for AD. But drug and non-drug treatments may help with both cognitive and behavioral symptoms.

The U.S. Food and Drug Administration (FDA) has approved two classes of drugs to treat cognitive symptoms of AD as following:

1. Cholinesterase inhibitors such as donepezil, rivastigmine, galantamine. All of these drugs are designed to prevent the breakdown of acetylcholine, a chemical messenger in the brain that is important for memory and other thinking skills.

2. An uncompetitive low-to-moderate affinity N-methyl-D-aspartate (NMDA) receptor antagonist such as memantine. This drug appears to work by regulating the activity of glutamate, one of the brain's specialized messenger chemicals involved in information

processing, storage and retrieval. Over stimulation of NMDA receptors from excess glutamate caused an excess accumulation of calcium in nerve cells, leading to disruption and death of cells. Memantine may protect cells against excess glutamate by partially blocking NMDA receptors.

Other drugs such as vitamin E are often prescribed as a supplementary treatment for AD, because they may protect brain cell damage from free radicals.

According to the behavioral symptoms, there are two distinct types of treatments for AD associated symptoms as following:

1. Non-drug strategies. Non-drug interventions should be tried first. In general, steps to managing behaviors include (1) identifying the behavior, (2) understanding its cause, and (3) adapting the care giving environment to remedy the situation.

2. Medications for treatment of behavioral symptoms

2.1 Antidepressant medications for relief mood and irritability such as citalopram, fluoxetine, paroxetine, sertraline, trazodone.

2.2 Anxiolytics for relief anxiety, restlessness, verbally disruptive behavior and resistance such as lorazepam, oxazepam.

2.3 Antipsychotic medications for treatment of hallucination, delusion, aggression, hostility and uncooperativeness such as olanzapine, quetiapine, risperidone, ziprasidone, haloperidol (Alzheimer's Association, 2006).

Effect of phytoestrogens on brain

Phytoestrogens are represented by hundreds of different type of molecules that are classified as non-steroidal in configuration with a customary diphenolic structure. They are abundant in fruits, vegetables, legumes, whole grains especially flaxseed, clover and soy products. They possess structure and function similar to 17β-estradiol structure. The three main classes of phytoestrogens are: (1) isoflavone (derived principally from soybeans and clover), (2) lignans (found in flaxseed in large quantities) and (3) coumestans (derived from sprouting plants like alfalfa). Phytoestrogens have been studied for their potential beneficial effects in the prevention of hormone–dependent cancers (e.g., breast cancer, prostate cancer), cardiovascular disease,

osteoporosis, alleviate the symptoms of menopause (e.g., hot flushes) as well as effect in brain (Lephart *et al*., 2002; Lee *et al*., 2004).

Influence of phytoestrogens on CNS have been widely investigated. Many studies reported the positive effects of phytoestrogen adminstration. Duffy *et al.* (2003) found that postmenopausal women receiving 60 mg of soy isoflavone per day for 12 weeks showed a significant improvement of cognitive function. It was also observed that postmenopausal women receiving 30 mg of isoflavone twice daily for 12 weeks showed a greater improvements in 3 task reflecting frontal lobe function: mental flexibility, planning and sustained attention as compared to those receiving placebo (File *et al*., 2002). In 2001, File and collaborates found that male and female students received 100 mg of isoflavone per day for 10 weeks showed a significant improvements in short-term and long-term memory and in mental flexibility. In 2006, Casini and collaborates demonstrated that soy isoflavone have positive effects for postmenopausal women by improving cognitive performance and mood. In animal studies, Pan et al. (1999a, 1999b) showed that dietary soya phytoestrogens increased BDNF in frontal cortex and NGF in the frontal cortex and hippocampus of OVX rats. In 2000, Pan and collaborates found that OVX rats consumed soy-isoflavone-containing diets for 10 months showed a dose-dependent improvement in their performance in radial arm maze tests. Furthermore, Lee et al. (2004) found that soy isoflavone caused an increase ChAT and a decrease AChE activity in male rats.

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CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Forty five male Wistar rats of body weight between 250-300 g were obtained from the National laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Animals were housed two per cage at the Faculty of Medicine, Srinakharinwirot University, Bangkok and acclimatized for at least 1 week prior to the experiment. All animals were in a controlled humidify room at a constant temperature of 25°C and maintained on 12-hour alternate light-dark cycle. They were allowed free access to food (CP company, Thailand) and drinking water.

2. Instruments

The following instruments were used in the experimentation.

1. Surgical equipments
2. Autopipettes of 20, 100, 200, 1000 and 5000 μl (Gibson, France)
3. Potter-Elvehjem homogenizer with pestle and glass homogenizing Vessel (Heidolph, Germany)
4. pH meter (Beckman Instrument, USA)
5. Sonicator (Elma, Germany)
6. Timer (Citzen®)
7. Vortex mixer (CT Laboratory, Thailand)
8. Metabolic shaker bath (Memmert, Germany)
9. Ultra-low temperature freezer (Forma Scientific Inc., USA)
10. Spectrophotometer (Shizumadzu, Japan)
11. Centrifuge (Beckman Instrument, USA)
12. Water bath

3. Chemicals

These following chemicals were used in the experimentation:

Acetyl CoA sodium salt, bovine serum albumin (BSA), choline chloride, cupric sulfate, dimethylsulfoxide (DMSO), 5,5'-dithiobisnitrobenzoic acid (DTNB), 4,4'dithiodipyridine (4PDS), ethylenediaminetetraacetic acid (EDTA) sodium salt, Folin & Ciocalteu's phenol reagent, neostigmine bromide, sodium carbonate (Na_2CO_3), sodium citrate, sodium phosphate dibasic anhydrous (Na_2HPO_4) and sodium phosphate monobasic anhydrous (NaH_2PO_4) were purchased from Sigma chemical Co., USA.

Acetylthiocholine iodide was purchased from Fluka chemic, Japan.

Hydrochloric acid (HCl), sodium arsenite, sodium chloride (NaCl), sodium hydroxide (NaOH) were purchased from Merck, Germany.

Estradiol valerate (Progynon depot®), was purchased from Schering AG Germany.

Except indicated, water used in this study was ultrapure water which was prepared by ELGASTAT MAXIMA UF® (ELGA Ltd, England).

4. *C. Comosa*

C. Comosa hexane and ethanolic extracts were kindly provided by Professor Dr. Apichart Suksamran, Faculty of Sciences, Ramkumhaeng University.

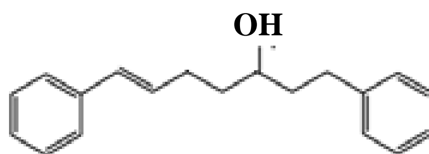
<u>Preparation of *C. comosa* extracts</u>

The rhizomes of plant were collected from Nakornpathom, Thailand. The dried rhizomes powder (30 kg) was extracted with n-hexane in a Soxhlet extractor to give a pale brownish viscous oil (1.01 kg). The marc was subsequently extracted with 95% ethanol, the ethanolic fraction was dried under vacuum in rotary evaporator and dried again with high vacuum pump to give a dark reddish-brown viscous oil (1.30 kg). For administration to animals, both hexane and ethanolic extracts were dissolved in corn oil.

<u>Chemical identification test</u>

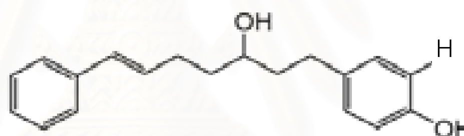
To find the marker of the extracts, *C. comosa* hexane extract was separated by thin layer chromatography (TLC), silica gel (Merck, Damstadt, Germany, catalog number 107729) were used, elucidated with hexane, hexane-dichloromethane and dichloromethane. The major constituent of this extract was 1,7-diphenyl-5-hydroxy-(1*E*)-

1-heptene (alnustone), which had a spectroscopy similar to earlier reported (Suksamrarn et al., 1997).

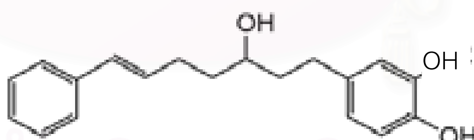


1,7-diphenyl-5-hydroxy-(1*E*)-1-heptene

C. comosa ethanolic extract was separated by thin layer chromatography (TLC), silica gel (Merck, Damstadt, Germany, catalog number 107729) were used, elucidated with hexane-dichloromethane, dichloromethane and dichloromethane-methanol. The major constituent of this extract was 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene which had a spectroscopy similar to earlier reported (Suksamrarn et al., 1997).



5-hydroxy-7-(4-hydroxyphenyl) -1-phenyl -(1*E*)-1-heptene



7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene

Methods

1. Animal treatment: an *ex vivo* study

Forty five rats were randomly divided into 6 treatment groups. Group 1-5 comprised of 8 rats and group 6 comprised of 5 rats as followings:

1. Control group: corn oil was given orally to each rat at 1 ml/kg/day for 30 days.

2. *C. comosa* group I: *C. comosa* hexane extract was given orally to each rat at dosage of 250 mg/kg/day for 30 days.
3. *C. comosa* group II: *C. comosa* hexane extract was given orally to each rat at dosage of 500 mg/kg/day for 30 days.
4. *C. comosa* group III: *C. comosa* ethanolic extract was given orally to each rat at dosage of 250 mg/kg/day for 30 days.
5. *C. comosa* group IV: *C. comosa* ethanolic extract was given orally to each rat at dosage of 500 mg/kg/day for 30 days.
6. Estradiol group: estradiol valerate was given intramuscularly to each rat at dosage of 10 μg/kg/day for 7 days.

Two rats in group 1-5 were given orally with corn oil or *C. comosa* extracts in each set of the experiment which started sequentially on the date 1, 4, 8, 11 and followed corn oil or extracts administration until finished 30 days. Rats in estradiol group were started giving estradiol during the last week before finishing the experiment of group1-5.

C. comosa for animal administration was prepared daily by dissolving 250 and 500 mg of *C. comosa* hexane or ethanolic extracts with 1 ml of corn oil to make a concentration of 250 and 500 mg/ml of *C. comosa* suspension.

2. Blood sampling for determination of estradiol concentration

At the end of the treatment, animals were fasted for 12 hours before anesthesized with diethyl ether by inhalation and then cervical dislocated. Blood samples were collected by heart puncture from left ventricle for determination of serum estradiol concentration.

Serum estradiol concentrations were analyzed by Professional Laboratories Management Corp. Co., Ltd., Bangkok.

3. Brain collection

<u>Reagents</u>

1. 0.9% w/v NaCl
2. liquid nitrogen

Procedure

1. After collecting blood samples, rats were decapitated. Whole rat brain was removed on ice.
2. The whole brain was rinsed with ice-cold 0.9% w/v NaCl, and then freezed with liquid nitrogen.
3. The whole brain was wrapped with aluminum foil and stored at -80°C until preparation of brain homogenate and enzyme activity assay.

4. Brain homogenate preparation

Reagents

1. 0.1 M sodium phosphate buffer, pH 7.4

   One liter of 0.1 M sodium phosphate buffer, pH 7.4 consisted of 11.36 g of Na_2HPO_4 and 2.4 g of NaH_2PO_4. The solution was adjusted to pH 7.4 with NaOH or HCl.

Procedure

1. The frozen whole brain was weighed, thawed and then cerebral cortex, basal forebrain and hippocampus were dissected on ice.
2. Each brain region was weighted and homogenized with ice cold 0.1 M sodium phosphate buffer, pH 7.4 to make a concentration of approximately 20 mg/ml of the homogenate.
3. Each brain region homogenate was aliquoted, kept in microtubes for analysis of protein concentrations and enzyme activity assay.

5. Determination of choline acetyltransferase activity

ChAT activity was determined using the method of Chao and Wolfgram (1971) and Lee et al. (2004). Acetyl CoA and choline choride were substrates of ChAT. ChAT in brain homogenate transferred acetate ion from acetyl CoA to choline. Activity of ChAT was determined by measuring the rate production of 4-thiopyridone (4-TP), which was a product of the reaction between CoA and 4PDS.

Reagents

1. 0.1 M sodium phosphate buffer, pH 7.4
2. 1.1 mM EDTA

   EDTA sodium salt 8.19 mg was dissolved with double distilled water and made up to 20 ml.
3. 3.0 M NaCl

   Sodium chloride 3.50 g was dissolved with double distilled water and made up to 20 ml.
4. 0.76 mM neostigmine bromide

   Neostigmine bromide 4.61 mg was dissolved with double distilled water and made up to 20 ml.
5. 1.0 M choline chloride

   Choline chloride 2.79 g was dissolved with double distilled water and made up to 20 ml.
6. 6.2 mM acetylCoA sodium salt

   AcetylCoA sodium salt 100 mg was dissolved with double distilled water and made up to 19.9 ml.
7. 2.5 mM sodium arsenite

   Thirty milliliters of 0.05 M sodium arsenite was added with double distill water and made up to 600 ml.
8. 1mM 4PDS

   4PDS 4.4 mg was dissolved with double distilled water and made up to 20 ml.
9. Double distilled water

Procedure

1. Three tubes were used for each brain homogenate sample. One tube was a sample blank and the others were sample tubes.
2. The following reagents were added into each tube for the reaction.

   - 20 μl of 0.1 M sodium phosphate buffer, pH 7.4
   - 20 μl of 1.1 mM EDTA
   - 20 μl of 3.0 M NaCl
   - 20 μl of 0.76 mM neostigmine bromide

- 20 μl of 1.0 M choline chloride
- 20 μl of 6.2 mM acetylCoA sodium salt
- 80 μl of double distilled water

3. All tubes were preincubated in metabolic shaker bath at 37°C for 5 minutes.
4. The reaction was initiated by an addition of 200 μl of brain homogenate. For the sample blank tube, 200 μl of boiled brain homogenate was added instead of brain homogenate.
5. The mixture was incubated for 20 minutes and boiled for 2 minutes for stopping the reaction.
6. Eight hundred microliters of 0.05 M sodium arsenite was added to each tube.
7. All tubes were centrifuged at 12,000 *g* for 5 minutes and 1 ml of the supernatant was collected.
8. Twenty microliters of 1 mM 4PDS was added to each tube and all tubes were allowed to stand at room temperature for 2 hours.
9. The absorbance of the solution was measured by spectrophotometer at 324 nm.

Calculation

Amount of 4-TP produced in the reaction was calculated using a molar extinction coefficient of 1.98×10^4 and the measured absorbance. Rates of the reaction were calculated by dividing amount of 4-TP in nmol by time of reaction (20 minutes), and amount of brain homogenate protein used (mg) in the reaction. Unit of ChAT activity was expressed as nmol/mg protein/min.

Verification of the method for determining of ChAT activity

Before determining ChAT activity of the brain homogenate samples, the method was verified for the linearity and the precision assays.

1. Linearity assay

Procedure

1. A cerebral cortex homogenate sample was used in the reaction by varying amount of homogenate protein (0.25, 0.5, 1, 1.5 and 2 mg of the cerebral cortex homogenate protein/400 μl of the reaction mixture).

2. The reaction was performed as described above, each amount of the protein sample was determined in duplicate.
3. The average absorbance of each sample was plotted against its amount of protein.
4. Coefficient of determination (r^2) was calculated.

2. Precision assay:

2.1 Intraday precision

<u>Procedure</u>

1. A cerebral cortex homogenate sample was used in the reaction by using 1 mg of the homogenate protein /400 μl of the reaction mixture.
2. The reaction was performed as described above repeatedly 5 times within the same day.
3. Percent coefficient of variation (%CV) of intraday precision was calculated as following:

$$\text{\% CV of intraday precision} = \frac{\text{SD of the absorbance of the reaction solutions assayed within 1 day}}{\text{Mean of absorbance of the reaction solutions assayed within 1 day}} \times 100$$

2.2 Interday precision

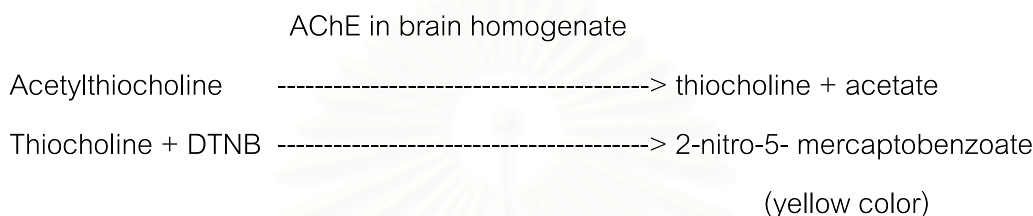
<u>Procedure</u>

1. A cerebral cortex homogenate sample was used in the reaction by using 1 mg of the homogenate protein /400 μl of the reaction mixture.
2. The reaction was performed as described above repeatedly 5 times within the same day and performed for 4 days.
3. Percent coefficient of variation (%CV) of intraday precision was calculated as following:

$$\text{\% CV of interday precision} = \frac{\text{SD of the absorbance of the reaction solutions assayed for 4 days}}{\text{Mean of absorbance of the reaction solutions assayed for 4 days}} \times 100$$

6. Determination of AChE activity

AChE activity was determined using the method of Ellman et al. (1961). The activity was determined by kinetic measuring an increase of the yellow color of the product produced from thiocholine that reacted with DTNB ion. Thiocholine was a degradation product of acetylthiocholine by AChE in the brain homogenate as following:



<u>Reagent</u>

1. 0.1 M sodium phosphate buffer, pH 7.4
2. 0.01 M DTNB

   DTNB 39.6 mg was dissolved with sodium phosphate buffer, pH 7.4 and made up to 10 ml
3. 0.075 acetylthiocholine iodide

   Acetylthiocholine iodide 0.108 g dissolved with double distilled water and made up to 5 ml.

<u>Procedure</u>

1. Brain homogenate of 0.4 ml was added into both cells containing 2.6 ml of 0.1 M sodium phosphate buffer, pH 7.4.
2. One hundred microliters of DTNB was added into both cells, mixed thoroughly.
3. Both cells were placed in a spectrophotometer and the absorbance was set to zero at 412 nm.
4. The reaction was started by an addition of 20 μl of 0.075 acetylthiocholine iodide in the sample cell. For the reference cell, 20 μl of sodium phosphate buffer, pH 7.4 was added in stead of 0.075 acetylthiocholine iodide. The solution in both cells was mixed thoroughly and measured spectrophotometrically as soon as possible.

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5. Change of the absorbance was recorded. The change of absorbance per min. was calculated. Each of the sample was determined in duplicate.

Calculations

Amount of 2-nitro-5- mercaptobenzoate produced in the reaction was calculated using a molar extinction coefficient of 1.36×10^4 and the measured absorbance. Rates of the reaction were calculated by dividing amount of 2-nitro-5- mercaptobenzoate in mol by time of reaction (4 minutes), and amount of brain homogenate protein used (mg) in the reaction. Unit of AChE activity was expressed as nmol/mg protein/min.

Verification of the method for determining of AChE activity

Before determining AChE activity of the brain homogenate samples, the method was verified for the linearity and the precision assays.

1. Linearity assay

Procedure

1. A cerebral cortex homogenate sample was used in the reaction by varying amount of homogenate protein (0.25, 0.5, 0.75, 1, 1.5, 2 mg of cerebral cortex homogenate protein/3120 μl of the reaction mixture).
2. The reaction was performed as described above, each amount of the protein sample was determined in duplicate.
3. The average change in absorbance was plotted against its amount of protein.
4. Coefficient of determination (r^2) was calculated.

2. Precision assay:

2.1 Intraday precision

Procedure

1. A cerebral cortex homogenate sample was used in the reaction by using 1 mg of cerebral cortex homogenate protein/3120 μl of the reaction mixture.
2. The reaction was performed as described above repeatedly 5 times within the same day.

3. Percent coefficient of variation (%CV) of intraday precision was calculated as following:

$$\%\ CV\ of\ intraday\ precision = \frac{\text{SD of the absorbance change of the reaction solutions assayed within 1 day}}{\text{Mean of absorbance change of the reaction solutions assayed within 1 day}} \times 100$$

2.2 Interday precision

Procedure

1. A cerebral cortex homogenate sample was used in the reaction by using 1 mg of cerebral cortex homogenate protein/3120 μl of the reaction mixture.
2. The reaction was performed as described above repeatedly 5 times within the same day and performed for 4 days.
3. Percent coefficient of variation (%CV) of intraday precision was calculated as following:

$$\%\ CV\ of\ interday\ precision = \frac{\text{SD of the absorbance change of the reaction solutions assayed for 4 days}}{\text{Mean of absorbance change of the reaction solutions assayed for 4 days}} \times 100$$

7. Effect of *C. comosa* on acetylcholinesterase activity in rat brain homogenate *in vitro*

Reagents

1. 0.1 M sodium phosphate buffer, (pH 7.4)
2. 0.01 M DTNB
3. 0.075 acetylthiocholine iodide
4. Double distilled water
5. DMSO
6. 1% DMSO

   DMSO was of 0.5 ml was added with double distilled water and made up to 50 ml.

7. 10 mg/ml of *C. comosa* hexane extract in 10% DMSO

   C. comosa hexane extract of 0.1 g was dissolved in 1 ml of DMSO and made up to 10 ml with double distilled water.

8. 1 mg/ml of *C. comosa* hexane extract in 1% DMSO

   One milliliter of 10 mg/ml *C. comosa* hexane extract was added with double distilled water and made up to 10 ml.

9. 0.01 mg/ml of *C. comosa* hexane extract in 1% DMSO

   One hundred microlitre of 1 mg/ml *C. comosa* hexane extract was added with 1% DMSO and made up to 10 ml.

10. 10 mg/ml of *C. comosa* ethanolic extract in 10% DMSO

    C. comosa ethanolic extract of 0.1 g was dissolved in 1 ml of DMSO and made up to 10 ml with double distilled water.

11. 1mg/ml of *C. comosa* ethanolic extract in 1% DMSO

    One milliliter of 10 mg/ml *C. comosa* ethanolic extract was added with double distilled water and made up to 10 ml.

12. 0.01 mg/ml of *C. comosa* ethanolic extract in 1% DMSO

    One hundred microlitre of 1 mg/ml *C. comosa* hexane extract was added with 1% DMSO and made up to 10 ml.

<u>Procedure</u>

1. Each 3000 μl of both cells comprised 1 mg protein of a brain homogenate, varied volume of diluted *C. comosa* hexane extract or *C. comosa* ethanolic extract containing *C. comosa* at a concentration of 0 μg/ml, 0.010 μg/ml , 0.10 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml of the reaction mixture and 0.1 M sodium phosphate buffer, pH 7.4 qs to 3000 μl
2. One hundred microliters of DTNB was added to the both cells, mixed thoroughly.
3. Both cells were placed in a spectrophotometer and the absorbance was set to zero at 412 nm.
4. The reaction was started by an addition of 20 μl of 0.075 acetylthiocholine iodide in the sample cell. For the reference cell, 20 μl of sodium phosphate buffer, pH 7.4 was added in stead of 0.075 acetylthiocholine iodide. The solution

in both cells was mixed thoroughly and measured spectrophotometrically as soon as possible.

5. Change of the absorbance was recorded. The change of absorbance per min. was calculated. Each of the sample was determined in duplicate.

Calculations

Amount of 2-nitro-5- mercaptobenzoate produced in the reaction was calculated using a molar extinction coefficient of 1.36×10^4 and the measured absorbance. Rates of the reaction were calculated by dividing amount of 2-nitro-5- mercaptobenzoate in nmol by time of reaction (4 minutes), and amount of brain homogenate protein used (mg) in the reaction. Unit of AChE activity was expressed as nmol/mg protein/min.

8. Determination of protein concentrations in brain homogenate

Brain homogenate protein concentrations were determined according to the method modified from the method of Lowry et al. (1951).

Reagents

1. 2% w/v Na_2CO_3
2. 0.5 M NaOH
3. 2% w/v sodium citrate
4. 1% w/v cupric sulfate
5. 1 mg/ml BSA in 0.5 M NaOH
6. Folin & Ciocalteu's phenol reagent
7. Working protein reagent. The solution was freshly prepared in a sufficient amount for all tubes in the assay (6.5 ml of the solution was required for each tube) This reagent comprised 2% w/v Na_2CO_3, 0.5 M NaOH, 2% w/v sodium citrate and 1% w/v cupric sulfate solutions in a 100:10:1:1 ratio, respectively.

Procedure

1. 16×125 mm Tube were labeled in duplicate for 7 standards (0, 50,100,150, 200, 250 and 300 μg) and for each unknown sample.

2. The following reagents were added into each standard solution tube

Standard tube	0	50	100	150	200	250	300	μg
1 mg/ml BSA	0	50	100	150	200	250	300	μl
0.5 M NaOH	500	450	400	350	300	250	200	μl

Each tube was mixed thoroughly, after addition of these reagents.

3. To each unknown sample tube, 490 μl of 0.5 M NaOH and 10 μl of brain homogenate sample were added then mixed thoroughly.
4. After 6.5 ml of working protein reagent was added to each tube in the assay, the tubes were allowed to stand at room temperature for 10 minutes.
5. While, 200 μl of Folin & Ciocalteu's phenol reagent was added to each tube in the assay, the tube was vortexed thoroughly for a minimum of 30 seconds
6. After the tubes were allowed to stand at room temperature for a minimum of 30 minutes, the absorbance of the solution was measured by spectrophotometer against the 0 μl standard at 500 nm.

Calculations

1. The average absorbance of each standard was plotted against its amount of protein. The best-fit regression line was drawn through the point. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve.
2. The protein concentration was expressed in a unit of mg/ml or μg/μl by dividing its amount of protein (from step 1) with the volume of brain homogenate used in the reaction.

9. Data analysis

All numeric quantitative data were presented as mean ± standard error of the mean (SEM). An one-way analysis of variance (ANOVA) and Student-Newman-Keuls test were used for statistical comparison at a significant level of $p < 0.05$.

CHAPTER IV

RESULTS

1. Verification of the methods used for determination of ChAT activity

1.1 Linearity assay

Linearity of the assay was performed by varying amount of cerebral cortex homogenate protein used in reaction (0.25, 0.5, 1, 1.5 and 2 mg of cerebral cortex homogenate protein / 400 μl of the reaction mixture). Coefficient of determination (r^2) between amounts of cerebral cortex homogenate protein and the corresponding absorbance was 0.9997 (Figure 6)

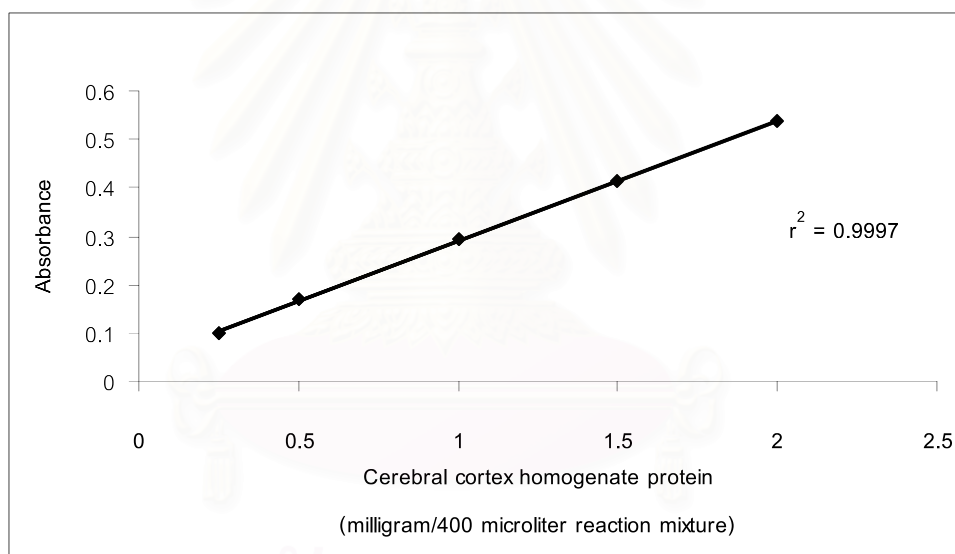

Figure 6 Linearity assay for the method used for determination of ChAT activity. Each point shown was mean of n = 2. (Procedure was demonstrated in the Materials and Methods).

1.2 Precision assay: Intraday and interday precision

Intraday and interday precision of the method used for determination of ChAT activity was performed by using 1 mg protein of a cerebral cortex homogenate as described in the Materials and Methods. Percent CV of the intraday precision of each day was 0.329, 3.436, 2.980 and 3.425 respectively and % CV of the interday precision was 2.357 (Table 1).

Table1 Intraday and interday precision of the method used for determination of ChAT activity

Date		Day1	Day2	Day3	Day4
Absorbance	1st	0.304	0.279	0.307	0.297
	2nd	0.302	0.291	0.294	0.302
	3rd	0.304	0.290	0.314	0.279
	4th	0.305	0.291	0.293	0.297
	5th	0.304	0.306	0.300	0.284
Mean of intraday precision		0.304	0.291	0.302	0.292
Standard deviation of intraday precision		0.001	0.010	0.009	0.010
% CV of intraday precision		0.329	3.436	2.980	3.425
Mean of interday precision			0.297		
Standard deviation of interday precision			0.007		
% CV of interday precision			2.357		

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2. Verification of the methods used for determination of AChE activity

2.1 Linearity assay

Linearity of the assay was performed by varying amount of cerebral cortex homogenate protein used in reaction (0.25, 0.5, 0.75, 1, 1.5 and 2 mg of cerebral cortex homogenate protein / 3120 μl of the reaction mixture). Coefficient of determination (r^2) between amounts of cerebral cortex homogenate protein and the corresponding absorbance change was 0.9913 (Figure 7).

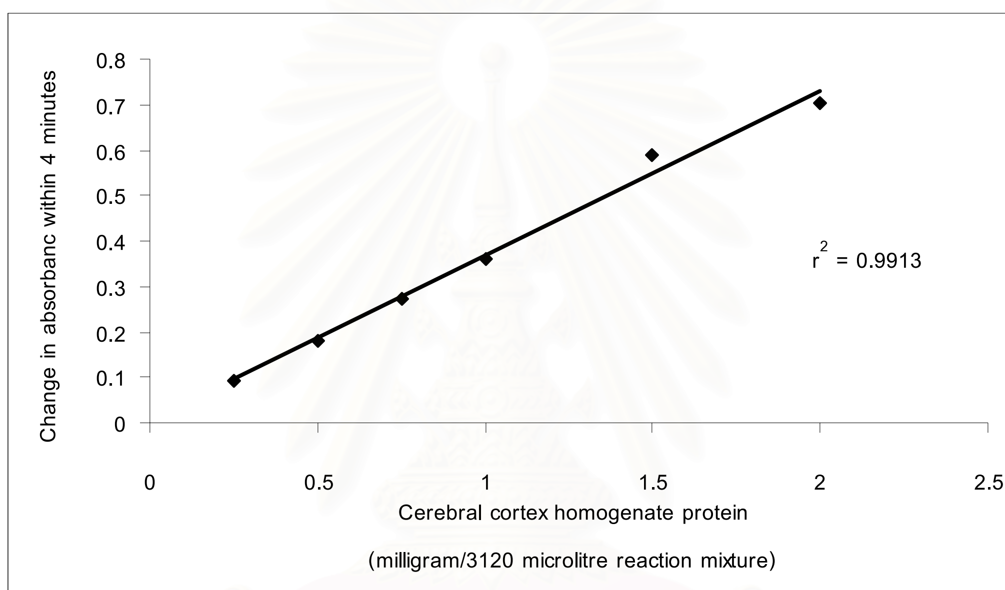

Figure 7 Linearity assay for the method used for determination of AChE activity. Each point shown was mean of n = 2. (Procedure was demonstrated in the Materials and Methods).

2.2 Precision assay: Intraday and interday precision

Intraday and interday precision of the method used for determination of AChE activity was performed by using 1 mg protein of a cerebral cortex homogenate as described in the Materials and Methods. Percent CV of the intraday precision of each day was 3.269, 1.698, 0.769, 1.176 respectively and % CV of the interday precision was 1.538 (Table 2).

Table 2 Intraday and interday precision of the method used for determination of AChE activity.

Date		Day1	Day2	Day3	Day4
Change in Absorbance within 4 minutes	1st	0.501	0.535	0.520	0.518
	2nd	0.536	0.538	0.516	0.512
	3rd	0.515	0.529	0.527	0.509
	4th	0.538	0.532	0.517	0.502
	5th	0.508	0.516	0.522	0.510
Mean of intraday precision		0.520	0.530	0.520	0.510
Standard deviation of intraday precision		0.017	0.009	0.004	0.006
% CV of intraday precision		3.269	1.698	0.769	1.176
Mean of interday precision		0.520			
Standard deviation of interday precision		0.008			
% CV of interday precision		1.538			

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3. Effects of *C. comosa* extracts on ChAT and AChE activities in rat brain: An *ex vivo* study

C. comosa hexane and ethanolic extracts were given orally to rats at doses of 250 and 500 mg/kg/day once daily for 30 days. Body weight of each rat was recorded in the beginning and the end of the treatment (Table 3). Also, at the end of the treatment, various brain regions were dissected and weighed (Table 3) for calculating the approximate volume of buffer used for preparing of brain homogenates. Serum samples collected from rats of all groups were analyzed for estradiol concentration. The results showed that both *C. comosa* hexane and ethanolic extracts caused a dose-related increase of serum estradiol concentration. As expected, rats in estradiol group possessed a significant increase of serum estradiol concentration as compared to the control group. Serum estradiol concentration in rats of the estradiol group was significantly higher than all group of the *C. comosa* treated rats (Table 4).

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Table 3 Body weight and various brain region weight of rats used in the *ex vivo* study experiment.

Parameter	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
Initial body [a] weight (g)	344.64±14.62	300.89±9.05	328.93±8.97	318.01±6.96	322.76±8.59	413.60±8.81
Final body [b] weight (g)	396.21±13.85	317.00±11.78	333.17±11.60	348.10±9.28	346.92±11.60	381.58±8.76
Whole brain [c] weight (g)	2.04±0.05	1.97±0.03	1.94±0.06	1.89±0.08	2.00±0.03	2.00±0.02
CC [d] weight (g)	0.68±0.05	0.59±0.02	0.61±0.02	0.63±0.03	0.64±0.02	0.62±0.03
BF [e] weight (g)	0.060±0.0060	0.075±0.007	0.086±0.004	0.088±0.004	0.089±0.005	0.066±0.007
HP [f] weight (g)	0.12±0.015	0.12±0.009	0.14±0.0036	0.14±0.0080	0.14±0.0072	0.12±0.01

Data shown were mean ± SEM (n=5-8)

[a] Body weight of rats at the beginning of *C. comosa* extracts administration

[b] Body weight of rats at the time of sacrification

[c] Whole brain weight of rats at the time of sacrification, before dissection of brain regions

[d] Cerebral cortex weight of rats at the time of sacrification, before preparation of brain homogenate

[e] Basal forebrain weight of rats at the time of sacrification, before preparation of brain homogenate

[f] Hippocampus weight of rats at the time of sacrification, before preparation of brain homogenate

Table 4 Effect of *C. comosa* on serum estradiol concentration

Parameter	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
Estradiol (pmol/L)	111.11±27.17	571.80±76.09*	1184.50±227.94* +	494.50±139.91*	825.40±211.02*	5568.40±608.91*∆

Data shown were mean ± SEM (n=4-8)

* $p<0.05$: *C. comosa* treated group, estradiol group vs control group

$^{+}$ $p<0.05$: *C. comosa* group II vs *C. comosa* group I

$^{\Delta}$ $p<0.05$: estradiol group vs *C. comosa* treated group

3.1 Effects of *C. comosa* extracts on ChAT activity

In cerebral cortex, *C. comosa* ethanolic extract caused a significant dose-dependent increase of ChAT activity while *C. comosa* hexane extract and estradiol valerate did not cause any significant change on this enzyme activity (Figure 8).

In basal forebrain, both hexane and ethanolic extracts of *C. comosa* as well as estradiol valerate did not exhibited any significant effect on ChAT activity (Figure 9).

In hippocampus, *C. comosa* ethanolic extract caused a significant increase of ChAT activity while *C. comosa* hexane extract and estradiol valerate did not cause any significant change on this enzyme activity (Figure10).

Regional differences in the level of ChAT activity were shown with the highest activity in basal forebrain followed by hippocampus, and cerebral cortex, respectively (Figure 11).

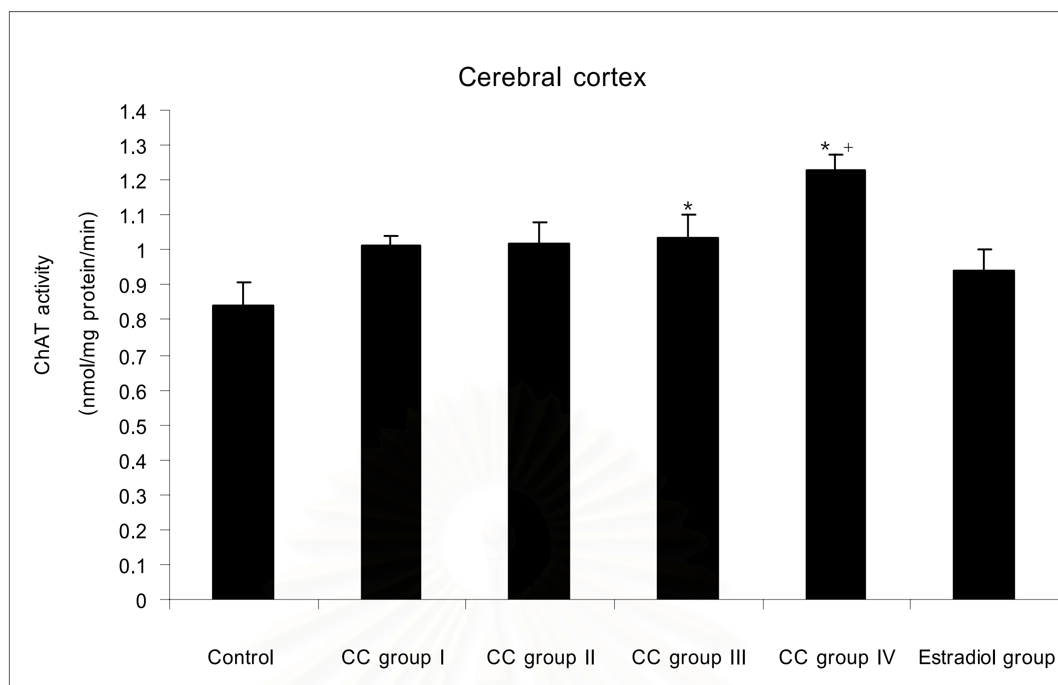

Figure 8 Effects of *C. comosa* extracts on ChAT activity in rat cerebral cortex.

The individual bar graph represented mean of ChAT activity with standard error of the mean (n=5-8). Rats were administered orally with 1 ml/kg/day of corn oil (Control group), 250 and 500 mg/kg/day of *C. comosa* hexane extract, 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (CC group I, CC group II, CC group III, CC group IV, respectively) for 30 days and intramuscularly with 10 μg/kg/day of estradiol valerate for 7 days (Estradiol group).

* p<0.05: *C. comosa* treated group vs control group

+ p <0.05: *C. comosa* 500 mg/kg/day vs *C. comosa* 250 mg/kg/day

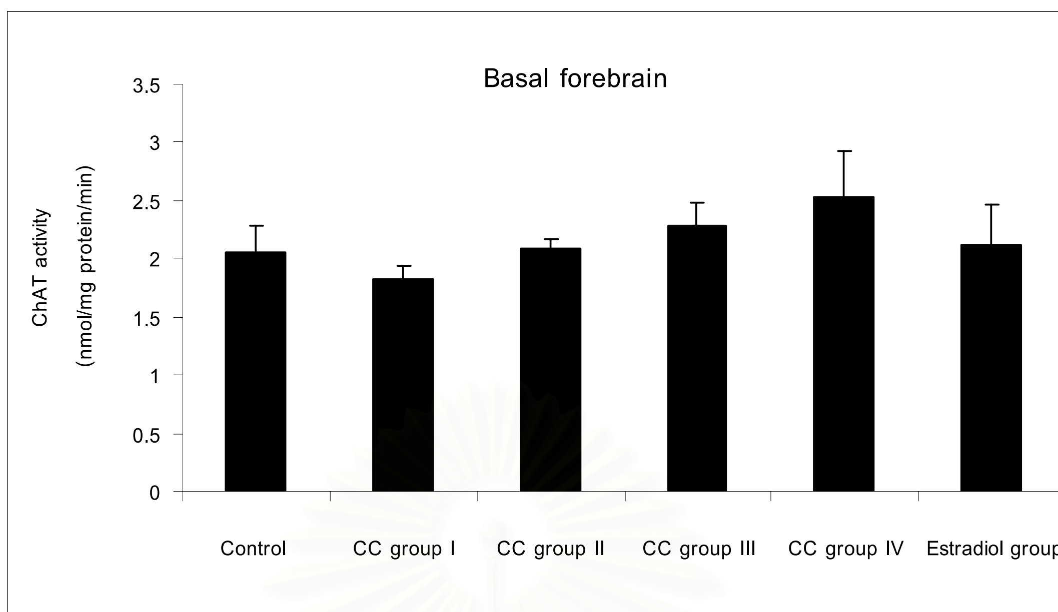

Figure 9 Effects of *C. comosa* extracts on ChAT activity in rat basal forebrain.

The individual bar graph represented mean of ChAT activity with standard error of the mean (n=5-8). Rats were administered orally with 1 ml/kg/day of corn oil (Control group), 250 and 500 mg/kg/day of *C. comosa* hexane extract, 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (CC group I, CC group II, CC group III, CC group IV, respectively) for 30 days and intramuscularly with 10 μg/kg/day of estradiol valerate for 7 days (Estradiol group).

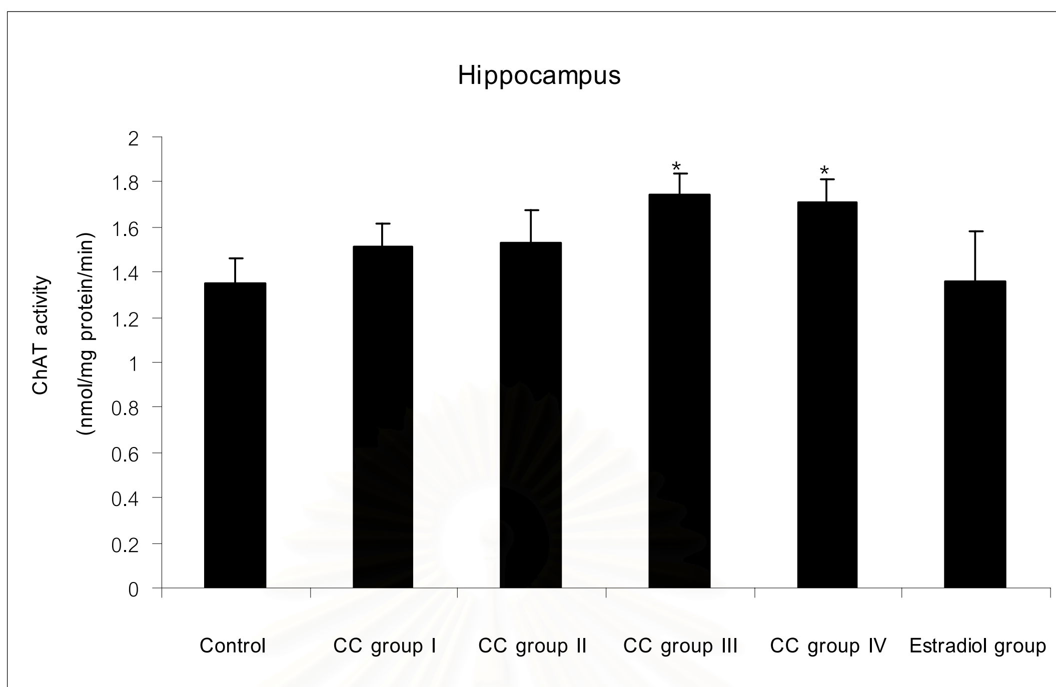

Figure 10 Effects of *C. comosa* extracts on ChAT activity in rat hippocampus.

The individual bar graph represented mean of ChAT activity with standard error of the mean (n=5-8). Rats were administered orally with 1 ml/kg/day of corn oil (Control group), 250 and 500 mg/kg/day of *C. comosa* hexane extract, 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (CC group I, CC group II, CC group III, CC group IV, respectively) for 30 days and intramuscularly with 10 μg/kg/day of estradiol valerate for 7 days (Estradiol group).

* $p<0.05$: *C. comosa* treated group vs control group

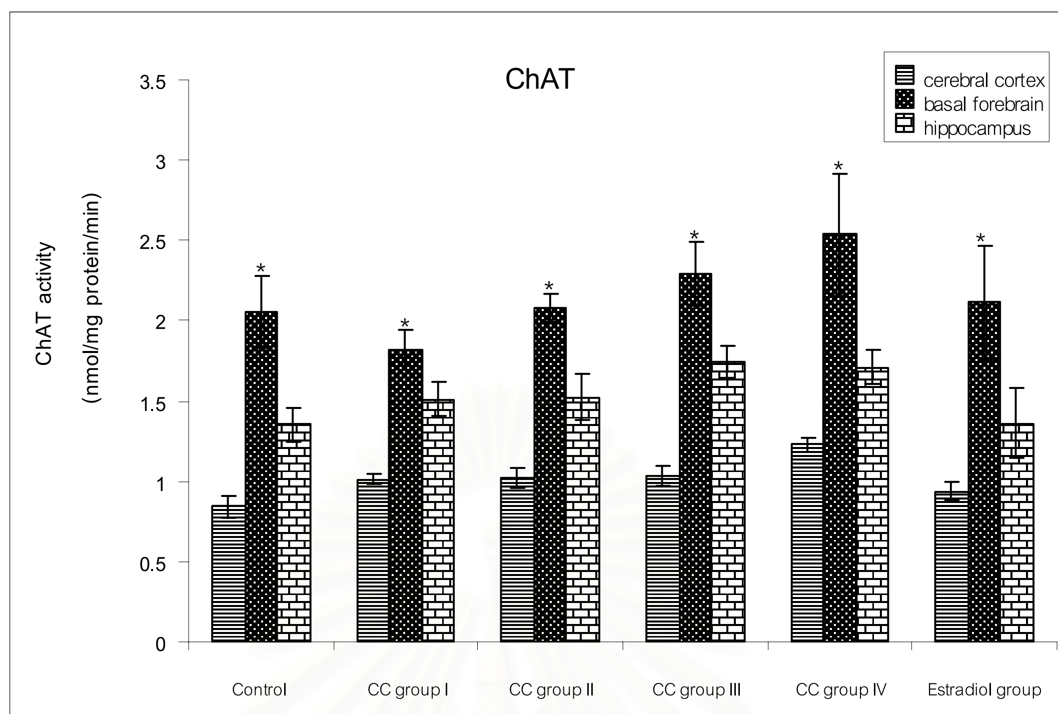

Figure 11 ChAT activity in different brain regions of the normal rats and rats treated with *C. comosa* extracts and estradiol.

The individual bar graph represented mean of ChAT activity with standard error of the mean (n=5-8). Rats were administered orally with 1 ml/kg/day of corn oil (Control group), 250 and 500 mg/kg/day of *C. comosa* hexane extract, 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (CC group I, CC group II, CC group III, CC group IV respectively) for 30 days and intramuscularly with 10 μg/kg/day of estradiol valerate for 7 days (Estradiol group).

* $p<0.05$: basal forebrain vs cerebral cortex, hippocampus

3.2 Effects of *C. comosa* extracts on AChE activity

Hexane and ethanolic extracts of *C. comosa* at both dasage regimens used in this study as well as estradiol valerate did not affect AChE activities in cerebral cortex, basal forebrain and hippocampus (Figure 12, 13, 14).

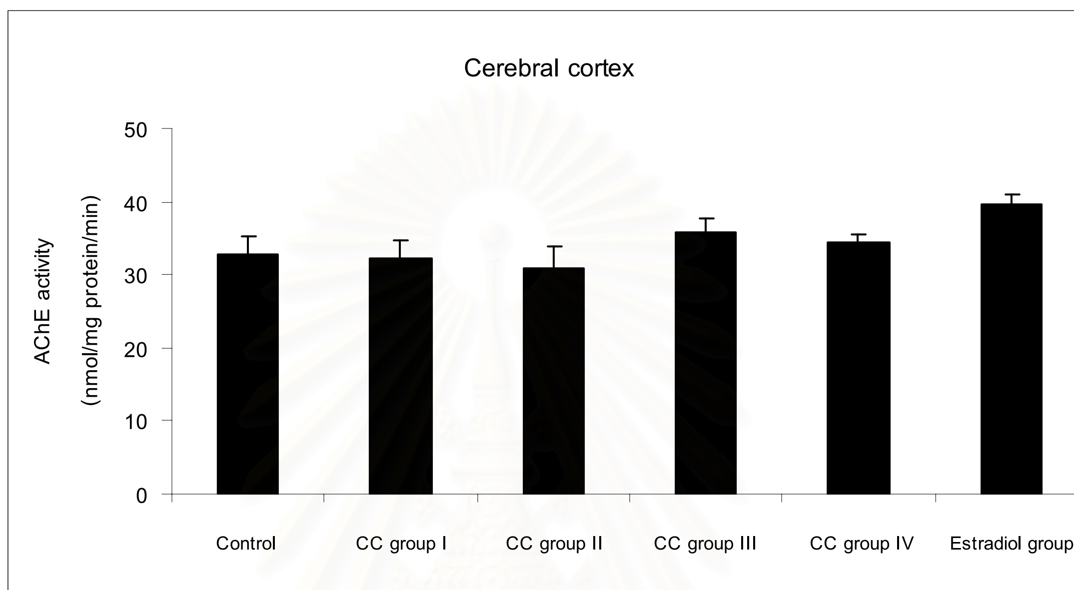

Figure 12 Effects of *C. comosa* extracts on AChE activity in rat cerebral cortex.
The individual bar graph represented mean of AChE activity with standard error of the mean (n=5-8). Rats were administered orally with 1 ml/kg/day of corn oil (Control group), 250 and 500 mg/kg/day of *C. comosa* hexane extract, 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (CC group I, CC group II, CC group III, CC group IV, respectively) for 30 days and intramuscularly with 10 μg/kg/day of estradiol valerate for 7 days (Estradiol group).

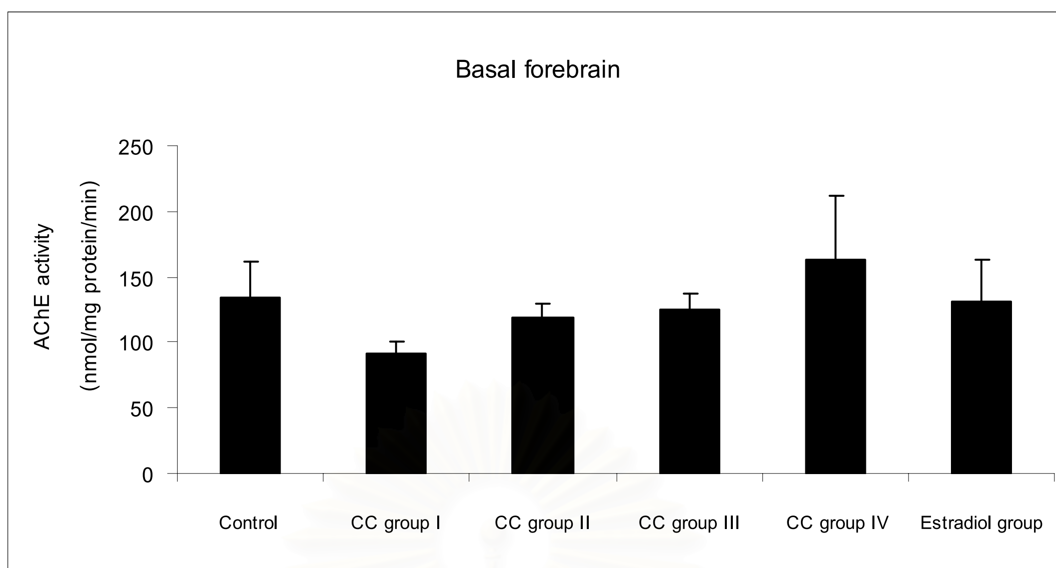

Figure 13 Effects of *C. comosa* extracts on AChE activity in rat basal forebrain

The individual bar graph represented mean of AChE activity with standard error of the mean (n=5-8). Rats were administered orally with 1 ml/kg/day of corn oil (Control group), 250 and 500 mg/kg/day of *C. comosa* hexane extract, 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (CC group I, CC group II, CC group III, CC group IV, respectively) for 30 days and intramuscularly with 10 μg/kg/day of estradiol valerate for 7 days (Estrdiol group).

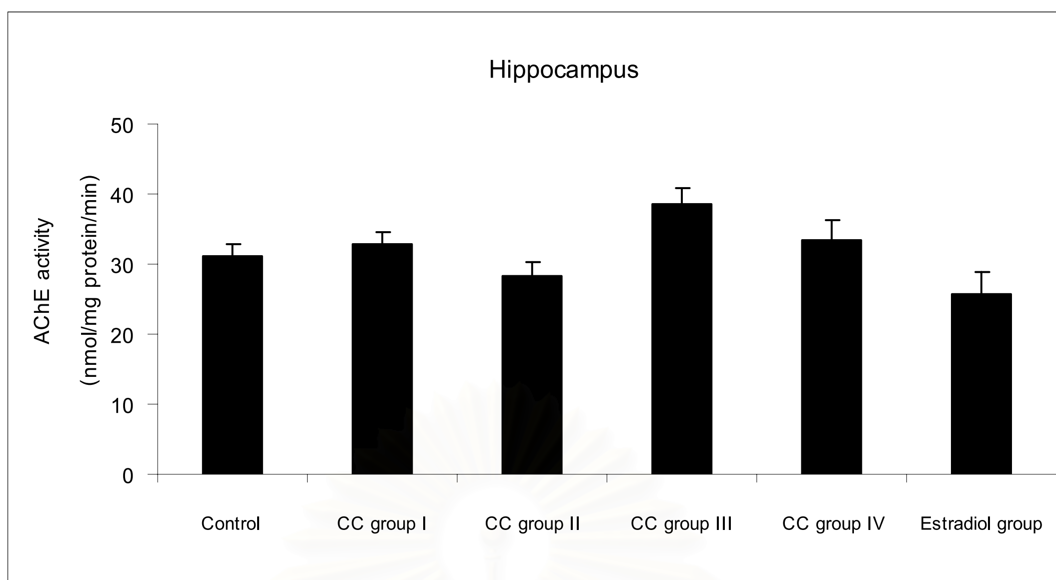

Figure 14 Effects of *C. comosa* extracts on AChE activity in rat hippocampus.

The individual bar graph represented mean of AChE activity with standard error of the mean (n=5-8). Rats were administered orally with 1 ml/kg/day of corn oil (Control group), 250 and 500 mg/kg/day of *C. comosa* hexane extract, 250 and 500 mg/kg/day of *C. comosa* ethanolic extract (CC group I, CC group II, CC group III, CC group IV, respectively) for 30 days and intramuscularly with 10 μg/kg/day of estradiol valerate for 7 days (Est.valerate group).

4. Effects of *C. comosa* extracts on AChE activities in rat brain homogenate: An *in vitro* study

C. comosa hexane and ethanolic extract at the concentration used in the reaction mixture ranging from 0 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml, 10 μg/ml and 100 μg/ml did not affect AChE activities in brain homogenates of cerebral cortex, basal forebrain and hippocampus (Table 5, 6)

Table 5 Effect of *C. comosa* hexane extract on rat brain homogenates studying *in vitro.* Each point represented mean of AChE activity (n=2) in brain homogenate of cerebral cortex, basal forebrain and hippocampus.

Brain region	Concentration(μg/ml)					
	0	0.01	0.1	1	10	100
Cerebral cortex	29.77	29.77	29.22	30.40	29.68	30.31
Basal forebrain	96.41	96.53	96.24	97.33	96.12	94.35
Hippocampus	24.72	25.26	23.63	23.63	23.77	24.78

Unit expressed as nmol/mg protein/min

Table 6 Effect of *C. comosa* ethanolic extract on rat brain homogenates studying *in vitro.* Each point represented mean of AChE activity (n=2) in brain homogenate of cerebral cortex, basal forebrain and hippocampus.

Brain region	Concentration(μg/ml)					
	0	0.01	0.1	1	10	100
Cerebral cortex	29.77	29.82	28.50	29.11	29.31	28.85
Basal forebrain	108.91	109.43	106.62	106.62	109.83	108.63
Hippocampus	19.70	20.25	20.33	20.42	19.64	18.98

Unit expressed as nmol/mg protein/min

CHAPTER V

DISCUSSION AND CONCLUSION

This study was performed primarily to investigate effects of *C. comosa* extracts on ChAT and AChE activities in rat brain. Both enzymes are related to the level of brain ACh, the neurotransmitter which play an important role in learning and memory process in cholinergic neurons. ACh is synthesized by ChAT and degraded by AChE. Effects of estrogen on the expression of these cholinergic enzymes have been studied for the possibly contribution of estrogen on brain beside its reproductive action (Mcewen and Alves, 1999). Several previous studies reported that *C. comosa* exhibited estrogenic-like effects (Piyachaturawat *et al.,* 1995a, 1995b, 1998a, 1999a). Whether or not *C. comosa* demonstrated the positive effects on brain, similar to estrogen, have never been investigated. The main objective of this study, thus to investigate effect of *C. comosa* extracts on the activities of ChAT and AChE enzymes in order to provide a preliminary information of *C. comosa* extracts on the cholinergic enzymes in various regions of rat brain. If the result showed that *C. comosa* extracts caused an increase of ChAT activity and/or a decrease of AChE activity, this would be an information indicated that *C. comosa* extracts possibly exhibited a positive effects on ACh level resulting in the positive effect on cholinergic nervous system.

This study was performed in male rats by giving *C. comosa* hexane and ethanolic extracts orally at the dosages of 250 and 500 mg/kg/day for 30 days. These doses of the hexane extract were shown to exhibit estrogenic effect in rats such as uterotropic effects, while of the alcoholic extract were shown to possess lipid-lowering effects (Piyachaturawat *et al*., 1995a, 1995b, 1998a, 1999a, 1999b). Estradiol valerate was also given intramuscular at 10 μg/kg/day for 7 days to rats in the estrogen reference group. This dosage regimen of estradiol was reported to produce serum estradiol level as found physiologically in female rats (Piyachaturawat *et al*., 1999a). The results showed that serum estradiol concentrations of rats receiving *C. comosa* hexane and ethanolic extracts were significant higher than the control rats and the increase of serum estradiol level was dose-related in the *C. comosa* hexane extract group. Even

though an increase of serum estradiol in ethanolic extract group was not dose-dependent, a tendency of dose-related increase of serum estradiol was shown. Serum estradiol concentration of rats given estradiol valerate was significant higher than the control rats and the increase was much higher than rats receiving both extracts of the *C. comosa.* An increase of serum estradiol following *C. comosa* administration might be one of the explanation for the estrogenic-like effects of this plant extracts reported earlier by many previous studies. The mechanism by which both extracts of *C. comosa* caused an increase of serum estradiol need to be further investigated.

In this study, effects of *C. comosa* hexane and ethanolic extracts on rat brain ChAT and AChE activities were investigated in three brain regions such as basal forebrain, cerebral cortex and hippocampus, the brain regions where normally contain cholinergic neurons. Basal forebrain is a primary source of the cholinergic system that project to the hippocampus and cerebral cortex, the brain regions which associated with ACh that play an importance role in learning and memory (Bear *et al*., 2007).

In this study, ChAT activity was determined using the method modified from the methods of both Chao and Wolfgram (1971) and Lee et al. (2004). For AChE enzyme, the activity was determined using the method of Ellman et al. (1961) which was reported for the determining of AChE activity in many tissues such as lung, liver, stomach, heart and muscle. Due to the method modifications, both methods were verified before using for the determination of ChAT and AChE activities in rat brain tissue in this study. Linearity and precision assays were performed. The results showed that linearity between amount of the brain tissue protein and the absorbance of the product produced from the reaction of ChAT and AChE activity assays were highly correlated with the r^2 of 0.9997 and 0.9913, respectively. Regarding the precision assay, high precision of both ChAT and AChE activity assays were shown by small % CV. The % CVs of intraday and interday precision of ChAT activity assay were 2.543% and 2.357% whereas % CVs of intraday and interday precision of AChE activity assay were 1.728% and 1.538%, respectively. These results indicated the high precision of the analysis method of both enzymes. Generally, precision of analytical methods should be limited by the % CV of less than 15% (U.S. food and drug administration, 2001).

Results from this study showing that *C. comosa* ethanolic extract caused an increase of ChAT activity at the dosages of both 250 and 500 mg/kg/day in cerebral cortex and hippocampus but not in basal forebrain. No effects of *C. comosa* hexane extract on ChAT activity were seen in all three regions of rat brain. Different constituents contained in ethanolic extract and hexane extract were most reasonably contributed to these different results. Even though this is the first report of positive effect of *C. comosa* extract on ChAT activity, other phytoestrogen-containing plant was also demonstrated this effect. Lee et al. (2004) found that soy isoflavone giving to male rats at the dosage of 0.3 g/kg of soy diet/day for 16 weeks caused an increase of ChAT activity in cerebral cortex and basal forebrain. An increase of ChAT activity in cerebral cortex and hippocampus by *C. comosa* ethanolic extract need to be confirmed at the level of enzyme protein concentration via immunoblotting which is interesting to be further explored. The mechanism(s) underlying the effect of *C. comosa* on ChAT activity in rat was not explored in the present study. Some chemical constituents in *C. comosa* or their metabolites may directly promote ChAT gene expression or they may possess a similar effect as estrogen which exhibit an increase of brain derived neurotrophic factor (Singh *et al*., 1995) or enhances ChAT activity post-translationally by itself (Nonner *et al*., 1996).

In this study, estradiol valerate did not exhibit any significant effects on ChAT activity in all three regions studied. This result was inconsistent to the results reported by Luine (1985), Singh et al. (1994) and Wu et al. (1999). In those studies, estradiol was found to increase or reverse ChAT activity in hippocampus (Luine, 1985; Singh *et al*. 1994 and Wu *et al*., 1999) and some area of basal forebrain nuclei (Luine, 1985). In the studies of Luine (1985), Singh et al. (1994) and Wu et al. (1999), they used female OVX rats supplemented with estradiol whereas in this study male rats were used with estradiol administration so as to similarly imitate the physiological estradiol level found normally in female rat. No effect of estrogen on ChAT activity in male rats in this study seem to be consistent to the result in male rats reported by Luine (1985) even though male rats in that study were castrated. This inconsistent results between female OVX rat and male rat might be explained by the sex difference in the response of brain regions follow estrogen administration. Also the different dosage regimen and the different form

of the estradiol given might contributed to this different result. In basal forebrain, both hexane and ethanolic extracts of *C. comosa* did not exhibit a significant increase effect on ChAT activity. Normally basal forebrain consists of various regions such as NBM, HDB, MS and substantia innominata. Several studied found that estrogen caused an increase of ChAT mRNA in MS and NBM but not the HDB (Gibbs *et al*., 1994; Gibbs, 1996). In contrast, Luine (1985) found that estrogen significant increased ChAT activity in HDB. The reason for these discrepancies is not clear but may be related to the difference of brain dissection in the region of basal forebrain among studies.

Both hexane and ethanolic extracts of *C. comosa* did not cause any significant effect on AChE activity in all three rat brain regions in this study. No effect of estradiol on AChE activity in this study inconsistent with a previous study of Das et al. (2000). In that study, they found that estradiol propionate treated OVX rat at dose of 1 μg/rat/day (body weight of rats range between 175-200 g) for 8 day caused a decrease of AChE activity in thalamus, medulla and hippocampus. One other study of Lee et al. (2004) demonstrated that soy isoflavone added in food for male rats at the concentration of 0.3 g/kg of soy diet/day for 16 weeks caused a decrease of AChE activity in cerebral cortex, basal forebrain and hippocampus. In the case of *C. comosa*, no effects of this plant extracts on AChE in rat brain provided a preliminary information that if *C. comosa* extracts possessed positive effect on cholinergic system, a decrease effect of these extracts on AChE, the degradative enzyme of ACh, was ruled out as the possible mechanism in this issue. In the case of estradiol, no decrease effect on AChE enzyme was shown in this study which is inconsistent to a similar previous study of Das et al (2000) as mentioned above. This different result might be complicated with the difference model of studies used between studies such as sex of rat, estrogen dosage regimen, estrogen ester form, duration of the treatment etc.

In conclusion, effects of *C. comosa* hexane and ethanolic extracts on ChAT and AChE activity in three rat brain regions (cerebral cortex, basal forebrain and hippocampus) were investigated in this study. Both extracts were given orally to rats at the doses of 250 and 500 mg/kg/day for 30 days. *C. comosa* ethanolic extract significantly increased ChAT activity in cerebral cortex and hippocampus but not in basal forebrain. *C. comosa* hexane extract did not exhibit any significant effect on this

enzyme activity. Both hexane and ethanolic extracts of *C. comosa* did not demonstrate any significant effect on AChE enzyme. An enhancement of ChAT activity in brain by *C. comosa* ethanolic extract provide a preliminary information that this extract might possess a beneficial effect on cholinergic nervous system regarding the deficiency of brain ACh which is associated with learning and memory. Confirmation of the enhancement of ChAT activity by *C. comosa* ethanolic extract in the level of protein enzyme with immunoblotting should be explored. Behavioral test should be further performed for investigating the overall outcome of *C. comosa* ethanolic extract on learning and memory associated cholinergic nervous system.

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APPENDICES

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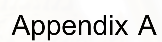

Appendix A

Body weight, brain weight, and estradiol concentration

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Table A1 Initial body weight of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	305.60	283.40	305.50	292.20	313.40	430.0
2	305.00	279.20	285.80	301.50	305.00	393.4
3	406.7	281.50	343.60	344.50	323.70	412.0
4	339.60	289.70	314.00	338.90	348.20	395.6
5	335.10	295.80	360.70	297.40	287.50	437.0
6	387.00	318.00	332.60	331.20	321.30	-
7	333.50	355.50	336.20	315.00	365.30	-
8	-	304.00	353.00	323.40	317.70	-
Average	344.6429	300.8875	328.9250	318.0125	322.7625	413.6000
SEM	14.6237	9.0516	8.9665	6.9613	8.5896	8.8066

Unit expressed as g

Table A2 Final body weight of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	358.50	327.00	296.00	315.00	367.00	398.40
2	377.50	301.50	333.20	336.40	314.00	361.30
3	402.50	279.50	358.00	317.50	341.70	384.60
4	407.50	305.00	311.00	374.50	372.00	361.40
5	369.00	315.50	387.5	347.50	314.00	402.20
6	469.20	307.50	321.00	357.00	355.00	-
7	389.30	307.50	325.5	391.30	401.00	-
8	-	392.50	-	345.60	310.60	-
Average	396.2143	317.0000	333.1714	348.1000	346.9125	381.5800
SEM	13.8485	11.7819	11.5967	9.2754	11.6005	8.7628

Unit expressed as g

Table A3 Whole brain weight of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	1.88	1.9	1.67	1.93	1.89	2.06
2	1.79	1.89	1.86	1.58	1.9	2.03
3	2.04	1.91	2.07	2.01	1.91	1.94
4	2.12	1.89	2.00	1.55	2.02	2.01
5	2.18	2.08	1.89	1.98	1.99	1.97
6	2.18	2.00	1.96	2.14	2.10	-
7	2.09	2.04	2.13	2.04	2.15	-
8	2.05	2.01	-	1.86	2.05	-
Average	2.0412	1.9650	1.9400	1.8863	2.0013	2.0020
SEM	0.0493	0.0269	0.0575	0.0758	0.0343	0.0213

Unit expressed as g

Table A4 Cerebral cortex weight of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	0.69	0.52	0.51	0.51	0.64	0.56
2	0.88	0.63	0.55	0.64	0.56	0.75
3	0.63	0.67	0.66	0.66	0.75	0.61
4	0.82	0.64	0.69	0.80	0.68	0.63
5	0.56	0.65	0.55	0.59	0.65	0.57
6	0.72	0.49	0.67	0.67	0.61	-
7	0.42	0.54	0.65	0.66	0.66	-
8	0.72	0.59	-	0.53	0.60	-
Average	0.6800	0.5912	0.6114	0.6325	0.6437	0.6240
SEM	0.0513	0.0237	0.0273	0.0323	0.0203	0.0340

Unit expressed as g

Table A5 Basal forebrain weight of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	0.06	0.05	0.07	0.08	0.06	0.08
2	0.04	0.08	0.09	0.08	0.09	0.05
3	0.05	0.06	0.08	0.08	0.10	0.05
4	0.04	0.07	0.08	0.07	0.10	0.07
5	0.07	0.11	0.10	0.10	0.09	0.08
6	0.06	0.09	0.09	0.09	0.09	-
7	0.07	0.07	0.09	0.10	0.08	-
8	0.09	0.07	-	0.10	0.10	-
Average	0.0600	0.0750	0.0857	0.0875	0.0888	0.0660
SEM	0.0060	0.0065	0.0037	0.0041	0.0048	0.0068

Unit expressed as g

Table A6 Hippocampus weight of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	0.07	0.12	0.13	0.15	0.15	0.15
2	0.06	0.12	0.14	0.17	0.13	0.09
3	0.09	0.14	0.16	0.11	0.17	0.12
4	0.14	0.14	0.15	0.16	0.14	0.11
5	0.18	0.14	0.14	0.13	0.16	0.13
6	0.14	0.09	0.14	0.14	0.12	-
7	0.10	0.08	0.14	0.12	0.13	-
8	0.14	0.15	-	0.11	0.11	-
Average	0.1150	0.1225	0.1429	0.1363	0.1388	0.1200
SEM	0.0146	0.0090	0.0036	0.0080	0.0072	0.0100

Unit expressed as g

Table A7 Estradiol concentration of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	96.4	830	1,806	607	683	6,811
2	86.8	613	1,221	773	1,010	6,575
3	50.4	-	-	-	-	3,369
4	133	-	-	-	-	5,642
5	75.6	-	954	983	1,432	5,445
6	84.2	531	757	199	142	-
7	70.5	364	-	219	-	-
8	292	521	-	186	860	-
Average	111.1125	571.8000	1184.5000	494.5000	825.4000	5568.4000
SEM	27.1661	76.0890	227.9402	139.9099	211.0155	608.9049

Unit expressed as pmol/L, Missing value (-) was due to blood insufficiency

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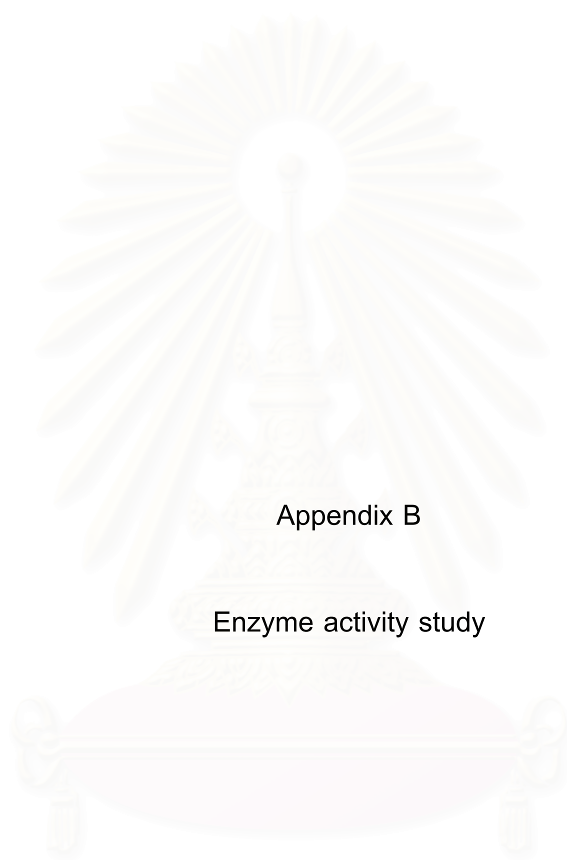

Appendix B

Enzyme activity study

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Table B1 ChAT activity in cerebral cortex of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	0.76	1.10	1.26	0.77	1.08	1.16
2	0.58	0.95	1.23	1.17	1.16	0.97
3	0.77	1.06	0.97	1.04	1.18	0.87
4	0.76	1.07	0.84	0.96	1.21	0.84
5	0.76	1.09	0.88	0.93	1.10	0.86
6	1.04	0.90	0.93	0.85	1.41	-
7	0.91	0.89	1.00	1.30	1.36	-
8	1.17	1.04	-	1.25	1.32	-
Average	0.8438	1.0125	1.0157	1.0338	1.2275	0.9400
SEM	0.0662	0.0303	0.0626	0.0676	0.0432	0.0594

Unit expressed as nmol/mg protein/min

Table B2 ChAT activity in basal forebrain of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	1.94	2.14	2.50	2.63	4.94	1.94
2	2.52	2.19	1.95	2.72	1.60	1.81
3	1.64	1.54	2.12	1.97	3.02	3.51
4	3.33	1.59	2.09	2.85	2.69	1.77
5	2.02	2.29	1.77	1.66	1.98	1.53
6	2.02	1.35	1.96	1.40	1.67	-
7	1.17	1.74	2.19	2.83	2.15	-
8	1.82	1.70	-	2.25	2.23	-
Average	2.0575	1.8175	2.0829	2.2888	2.5350	2.1120
SEM	0.2263	0.1220	0.0870	0.1981	0.3830	0.3557

Unit expressed as nmol/mg protein/min

Table B3 ChAT activity in hippocampus of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	1.53	1.02	1.53	2.23	2.08	1.89
2	1.09	1.51	1.21	1.87	1.77	1.79
3	0.96	1.37	1.85	1.78	1.69	1.42
4	1.02	1.37	1.06	1.81	1.42	0.79
5	1.34	1.44	1.30	1.71	1.17	0.92
6	1.49	1.58	1.54	1.74	2.05	-
7	1.73	1.87	2.19	1.28	1.82	-
8	1.66	1.94	-	1.54	1.67	-
Average	1.3525	1.5125	1.5257	1.7450	1.7088	1.3620
SEM	0.1053	0.1038	0.1475	0.0960	0.1074	0.2222

Unit expressed as nmol/mg protein/min

Table B4 AChE activity in cerebral cortex of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	30.33	30.90	35.72	29.28	32.70	36.01
2	40.03	35.34	31.66	32.63	33.14	41.60
3	31.00	38.80	32.96	30.92	36.23	37.20
4	25.10	40.67	36.37	32.15	34.96	42.07
5	33.89	36.37	32.43	39.77	40.58	41.66
6	36.16	20.07	33.03	38.42	31.13	-
7	23.46	28.17	13.81	42.93	31.67	-
8	42.97	27.86	-	40.84	35.91	-
Average	32.8675	32.2725	30.8543	35.8675	34.5400	39.7080
SEM	2.4054	2.4143	2.9151	1.8348	1.0906	1.2832

Unit expressed as nmol/mg protein/min

Table B5 AChE activity in basal forebrain of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	114.98	79.82	174.42	142.27	503.41	110.23
2	126.51	144.77	99.76	147.60	108.69	103.86
3	94.77	66.36	124.57	138.90	119.84	260.21
4	306.34	83.25	134.05	161.99	130.58	85.43
5	146.33	122.13	84.49	67.79	114.83	94.81
6	129.44	70.81	108.29	85.02	75.87	-
7	37.13	86.37	104.02	146.56	124.52	-
8	117.24	75.67	-	105.32	122.42	-
Average	134.0925	91.1475	118.5143	124.4312	162.5200	130.9080
SEM	27.2211	9.7435	11.1563	12.0204	49.0590	32.5956

Unit expressed as nmol/mg protein/min

Table B6 AChE activity in hippocampus of an individual rat

Rat No.	Group					
	Control group	*C.comosa* group I	*C.comosa* group II	*C.comosa* group III	*C.comosa* group IV	Estradiol group
1	31.66	27.72	32.72	46.70	-	31.27
2	29.79	31.71	31.80	49.32	44.55	33.06
3	19.96	26.69	28.21	31.70	29.60	27.40
4	31.55	29.72	23.75	36.92	27.14	18.63
5	37.91	32.79	29.20	34.96	27.01	18.24
6	31.21	36.99	32.89	42.13	43.74	-
7	30.89	35.55	19.96	32.57	32.17	-
8	35.38	41.43	-	34.29	29.67	-
Average	31.0438	32.8250	28.3614	38.5738	33.4114	25.7200
SEM	1.8471	1.7597	1.8510	2.3589	2.8499	3.1122

Unit expressed as nmol/mg protein/min

Table B7 AChE activities in rat brain homogenate: An *in vitro* study

Concentration (μg/ml)	*C.comosa* hexane extract			*C.comosa* ethanolic extract		
	Cerebral cortex	Basal forebrain	Hippocampus	Cerebral cortex	Basal forebrain	Hippocampus
0	29.77	96.41	24.72	29.77	108.91	19.70
0.01	29.77	96.53	25.26	29.82	109.43	20.25
0.1	29.22	96.24	23.63	28.50	106.62	20.33
1	30.40	97.33	23.63	29.11	106.62	20.42
10	29.68	96.12	23.77	29.31	109.83	19.64
100	30.31	94.35	24.78	28.85	108.63	18.98

Unit expressed as nmol/mg protein/min

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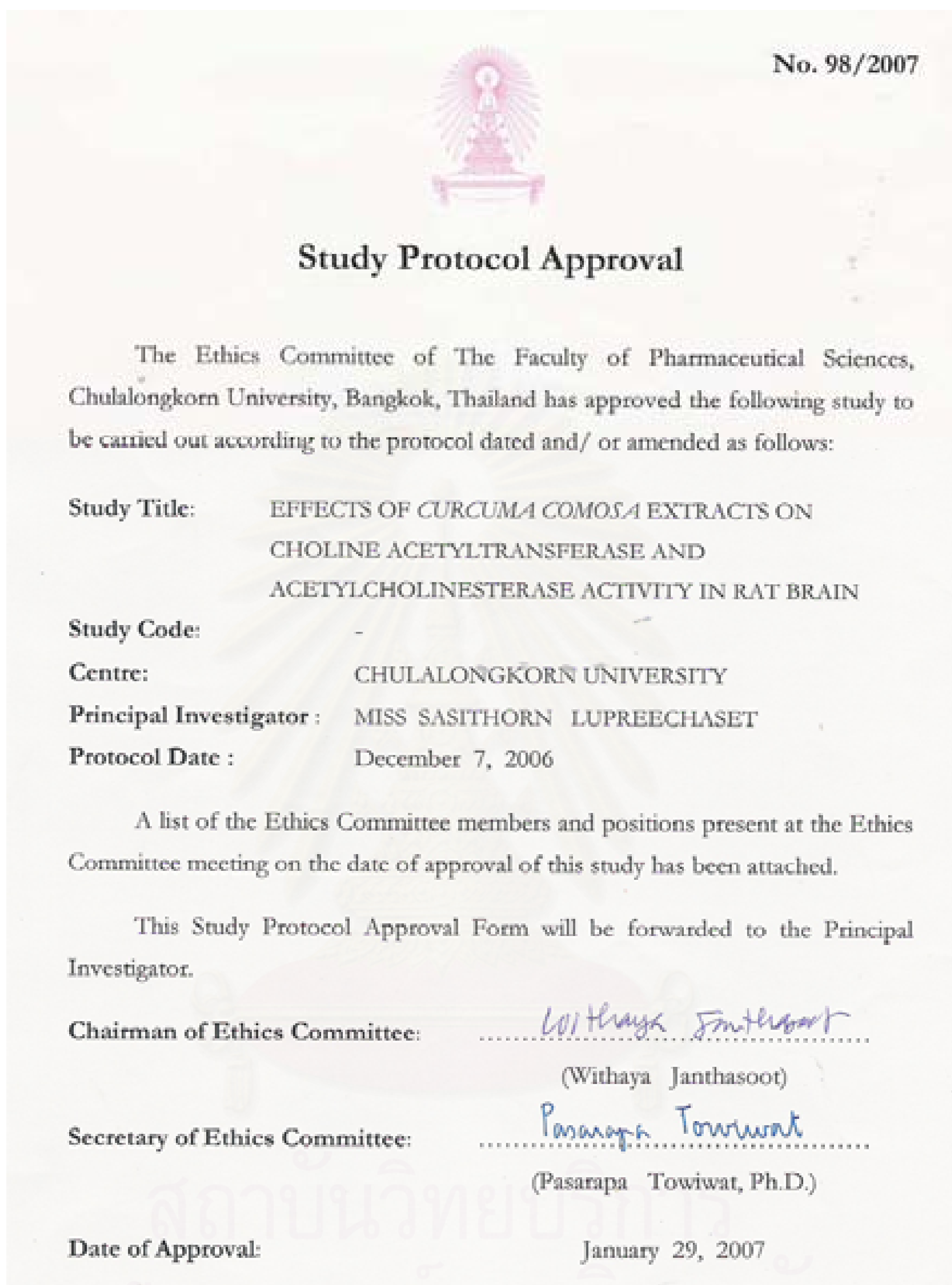
No. 98/2007

Study Protocol Approval

The Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol dated and/ or amended as follows:

Study Title: EFFECTS OF *CURCUMA COMOSA* EXTRACTS ON CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE ACTIVITY IN RAT BRAIN

Study Code: -

Centre: CHULALONGKORN UNIVERSITY

Principal Investigator : MISS SASITHORN LUPREECHASET

Protocol Date : December 7, 2006

A list of the Ethics Committee members and positions present at the Ethics Committee meeting on the date of approval of this study has been attached.

This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Chairman of Ethics Committee: ....

(Withaya Janthasoot)

Secretary of Ethics Committee: ....

(Pasarapa Towiwat, Ph.D.)

Date of Approval: January 29, 2007

Figure 15 Study Protocol Approval by Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

CURRICULUM VITAE

Miss Sasithorn Lupreechaset was born in June 29, 1978 in Mahasarakham, Thailand. She graduated with a B.pharm in 2001 from the Faculty of pharmacy, Srinakharinwirot University, Bangkok, Thailand.

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