ผลของสารสกัดเอทานอลจากบัวบกต่อความบกพร่องในการเรียนรู้และความจำที่ถูกเหนี่ยวนำโดยภาวะสมองขาดเลือด และถูกเหนี่ยวนำโดยสารสะโคโพลามีนในหนูถีบจักร

นางสาวเสาวลักษณ์ ดอกนาค

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF ASIATIC PENNYWORT (CENTELLA ASIATICA) ETHANOL EXTRACT ON IMPAIRMENT OF LEARNING AND MEMORY INDUCED BY CEREBRAL ISCHEMIA AND INDUCED BY SCOPOLAMINE IN MICE.

Miss Saowalak Doknark

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เสาวลักษณ์ ดอกนาค : ผลของสารสกัดเอทานอลจากบัวบกต่อความบกพร่องในการเรียนรู้และความจำที่ถูกเหนี่ยวนำโดยภาวะสมองขาดเลือด และถูกเหนี่ยวนำโดยสารสะโคโพลามีนในหนูถีบจักร. (EFFECTS OF ASIATIC PENNYWORT (CENTELLA ASIATICA) ETHANOL EXTRACT ON IMPAIRMENT OF LEARNING AND MEMORY INDUCED BY CEREBRAL ISCHEMIA AND INDUCED BY SCOPOLAMINE IN MICE.) อ. ที่ปรึกษา : รศ.ดร. บุญยงค์ ตันติสิระ, อ. ที่ปรึกษาร่วม : รศ.ดร. มยุรี ตันติสิระ : 67 หน้า. 974-17-3737-8.

งานวิจัยนี้ เป็นการศึกษาผลของสารสกัดเอทานอลจากบัวบก ซึ่งเป็นพืชสมุนไพร ต่อความบกพร่องในการเรียนรู้และความจำในหนูถีบจักรที่ถูกเหนี่ยวนำโดยการทำให้สมองอยู่ในภาวะขาดเลือด จากการผูกหลอดเลือดคอมมอนคาโรติดทั้งสองข้าง หรือจากการได้รับสารสะโคโพลามีน พบว่าหนูที่อยู่ในภาวะขาดเลือดในสมองใช้เวลาในการหาแท่นพักนานขึ้นเมื่อทดสอบด้วยวิธีมอรีสวอเตอร์เมส นอกจากนี้เมื่อทดสอบด้วยวิธีสะเต็บดาวน์พบว่า หนูใช้เวลาอยู่บนแท่นพักลดลง และจำนวนครั้งที่ก้าวลงจากแท่นพักเพิ่มขึ้นซึ่งหมายถึงหนูในกลุ่มนี้ เกิดความบกพร่องในการเรียนรู้และความจำ แต่เมื่อให้สารทดสอบทางปากในขนาด 300, 1000 หรือ 1500 มิลลิกรัมต่อกิโลกรัมน้ำหนักต่อวัน มีผลทำให้หนูใช้เวลาในการหาแท่นพักลดลง เมื่อทดสอบด้วยวิธีมอรีสวอเตอร์เมส และจากการทดสอบด้วยวิธีสะเต็บดาวน์ หนูใช้เวลาอยู่บนแท่นพักเพิ่มขึ้น และจำนวนครั้งที่ก้าวลงจากแท่นพักลดลง เมื่อเทียบกับหนูในกลุ่มควบคุม แสดงว่าสารทดสอบสามารถแก้ไขความบกพร่องในการเรียนรู้และความจำที่เกิดจากภาวะสมองขาดเลือดได้ และยังพบว่าสารทดสอบทุกขนาดไม่มีผลต่ออัตราการเคลื่อนไหวของหนูที่อยู่ในภาวะสมองขาดเลือดแต่อย่างใด นอกจากนั้นยังพบว่าสารทดสอบทุกขนาด สามารถลดระดับ เอ็มดีเอ (ดัชนีวัดระดับออกซิเดทีพสเตรท) ที่เพิ่มขึ้นในสมองหนูที่อยู่ในภาวะสมองขาดเลือดได้

ในทางตรงกันข้ามไม่พบว่ามีการเพิ่มขึ้นของระดับ เอ็มดีเอ ในหนูถีบจักรที่ได้รับการเหนี่ยวนำให้เกิดความบกพร่องในการเรียนรู้และความจำด้วยสารสะโคโพลามีน และสารทดสอบทุกขนาดไม่สามารถแก้ไข ความบกพร่องในการเรียนรู้และความจำที่เกิดขึ้นในกลุ่มการทดลองนี้ แสดงว่าสารทดสอบไม่น่าจะมีการออกฤทธิ์ต่อระบบโคลิเนอร์จิก ในระบบประสาทส่วนกลาง

อาจกล่าวได้ว่าสารสกัดเอทานอลจากบัวบก สามารถแก้ไขความบกพร่องในการเรียนรู้และความจำในหนูที่อยู่ในภาวะขาดเลือดในสมองจากคุณสมบัติต้านออกซิเดชั่น ดังนั้นสารสกัดเอทานอลจากบัวบก อาจสามารถแก้ไขภาวะความจำบกพร่องที่เกิดจากภาวะออกซิเดทีพสเตรทได้ สิ่งที่น่าสนใจในการศึกษาส่วนต่อไปคือ การหาสารประกอบสำคัญในสารสกัดเอทานอลในบัวบกที่มีผลต่อการเรียนรู้และความจำ

ลายมือชื่อนิสิต.....

สาขาวิชาสรีรวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4489118720: MAJOR PHYSIOLOGY

KEY WORD: *Centella asiatica* / Learning / Memory / Cerebral ischemia / Scopolamine/ Morris water maze / Step down test / Locomotor activity / Lipid peroxide

SAOWALAK DOKNARK : EFFECTS OF ASIATIC PENNYWORT (CENTELLA ASIATICA) ETHANOL EXTRACT ON IMPAIRMENT OF LEARNING AND MEMORY INDUCED BY CEREBRAL ISCHEMIA AND INDUCED BY SCOPOLAMINE IN MICE.

THESIS ADVISOR : Assosiate Professor Boonyong Tantisira, Ph.D., THESIS COADVISOR : Assosiate Professor Mayuree Tantisira, Ph.D. 67 pp. ISBN 974-17-3737-8

The effects of crude ethanolic extracts of *Centella asiatica* (CE), a traditional medical plant, on learning and memory impairment induced by either cerebral ischemia (bilateral common carotid arteries: 2VO) or scopolamine, were investigated in mice. The 2VO caused an increase in the latency to find the platform in learning trial in Morris water maze (MWM), a reduction of step-down latency and an increment of number of errors in step-down test indicating that the 2VO could impair learning and memory. Treatment with oral administration of CE (300, 1000 and 1500 mg/kg. day body weight (B.W.)) once daily markedly shortened the latency of escaping onto the platform in MWM, prolonged the step-down latency and decreased the number of error significantly as compared with 2VO-operated mice. These results suggested that CE could improve learning and memory impairment induced by cerebral ischemia. In addition, the CE administration at all doses did not show any significant effect on spontaneous locomotor activity in 2VO mice. Furthermore, CE in doses of 100, 300, 1000 or 1500 mg/kg/day B.W. significantly reduced malondialdehyde (MDA) level in the brain of 2VO mice.

On the other hand, scopolamine, though, caused an impairment of learning and memory did not increase the level of MDA. The administration of CE (100, 300, 1000 or 1500 mg/kg/day B.W.) did not attenuate learning and memory impairment induced by scopolamine in mice. This result suggested that CE might have no cholinomimetic activity in the CNS.

Therefore, it is suggestive that antioxidative property of CE could, at least partly, contribute to its positive effect on memory deficit in 2VO mice. Thus, CE might be beneficial for memory impairment in which oxidative stress is an underlying cause. Further study is needed to identify the nature of compound (s) accounted for the positive effect of CE on memory.

Student's signature.....

Field of study Physiology Advisor's signature.....

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2VO	=	Two vessel occlusion
Ach	=	Acetylcholine
AD	=	Alzheimer's disease
B.W.	=	Body weight
^{0}C	=	Degree Celsius
CBF	=	Cerebral blood flow
CE	=	Crude ethanol extract of *Centella asiatica*
ChAT	=	Choline acetyltransferease
CNS	=	Central nervous system
cm	=	Centimeter
dc	=	Direct current
et al.	=	et alii (and other)
GC/MS	=	Gas chromatography/mass spectrometry
h	=	Hours
HPLC	=	High performance liquid chromatography
Hz	=	Hertz
kg	=	Kilogram
L.	=	Linn.

i.p.	=	Intraperitoneal injection
MDA	=	Malondialdehyde
min	=	Minute
mg	=	Milligram
ml	=	Milliliter
mm	=	Millimeter
ms	=	Milliseconds
MWM	=	Morris Water Maze
nmol/g tissue	=	nanomoles per gram tissue
p.o.	=	Perorally
Scop	=	Scopolamine
sec	=	Seconds
S.E.M	=	Standard error of the mean
TBARs	=	2-Thiobarbituric acid reactive substance
w/w	=	Weight by weight

CHAPTER I

INTRODUCTION

1. *Centella asiatica* (L.)

Centella asiatica (L.) Urban (Umbelliferae), has been used as a traditional herbal medicine in Asian countries for hundreds of years (Figure 1.). It is a perennial, herbaceous creeper growing to 50 cm with fan shaped leaves, a slender and rooting at the nodes (Cheng and Koo, 2000; Hamid et al., 2002). The therapeutic use of this herbal remedy with its wide range of applications has been well documented in South East Asia and India for centuries. And the plant continues to be used with the framework of folk medicine (Brinkhaus et al., 2000).

Figure 1. *Centella asiatica* (L.) Urban (Umbelliferae).

1.1 Names and synonyms

Centella asiatica (L.) Urban is a genus of the plant family Apiaceae (Umbelliferae) (see Table 1), which contains 20 different species. Apart from this name, which is usually employed in scientific work, the synonym *Hydrocotyle asiatica* L. is the designation most commonly found. In German, the medicinal plant is also known by the colloquial name of “Indischer Wasserernabel”, Further names are: Indian Pennywort (English), Hydrocotyle Asiatigue (French), Idrocotyle (Italian), Brahma-manduki and

Brahmi-Buti (Hindi), Tsubo-kusa (Japanese), Tungchian and Luei Gong Gen (Chinese) (Brinkhaus et al., 2000)., and Bua-Bok (Thailand) (Nunthana Sitthichai, 2003).

Table 1. Systematic classification (Taxonomy) of *Centella asiatica*.

Classification	Name
Kingdom	Eukaryota
Subkingdom	Embryophyta
Division	Spermatophyta
Subdivision	Angiospermae
Class	Dicotyledoneae
Subclass	Rosidae
Superorder	Aralianae
Order	Araliales (Umbelliflorae)
Family	Apiaceae or Umbelliferae
Subfamily	Hydrocotyle
Genus	Centella
Species	Centella asiatica

1.2 Botany

Centella asiatica is perennial creeping plant that flowers between August and September; its flowers are of a light violet color. The gray to brownish-green plant has a smell that is reminiscent of tobacco leaves, and a mildly bitter taste. The leaves have long petioles arising rosette-like form a common base (the node), and the individual “leaf rosettes” (the nodes) are connected by slender aerial stolons or runners.

The leaves are thin and soft, with palmate nerves, hairless or with only a few hairs, and measure about 2 to 5 cm in diameter. The leaf margin is crenate or slightly lobed. The petioles are between 5 and 15 cm in length, slender and hairless or bear only a few scattered hairs. The short-pediceled umbels arise in the leaf axils. The 2 to 5 fruits of each umbel are enclosed within a pericarp comprising 1 to 2 cm-large elliptical bracts. The 2 schizocarps are attached together by narrow connecting ridges. Their surfaces show a clearly reticulate pattern.

Centella asiatica grows in moist habitats at altitudes between 0 and 2500 metres above sea level, and tolerates dense shade. For cultivation purposes, the stolons together with stems and roots are employed. The stolons are placed in moist sand or moist earth, and the young plants are ready for transplantation after about one to two weeks. In particular when growing in moist and shady habitats, the plant can be harvested 6 months after planting, and at any time throughout the year (Brinkhaus et al., 2000).

1.3 Chemical constituents

In addition to about 0.1% essential oils and other volatile constituents, *Centella asiatica* contains a wide range of other substances. These derive from the metabolism of phenylpropane and acetate, and belong to the flavonoids and terpenes (Brinkhaus et al., 2000).

The substances of interested therapeutic are the saponin-containing triterpene acids and their sugar esters, the most important being: asiaticoside, asiatic

acid and masdecassic acid. There are pentacyclictriterpenes (see Figure 2) (Inamdar et al.,. 1996; Brinkhaus et al., 2000).

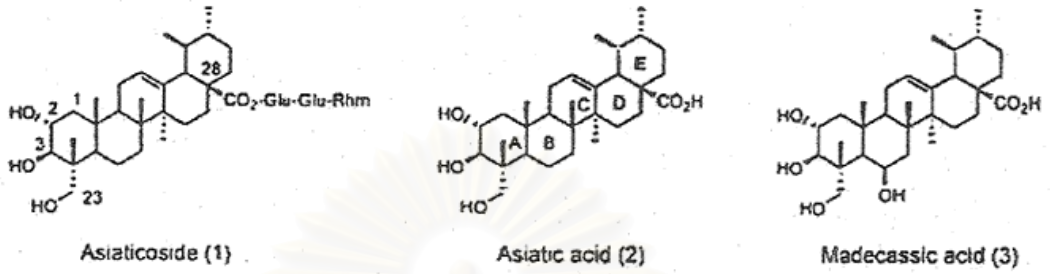

Figure 2. Structure of asiatic acid (1), asiaticoside (2), Madecassic acid (3) (Inamdar et al., 1996).

2. Traditional applications

The spectrum of indications for use of *Centella asiatica* in the field of traditional medicine is extremely wide. Historical sources and records of present-day healers practical folk medicine show that the phytotherapeutic applications of *Centella asiatica* encompass the same specialty-related indications as apply to the usage of the plant in modern phytomedicine. Many historical traditional indications continue to apply in modern traditional medicine in Asia. This is particularly true both of Ayurveda medicine – a system of medicine that is supported by the WHO for primary care in rural area – and of folk medicine in the non – urbanized parts of Asia. *Centella asiatica* has been used in diseases of gastrointestinal, dermatological, infectious, nephrological, urogenital, neurobiological, psychiatric and other (Brinkhaus et al., 2000).

3. Pharmacological activities

Asiaticoside isolated from the plant *Centella asiatica*, has been, therefore, studied for its wound healing activity to promote definite histological changes (May, 1967), enhanced induction of antioxidant levels at an initial stage of healing (Shukla, Rasik, Dhawan, 1999). In addition, it exhibits wound healing activity in normal as well as in diabetic animals (Shukla et al., 1999). *Centella* extract prevented ethanol induced gastric mucosal lesions by strengthening the mucosal barrier and reducing the damaging effects of free radicals (Cheng and Koo, 2000). The methanolic extract of *Centella asiatica* has shown significant cytotoxicity to Ehrlich ascites tumour cells and Daltonjs lymphoma ascites tumour cells (Babu, Kuttan and Padikkala, 1995). BR-16A (Mentat^{R}) is a herbal medication derived from Ayurveda and claimed to enhance cognition and to ameliorate various forms of brain deficits. It contains over 20 different ingredients of which Jal-brahmi (*Bacopa monnieri*) MandooKaparni (*Centella asiatica*) Ashwagandha (*Withania somnifera*) Shankapushpi (*Evolvulus alsinoides*) Jatamansi (*Nardostachys jatamansi*) Vach (*Acorus calamus*) Malkangni (*Celastrus paniculatus*) and Sonth *(Zingiber officinale*) (Handu and Bhargava, 1997). BR-16A has been shown to improves memory and learning in mice, and attenuate the amnesic effects of scopolamine and electro-convulsive shock (Kulkarni and Verma, 1992) In addition, it prevented the cognitive deficits produced by subchronic aluminium administration (Handu and Bhargava, 1997) The *Centella* extract has shown to have central nervous system depressant activity that it offers good behavioural radioprotection against conditioned taste aversion in rat (Shobi and Goel, 2001). The earlier study have demonstrated that the aqueous extract of *Centella asiatica* has cognition-enhancing properties with an associated decrease in the brain oxidative stress parameters of the normal rat (Veerendra Kumar and Gupta, 2002). Further studies showed that *Centella asiatica* prevented the cognitive impairment and attenuated the oxidative stress induced by PTZ kindling (Gupta, Veerendra Kumar and Srivastava, 2003).

In traditional practices of medicine, plants have been used to enhance cognitive function and to alleviate other symptoms associated with Alzheimer's disease (AD) (Howes and Houghton, 2003). Recent research suggested that, ethanol is the best solvent for extracting antioxidative compounds form *Centella asiatica* ((Hamid, Shah and

Muse et al, 2002). Oxidative stress due to increase in free radical generation or impaired endogenous antioxidant mechanism is an important factor that has been in AD and cognitive deficits seen in elderly (Smith, 2000). Thus the efforts have been directed to find therapeutic agents such as *Centella asiatica* that could reduce the oxidative stress and improve the learning and memory.

4. Alzheimer's disease (AD)

Dementia refers to group of disorders characterized by global cognitive decline, including the decline in memory. Many diseases are known to cause dementia and the new ones are still being recognized. The term dementia does not imply a prognosis. Some dementias are fully treatable, some are partially treatable, and others do not have effective treatment at the present time. The most common dementia is AD, which was first recognized by Alois Alzhiemer in 1906 (Holroyd and Shepherd, 2001).

The emergence of AD as one of the major public health issues for the 21st century is being shaped by the changing trends in population demography, health economics, and the overall health status of the world's population. Among the many factors influencing the magnitude and nature this public health problem are: (1) an increasing number of older individuals at risk for AD; (2) the rising cost of long-term care; and (3) the increase in the duration of AD disability (since people are living longer).

It is estimated that by the year 2030, there will be nearly 64 million people $\geq$ 65 years old in the United States; most of this population will be well beyond the age of 85. Some of the more rigorous current studies with the older populations indicate that mortality rates do not increase in a linear fashion with age; the rate increase in both disabilities and mortality appears to gradually slow down or decline in the "oldest-old" age groups. The implication for the future is that the pool of individuals at risk for neurodegenerative disorders such as AD will increase substantially (Khachaturian, 1998; Holroyd and Shepherd, 2001).

4.1 Pathophysiology

There has been an explosion of research in the understanding of the pathophysiology of AD over the past 10 years.

4.2 Genetics

There is a clear evidence for genetic risk of AD. Some families demonstrate autosomal dominant inheritance of AD with mutations located on chromosome 21, a locus that encodes the amyloid precursor protein. Other mutations have been described on chromosomes 1 and 14, for gene loci encoding for presenilin proteins. These findings are extremely interesting, as the build-up of amyloid proteins is a diagnostic pathologic feature in AD. Such familial inheritances account for only 2% of all AD case. However, considerable research has focused on the finding that apolipoprotein E type 4 (APOE4) is associated with late-onset AD both in sporadic and familial cases. Interestingly, this association is dose-dependent, in that persons who are homozygous for type 4 allele have a greater risk for AD than persons that are heterozygous. The association between the APOE4 allele and AD may account for 30-40% or even more of all AD. Importantly, it should be emphasized that the APOE4 allele is neither necessary nor sufficient for development of the illness, which has led the American College of Medical Genetics and American Society of Human Genetics to conclude that testing for the presence of APOE should not be used as a predictive test for Alzheimer's disease at this time. Recently, AD has been linked both to chromosome 12 and to mutations on the maternally transmitted mitochondria. Given the multiple linkages being characterized across different families, it is clear that AD is a heterogeneous disorder (Pratico and Delanty, 2000; Holroyd and Shepherd, 2001).

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4.4 Neurochemistry and Neuropathology

AD is characterized neuropathologically by neuritic plaques and neurofibrillarly tangles, which are abnormal amyloid proteins present between and within neurons. Loss of neurons occurs especially in areas of cerebral cortex and the hippocampus. The pathogenesis involves the deposition of beta-amyloid into plaques,

causing inflammation and oxidative stress. These processes then cause microtubular dysfunction in which neurons are unable to send nutrients along the axon, leading to neuronal death. Excess productions of free radicals also lead to further neuronal degeneration. Amyloid is an insoluble substance that activates astrocytes, microglia, and other components of an inflammatory response, leading to future damage. Neurofibrillary tangles within neuronal cells are abnormally phosphorylated, causing them to become cross linked and insoluble and are felt to contribute to neuronal death (Francis, 1999; Pratico and Delanty, 2000.)

The pathologic changes of plaques and tangles often begin medial temporal lobe and cortical association areas, which project back to the nucleus basalis, where most cholinergic neurons are located. Subsequent death of cholinergic neurons causes decrease in neurotransmitter acetycholine. Acetycholine (Ach) is believed to be involved in memory. Although the cholinergic deficit is believed to be major neurotransmitter abnormality in this disorder, losses of serotonergic and adrenergic neurons may contribute to psychiatric and personality changes in illness (Pratico and Delanty, 2000; Holroyd and Shepherd, 2001).

4.5 Free radical injury in Alzheimer's disease

The brain may be particularly vulnerable to oxidative damage (Nakashima, 1999), because it has high energy requirements and a high oxygen consumption rate. It is rich in peroxidizable fatty acids and contains high levels of transition metals, which may catalyze the formation of the reactive hydroxyl radical. Furthermore the brain has a relative deficit of antioxidant defenses compared with other organs. There is a growing body of evidence suggesting that oxidative injury is involved in the pathogenesis of AD. Oxidantive stress to the central nervous system predominantly manifests as lipid peroxidation because of its high lipid content and unusually high concentration of polyunsaturated fatty acids that are particularly susceptible to oxidation (pratico and Delanty, 2000).

5. Animal models of Alzheimer's disease

5.1 Cerebral ischemia model

A reduction in cerebral blood flow (CBF) has been shown to closely related to brain dysfunction after stroke. Several studies have demonstrated that this reduction in CBF precedes the onset of symptoms in multi-infarct dementia, but also in other types of dementia, including AD. The degree of disruption of CBF is correlated to the severity of these diseases (Ohta et al., 1997).

Since it has been anticipated that there is dynamic interaction between hypertension, reduced CBF, cerebrovascular pathology, cognitive performance and memory capacity, experimental models were established to investigate the causal relationship between these factors. The laboratory animal models offer the possibility to take correlation analysis between CBF, vascular parameters and cognitive performance accomplished in human studies one step further since correlative analysis offers only description of the coincidence of particular factors but not causality per se (De la Torre, 2000; Farkas and Luiten, 2000). Contemplates that chronically reduce cerebral blood flow can trigger the degeneration of the capillary ultrastructure in the brain. Creating a reduction of cerebral blood flow in laboratory animals can test such a presumed sequence of events best. The ligation of the different large arteries that supply the brain is routinely applied under experimental conditions to achieve various degree of cerebral hypoperfusion. The bilateral occlusion of common carotid arteries (two-vessel occlusion, 2VO) is a well characterized in rodent. The 2VO paradigm is frequently discussed in the context of AD because of apparent prevalence of cerebral hypoperfusion in the disease. Nonetheless, the 2VO experiments should not be literally interpreted as representative of a definite corotid artery occlusion in AD patients since the carotid flow in AD may be hampered but not totally blocked (Farkas and Luiten, 2000). The 2VO model stands for visualization of cerebrovascular and behavioral consequences of the reduce CBF (Corbett and Nurse, 1998; Farkas and Luiten, 2000), whatever its trigger may be. In fact, the cause of the lowered CBF in AD is not certain but the possibilities may very well include (cardio)vascular factors such as hypertension and arterosclerosis – even in the carotid sinus, as well as Aβ - induced vascular constriction, or degenerating neural

regulation (Farkas and Luiten, 2000) and increasing lipid peroxide (Kondo et al., 1997). Reported the degree of lipid peroxidation, which was measured after 20 min of reperfusion, also increased with the ischemia time (Sakamoto et al, 1991). The 2VO showed no shrinkage of the optic nerves, and exhibited a normal circadian rhythm of motor activity. These finding indicate that 2VO did not impair the visual system in these rats. Therefore, they suggest the 2VO induced a long lasting working memory impairment with minimal damage to the visual system (Ohta, 1997).

5.2 Scopolamine model

Brain damage also results from chemical neurotoxicity, either from drugs or environmental toxins (Yamada and Nebeshima, 2000). A large number of pharmacological studies have shown that Ach receptor antagonists such as systemic injections of scopolamine influence a wide variety of brain structures. Scopolamine reduces performance of animals in learning and memory tasks (Hasselmo, Wyble and Wallenstein, 1996; Palmer, 2002). The cognitive deterioration observed resembles the memory disturbance seen in AD. Scopolamine-induced amnesia has therefore been used as an experimental model for this illness (Andersen, Lindberg and Myhrer, 2002)

6. Animal studies of aging and cognitive decline

Age animals (Animal models of AD) that demonstrate impairments in learning and memory tasks are characterized as having smaller numbers of neurons and greater atrophy of surviving cholinergic neurons in the basal forebrain. Within the domain of spatial memory, for example, studies of aged rats have revealed a significant correlation between the number and size of histochemically detectable cholinergic neurons in the basal forebrain. These pathological have been shown to a wide variety of behavioral deficits in aged animals. For example, spatial navigation deficit in the Morris water maze as measured by increased escape latencies and short-term memory deficits in passive avoidance task (Muir 1997).

6.1 The Morris water maze (MWM)

Twenty years ago, a device was described to investigate spatial learning and memory in the laboratory. In the meanwhile, it has become one of the most frequently used laboratory tools in behavioral neuroscience. The device consists of a large circular pool filled with opaque water in which a small escape platform is hidden. During a number of training trials, animals learn to find the platform and escape from the pool. Surely one of the reasons for its success is its relative simple, it has been used in some of most sophisticated experiments in the study of the neurobiology and neuropharmacology of spatial learning and memory, as well, it has been used in the validation of rodent models for neurocognitive disorders. In the process, MWM testing gained a position at the very core of contemporary neuroscience research. Throughout the years, the task has been given various names, such as Morris swimming pool, Morris maze, water maze, spatial navigation task, etc. Here, its most common name, Morris water maze (MWM), because Richard Morris developed this test, will be used (Figure 4.) (Markowska et al.,1998; D' Hooge and De Deyn, 2001; Myhrer, 2003).

6.2 Passive avoidance

Fear-motivated avoidance tests are usually based on electric current as source of punishment. In many tests, the floor of the apparatus is made up by a grid that can be electrified. In so-called consummator conflict tests, the animal receives an electric shock when touching food or water. Avoidance tests are divided into two categories: passive avoidance and active avoidance. In passive avoidance, the animal has to refrain from executing a previously response, e.g., touch food or water, step down from and elevated position (to a grid floor) or step into a narrow an apparently safer place (with a grid floor). Step-down (Figure 4.) or step-through tests are most frequently used to measure passive avoidance behavior. The latency to refrain from performing the punished act expresses the ability to avoid (Myhrer, 2003).

7. Lipid peroxidation

Lipid peroxidation is the mechanism by which lipids are attacked by reactive oxygen species with sufficient energy to form a carbon radical that reacts with oxygen and results in a peroxyl radical, thus generating lipid peroxides. Markers of brain lipid peroxidation have been the most studied indices of oxidant stress in AD. A wide range of techniques is available to measure this process and the level of its end products. As a general rule, most of them work well when applied to in vitro systems, but they are misleading when applied to biological systems. It is always more appropriate to separate the different lipid peroxidation products of interest before assaying them, especially when complex mixtures are being studied, by using high performance liquid chromatography (HPLC) or gas chromatography/mass spectrometry (GC/MS) analysis.

In quantitative postmortem studies, Lipid Peroxidation has been quantitatively assessed by measuring malondialdehyde (MDA) levels by the thiobarbituric acid-reacting substances (TBARS) assay; Lipid hydroperoxides; aldehydes; and isoprostances. The majority of the published studies have used the TBARS test. It is easy to perform and inexpensive but also has significant shortcomings when used to assess Lipid Peroxidation in complex biological systems (Pratico and Delanty, 2000).

8. Therapeutic approaches in AD

Because the etiology and pathogenesis of A has not been clearly defined yet, and therefore, the therapeutic target central to the pathological process still needs to be found, the current strategies to help patients during the course of this devastating disease are directed against various factors and events that are associated with AD. The major histopathological hallmarks of this neurodegenerative disorder have already been known over 90 years and the modern cell and molecular biology has advanced the AD field. Although many landmark findings have been made in recent AD research, the cause or possibly the causes of AD are not known. Consequently, current hypothesis of AD's pathogenesis (e.g. use of Ach esterase inhibitors according to the Ach efficiency

hypothesis). In addition, clinical data can be accumulated by retrospective analysis of already concluded drug trials.

Since any successful treatment for AD in the future is dependent on an early diagnosis of the disease and since it has to be recalled that AD is currently still diagnosed by excluding other possible causes of the existing dementia such as vascular dementia.

8.1 Classic therapeutic targets

Because a substantial dysfunction and loss of cholinergic neurons occur in AD, until recently, the major therapeutic approach was to replace this existing neurotransmitter deficiency. The cholinergic system plays a central role in learning and memory. The presence of acetylcholine is not only necessary for the above-mentioned processes, but can also ameliorate learning deficits and restore memory following the nucleus basalis magnocellularis, the brain area that provides the major cholinergic innervation of the neocortex. Drugs like Cognex/tacrine hydrochloride and Arizept/donepizil are inhibitors of the enzyme cholinesterase that degrades the neurotransmitter Ach in the synaptic cleft. Ach levels, which are decreased in AD, can be locally increased by a block of its degradation. However, because AD is not just a disease of a neurotransmitter deficiency, but rather is characterized by a massive synaptic loss and neuronal degeneration, approaches targeting tropic factors, such as nerve growth factor, appeared worth while.

5.2 Antioxidants as Neuroprotectants

According to the oxidative stress hypothesis of AD, numerous approaches for an effective antioxidant neuroprotection have been developed. Antioxidant therapy is discussed for AD as well as for a variety of other neurodegenerative disorders. Numerous free radical scavengers have been used in experimental paradigms of neuronal cell death *in vitro* and *in vivo* such as vitamin E (α-tocopherol), the pineal hormone melatonin, the lazaroids (21-aminosteroids) or

mifepristone (RU486). All these antioxidants share their high lipophilicity as a consequence of their chemical structure and their free radical scavenging moieties.

5.3 The female sex hormone estrogen in neuroprotection

Estrogen may protect neurons against exogenous insults during the development of AD at various levels. Therefore, this endogenous hormone is an ideal candidate for an antioxidant neuroprotective compound. Chemical modifications of this molecule might be able to enhance the protective activities, for example, its antioxidant potential, and might also help to dissect the pure activity as a sex hormone form the neuroprotective effects. Such an approach would allow the design of appropriate drugs that do not cause feminization or other untoward hormonal effects, and would turn such estrogen-derivatives into a neuroprotective compound which can also be used in male AD patients.

5.4 Anti-Inflammatory drugs and alternative approaches

As pointed out earlier, Ad pathology has also a strong inflammatory component and, therefore, inflammatory mediators may have a role in the pathogenesis of AD and, consequently, some therapeutic implications. Initially, two preliminary clinical studies suggested that anti-inflammatory drugs decrease the rate of cognitive decline in AD and, therefore, increased the interest in the potential involvement of inflammatory mechanisms in disease progression. Results of more recent studies suggest that non-steroidal anti-inflammatory drugs (NSIADs) cause a delay in the onset or slow down progression of AD (Behl, 1999).

Aims and objectives

The present studies aim to establish the effect of *Centella asiatica* cultivated in Thailand on learning and memory. Total ethanol extract of *Centella asiatica* with its thin layer chromatogram correlates with those specified in Thai pharmacopia was used in animal models of memory deficit induced in mice by either 2VO or

scopolamine. In Morris water maze and step down test were used to evaluate the extent of memory deficit. In addition the effect of *Centella asiatica* on lipid peroxidatin was also performed.

CHAPTER II

MATERIALS AND METHODS

1. Experimental animals

All experiments were performed on male ICR mice, eight-week old, and weighing 30-35 g. All animals were obtained form National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand.

Prior to testing, they were housed 8 mice per cage for one week in the Animal House of the Faculty of Pharmaceutical Sciences, Chulalongkorn University and maintained on 12:12 light-dark cycle at controlled temperature (25$\pm$ 2 ^{0}C). They were allowed free access to both food pellets (C.P. Mice Feed) and water.

All behavioral experiments were carried out in a room adjacent to that in which the mice were housed under the same conditions of temperature and humidity.

The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. Experimental instruments

1. Rotary Evaporate (Rotavapor R-114, Buchi)
2. pH meter (Beckman, U.K.)
3. Stop watch (Galant, Switzerland)
4. Morris water maze set (Home made, Thailand)
5. Step down set (Home made, Thailand)
6. Locomotor activity set (UGO Basile, Comerico, Italy)
7. Automatic micropipette (Pipet-Lite™, U.S.A.)

8. Automatic mixer (Vertex, U.S.A.)
9. Homogenizer (Glas-Col, Terre Haute, U.S.A.)
10. Centrifugater (Sorvell, GLC-2B, U.S.A.)
11. Spectophotometer (Shimadzu, UV1201, Japan)
12. Conical centrifuge tube (Nunc, Denmark)

3. **Drugs and chemicals**

1. Fresh plant of *Centella asiatica* (Nonthaburee Province, Thailand)
2. Ethanol 95% (GPO, Thailand)
3. TLC (Silica gel form Merck, India)
4. Chloroform (Lab-scan LTD, Ireland)
5. Methanol (Lab-scan LTD, Ireland)
6. Asiaticoside (Extrasynthase, France)
7. Normal saline solution (Thai Nakron Patana Co., Ltd., Thailand)
8. Scopolamine hydrobromide (Sigma , U.S.A)
9. Tween 20 (The East Asiatic Co., Ltd., U.S.A.)
10. Pentobarbital sodium (Sigma, U.S.A)
11. Sodium hydrogen phosphate-2-hydrate (Sigma USA.)
12. Sodium dihydrogen phosphate-2-hydrate (Sigma USA.)
13. Acetic acid (Sigma USA.)

14. Sodium dodecyl sulfate (Sigma USA.)

15. Thiobarbituric acid (Sigma USA.)

16. N-butanol (Sigma USA.)

17. Pyridine (Sigma USA.)

18. 1, 1, 3, 3-Tetraethoxy-propane (Malondiadehyde) (Sigma USA.)

4. Plant material and preparation of the extract

Centella asiatica plants were procured form the local market in Nonthaburee Province, Thailand. The whole plants (96 kg) of *Centella asiatica* were washed with running tap water and leaves (58 kg) were separated for extraction. The leaves were dried. Dried plants (7.1 kg) were extracted with ethanol for 3 days. The pooled extracts were concentrated and then evaporated to dryness under vacuum. The percentage w/w yield of the crude ethanolic extract of *Centella asiatica* (CE) was12.68.The CE was further characterized by preparative Thin-layer Chromatogram with chloroform : methanol : water (15:7:1) solvent system. Spray the plate with *anisaldehyde TS* and heat. The chromatogram obtained form shows a purple sport and violet sport, corresponding to the asiaticoside and the Asiatic acid spots. Several other spots of different colours are observed.

5. Experimental methods

5.1 Experimental design

Animals were randomly divided into 2 groups. Cerebral ischemia to induce learning and memory impairment was performed on the first group where as the second group was given scopolamine to induce learning and memory impairment. One hour before behavior testing (MWM, step-down test and locomotor activity test), vehicle

(Tween 20/water 1:5) or CE (100, 300, 1000 or 1500 mg/kg B.W., dissolved in Tween 20+ water) were administrated orally through an intragastric feeding tube. Scopolamine was administered into the second group 30 min before behavior testing. Following the behavior test, the animals were sacrificed and whole brain was dissected for estimation of marker of oxidative stress (malondialdehyde).

5.2 Cerebral ischemia-induced learning and memory impairment

Mice were subjected to cerebral ischemia induced by 2VO plus hypotension (Figure 3). In brief, the mice were anesthetized with sodium pentobarbital (Nembutal sodium solution, 60 mg/kg, intraperitoneal injection). Under deep anesthesia, the neck skin of mice was vertically incised. The common carotid arteries were exposed, carefully separated form the adjacent veins and sympathetic nerves, and then occluded by artery clips. While the arteries were clamped, blood (0.3 ml) was withdrawn by cutting off the tip of the tail. Then, the artery clips were removed and cerebral blood flow was restored after 20 min. The skin incision was closed and the mice were kept in an air-condition room at 25 ^{0}C. Sham-operated mice were subjected to the same procedure without carotid clamping and bleeding. After 24 h, the following MWM were carried out (Xu et al., 2000; Watanabe H. et al, 2003).

To study the effects of Asiatic pennywort (*Centella asiatica*) ethanol extract on impairment of learning and memory induced by cerebral ischemia, six groups of animals were used: one group of sham-operated animals (n = 8) and one group of 2VO animals (n = 8) receiving vehicle four groups of 2VO animals (n = 8 per group) receiving 100, 300, 1000 or 1500 mg/kg B.W. of CE respectively by oral route for 8 consecutive days. In each group of six animals was performed for three behavioral test, MWM test, step-down test and spontaneous locomotor activity. MWM was tested for 5 consecutive days. The step-down test was performed 6 day after 2VO. Spontaneous locomotor activity test 8 day after 2VO. Following the spontaneous locomotor activity test, the animals were sacrificed for estimation of lipid peroxidation (Bejar, Wang and Weinstock, 1999).

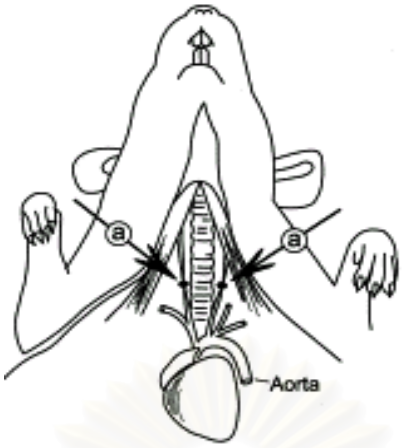

Figure 3. Experimental mice model of cerebral ischemia (bilateral common carotid artery occlusion, two vessel occlusion: 2VO); (a) the common carotid arteries

5.3 Scopolamine-induced learning and memory impairment

To study the effects of CE on impairment of learning and memory induced by scopolamine. Six groups of animals were used: groups of mice (*n* = 8 per group) were administered orally for 8 consecutive days, with vehicle or CE (100, 300, 1000 or 1500 mg/kg B.W.) followed 10min later by an intraperitoneal injection of scopolamine (0.5 mg/ kg) or normal saline (0.1 ml). AT 30 min after the injection of scopolamine all mice were subjected to tests group: in the same manner as described for 2VO (Bejar, Wang and Weinstock, 1999).

5.4 Behavior tests

5.4.1 Morris water maze (MWM)

After 24 h of cerebral ischemia, the MWM was performed. The procedure used was a modification of that described by Morris (1984). The MWM consisted of a circular pool (Figure 4), painting with black color, which was 70 cm in diameter and depth of 13 cm of water with water maintained at 25±1 ^{0}C. A platform (6 cm diameter) was situated 1 cm below the surface of the water. The pool was divided into four quadrants with platform in a fixed position in one quadrant. Daily swimming consisted of four trials in which the mice was placed in the water form four different starting points and the latency of escaping onto the platform was recorded. This was conducted for 5 consecutive days. A maximum of 60 sec was allowed during which the mice had to find the platform and climb onto it 15 sec (Watanabe H. et al, 2003).

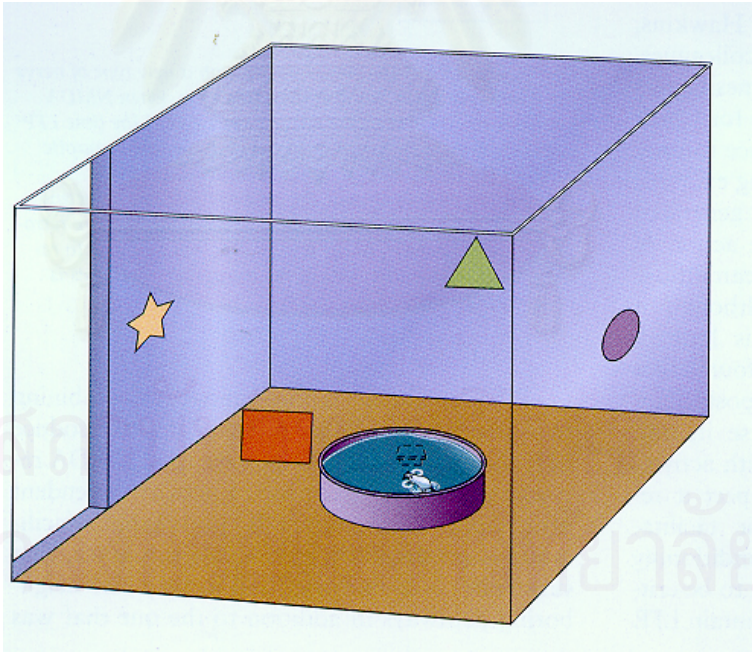

Figure 4. Equipment for Morris water maze test

5.4.2 Step-down test

A step-down passive avoidance was examined using apparatus consisted of plexiglass chamber (Figure 5). The inside dimensions of the activity cage are, length 35 cm; width 23 cm; and height 20 cm. The cage floor is made of evenly spaced stainless steel bars (3 mm diameter) that are spaced 11 mm apart, and a wooden platform (5 cm diameter, 4 cm height) set on grid in one corner. Electric stimulation was given through the grid connected with a scrambled shock generator (1 Hz, 1 ms, 36 V dc). Step-down experiment was started after 24 h of the last MWM testing; Mice were placed in the box to get adapted to environment for 3 min without electric shock. When electric shock was delivered, mice escaped form the grid floor back the platform. The duration of training test was 5 min and the shock was maintained for this period. Twenty-four hours after training, mice were placed on the platform for retention test. The electric shocks were still delivered for 5 min. step-down latency and number of errors was recorded. The time (step-down latency) that elapsed until the mice stepped down from the platform was recorded. If the mice did not step down from the platform within 300 s was recorded. An error was counted whenever the mice stepped down form the platform and the number of errors made in 5 min was recorded (Luo, Yin and Wei, 2003).

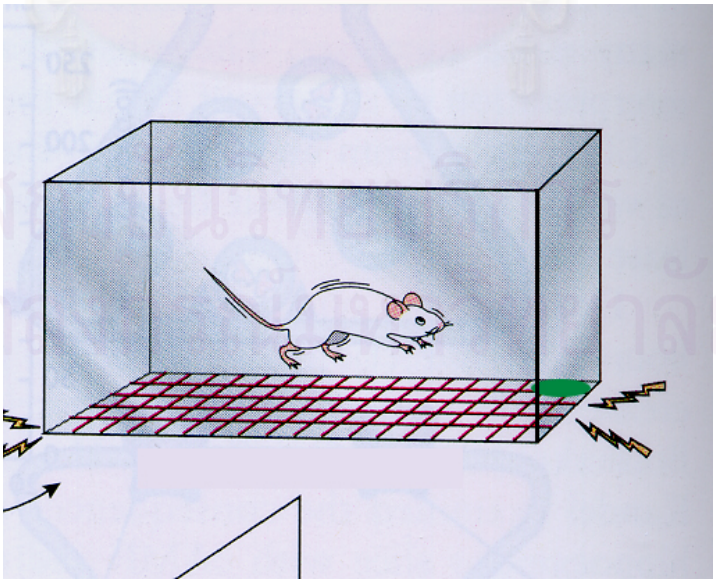

Figure 5. Equipment for Step-down test

5.4.3 Spontaneous locomotor activity test

Each animal was placed in an activity cage consisting of Plexiglass chamber and counting (Figure 6). The inside dimensions of the activity cage are, length 35 cm; width 23 cm; and height 20 cm. The cage floor is made of evenly spaced stainless steel bars (3 mm diameter) that are spaced 11 mm apart, connected to the circuit of counting unit. The registered numbers or counts of movements were recorded at 5 min intervals. The apparatus was placed in light and sound attenuated, and ventilated testing room with other behavioral testing apparatus (Jain et al., 2002; Gupta et al., 2003).

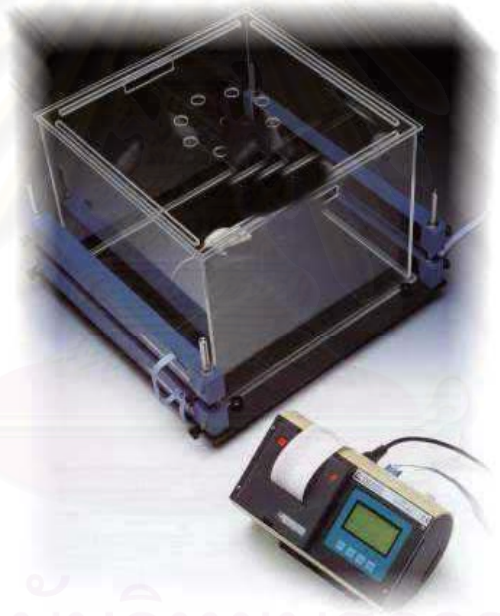

Figure 6. Activity meter

5.5 Lipid peroxidation assay

Following the behavioral testing, the animals were decapitated and the brains were quickly removed, cleaned with ice-cold saline and stored at –80 ^{0}C.

5.5.1 Tissue preparation

Brain tissue samples were thawed and homogenized with 10 times (W/V) ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates form rat brain were used for the determination lipid peroxidation

5.5.2 Measurement of lipid peroxidation

Malondialdehyde (MDA), a measure of lipid peroxidation, was measured as described by Gupta et al. (2003). The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodiumdodecyl sulphate (8.1%) were added to 0.1 ml of processed tissue samples, then heated at 100 ^{0}C for 60 min. the mixture was cooled with tap water and 5 ml of *n*-butanol/pyridine (15:1), 1 ml of distilled water was added. The mixture was vortexes vigorously. After centrifugation at 2500 rpm for 20 min, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer. The concentration of MDA is expressed as nanomoles/g tissue (Gupta et al, 2003).

6. Data analysis

The data of behavioral and biochemical tests are represented as mean value for the group $\pm$ standard error of mean (S.E.M.) in figures and were processed by one-way ANOVA followed by Tukey HSD post-hoc test for comparisons between control and different treated groups. *P* value of less then 0.05 was considered to be significant.

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RESULTS

Effects of *Centella asiatica* ethanol extract on impairment of learning and memory induced by cerebral ischemia

1. Effects of 2VO on spatial learning and memory performance in mice.

The MWM performance in 2VO- and sham-operated mice as measured by latency to reach the hidden platform during 5 days was summarized in Figure 7. Mice subjected to 2VO required a longer time to locate the hidden platform than sham-operated mice during the learning trials. The escape latency was significantly delayed in 2VO mice as compared to the sham-operated mice.

2. Effects of vehicle on spatial learning and memory performance in 2VO mice.

Tween 20/water 1:5, being used as a vehicle, clearly exerted no effect on MWM in Sham- and 2VO-operated mice as shown in Figure 8.

3. Effects of *Centella asiatica* on spatial learning and memory performance in 2VO mice.

Effects of treatment with CE at 100, 300, 1000 or 1500 mg/kg body weight (B.W.), p.o., compared to vehicle treated on MWM performance in 2VO-operated mice were shown in Figure 9. CE was orally given to animals 1 h before testing. The trial test was performed 24 h after the ischemia. CE administration markedly attenuated the memory deficits in 2VO mice. The latency of escaping onto the platform of CE treated mice was shortened than the vehicle-treated mice during the learning period for 5 days. A significant difference found between CE-treated and vehicle-treated mice.

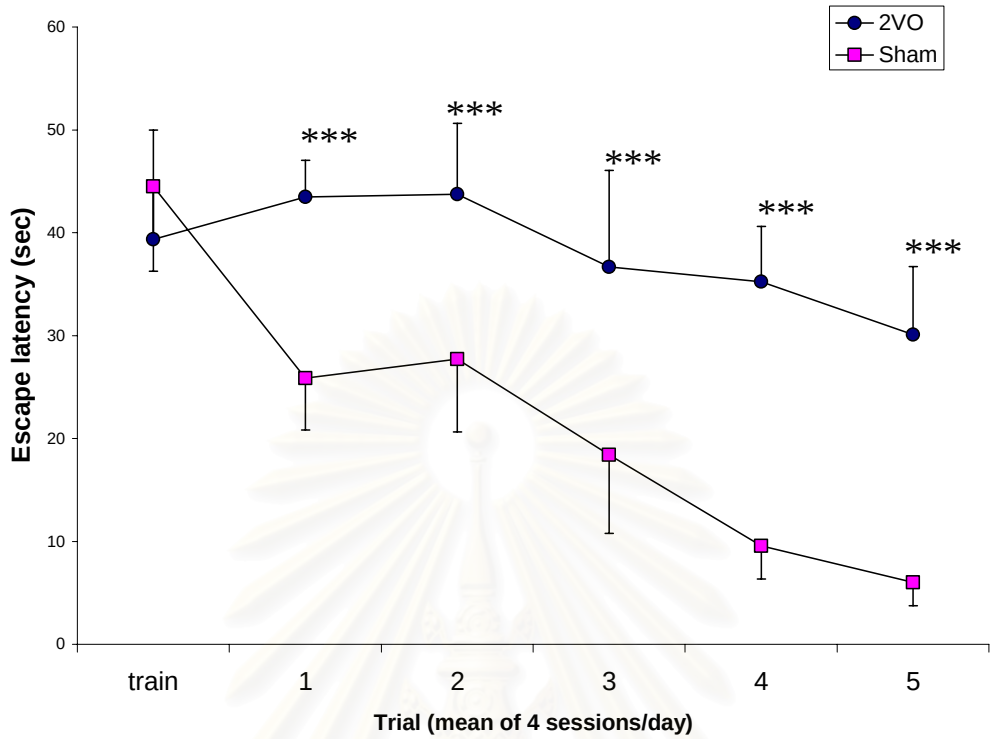


Figure 7 : The MWM performance in 2VO- and sham-operated mice. The escaping latency onto the platform was measured on 5 consecutive days. Each data point represents the mean± S.E.M. (*n*=8) of four trials.

***Significance of difference vs. sham-operated mice at $P < 0.001$

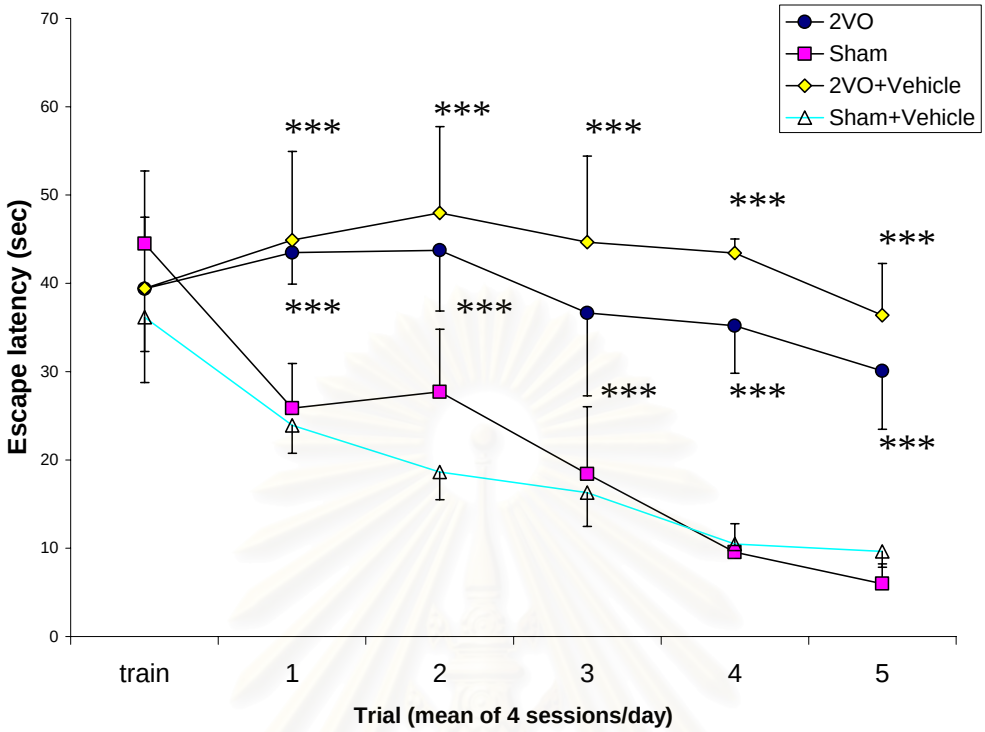


Figure 8 : Effects of vehicle on MWM performance in 2VO- and sham-operated mice. The escaping latency onto the platform was measured on 5 consecutive days. Each data point represents the mean ± S.E.M. (*n*=8) of four trials.

*** Significance of difference vs. sham-operated mice at $P < 0.001$

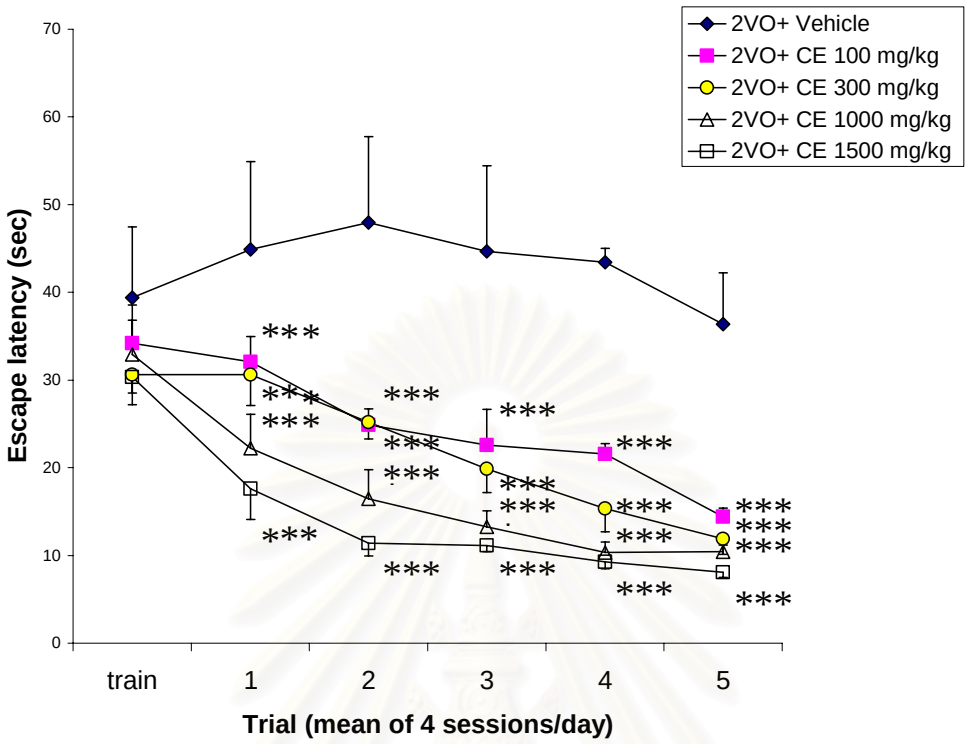


Figure 9 : Effects of CE on 2VO-induced disruption of memory in the MWM performance. Mice were orally given with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg BW. once daily. The escaping latency onto the platform was measured on 5 consecutive days. Each data point represents the mean± S.E.M. (*n*=8) of four trials.

*** Significance of difference vs. 2VO-operated mice at $P < 0.001$

4. Effects of 2VO on step-down passive avoidance in mice.

In experiments on the step-down test, 2VO caused a significant reduction in the step-down latency and marked increase in the step-down errors as shown in Figure 10.

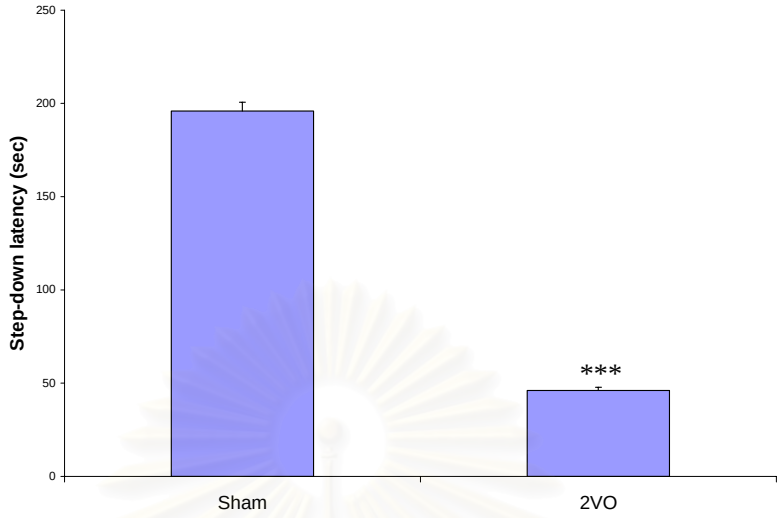
5. Effects of vehicle on step-down passive avoidance in 2VO mice.

Vehicle, used as a solvent for CE, clearly exerted no effect on step-down passive avoidance in sham-and 2VO-operatioed mice (Figure 11).

6. Effects of *Centella asiatica* on step-down passive avoidance in 2VO mice.

Effects of treatment with CE at 100, 300, 1000 or 1500 mg/kg B.W., p.o., reversed the reduction in step-down latency significantly and decrease significantly the step-down errors in 2VO-operated mice as shown in Figure 12.

(A)



(B)

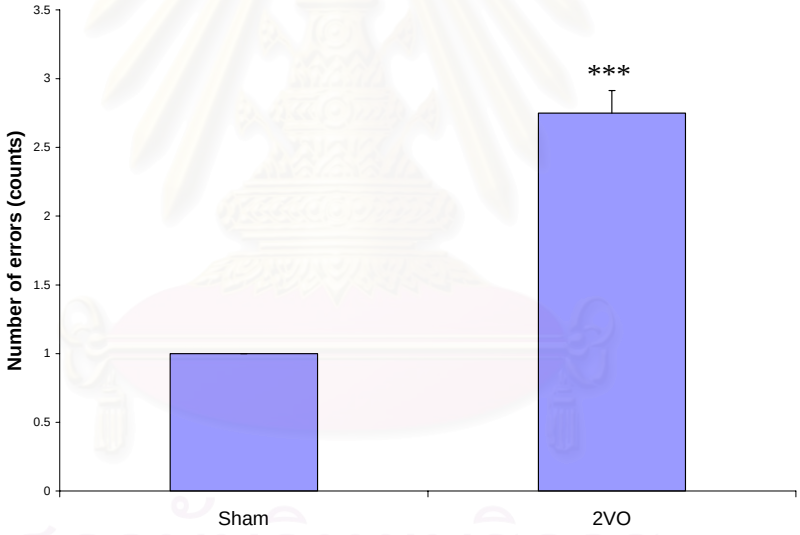
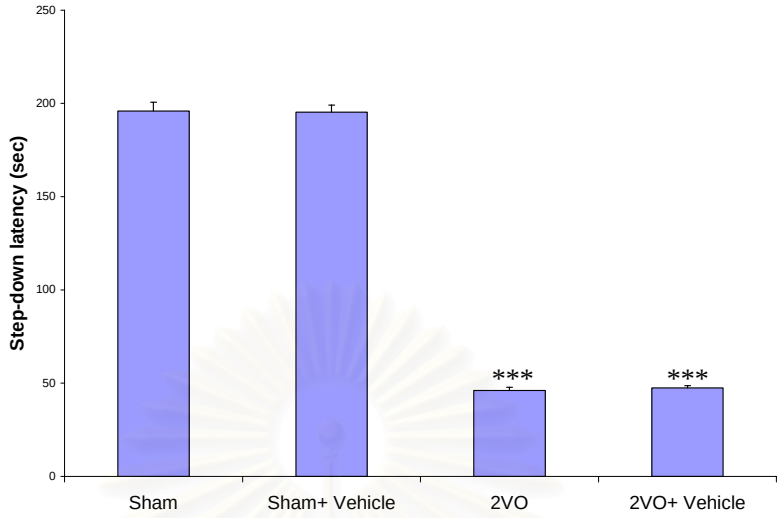


Figure 10 : Step-down test 2VO- and sham-operated mice. Step-down latency (A) and number of errors (B) were expressed as the mean ± S.E.M. (*n*=8).

***Significance of difference vs. sham-operated mice at $P < 0.001$

(A)



(B)

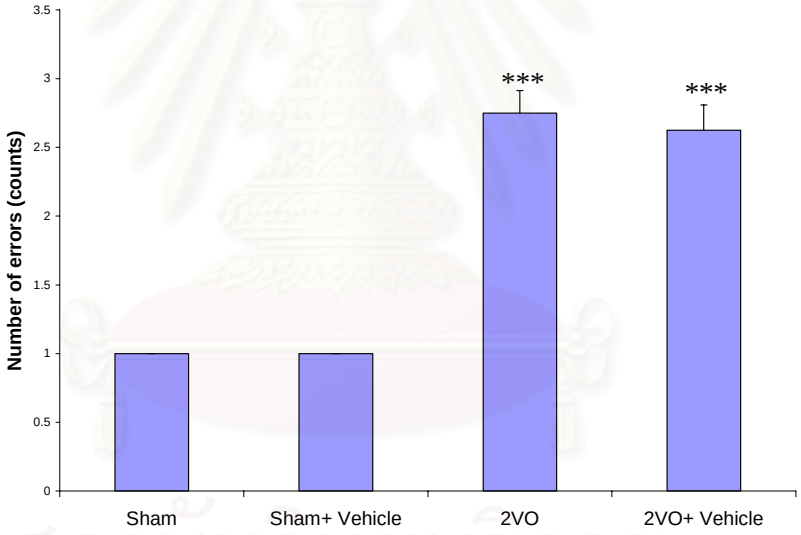
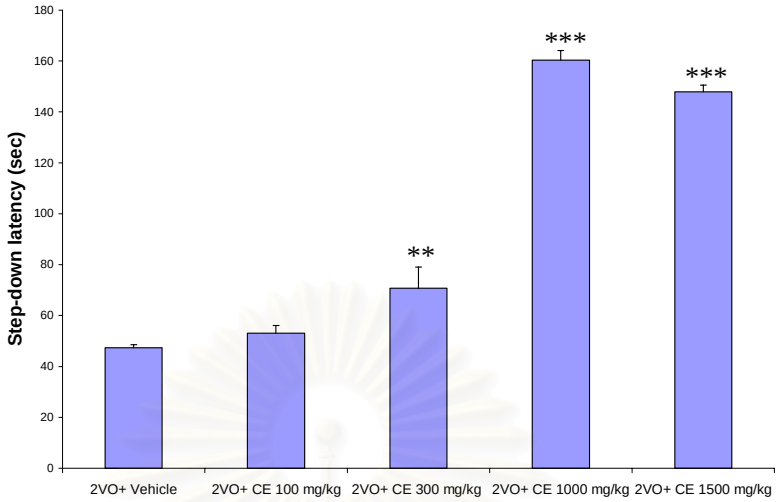


Figure 11 : Effects of vehicle on step-down test in 2VO- and sham-operated mice. Step-down latency (A) and number of errors (B) were expressed as the mean ± S.E.M. (*n*=8).

***Significance of difference vs. sham-operated mice at $P < 0.001$

(A)



(B)

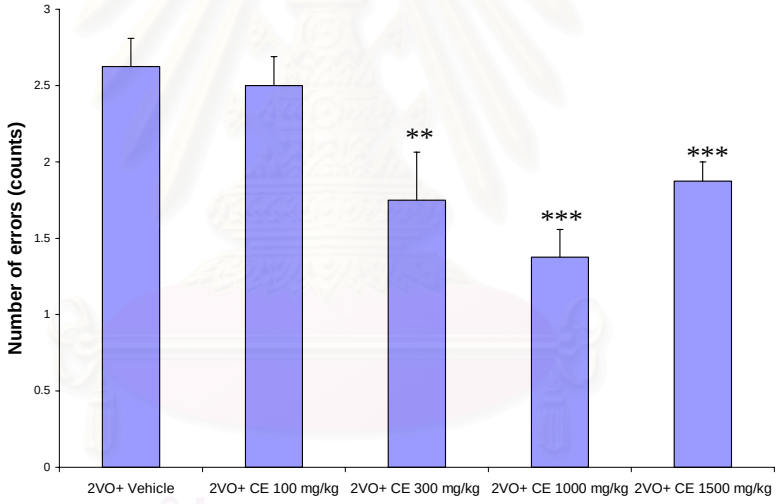


Figure 12 : Effects of CE on 2VO-induced disruption of memory in step-down test. Mice were orally given with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg BW. once daily. Step-down latency (A) and number of errors (B) were expressed as the mean $\pm$ S.E.M. (n=8).

, * Significance of difference vs. 2VO-operated mice at $P < 0.01$, $P < 0.001$, respectively.

7. Effects of 2VO on spontaneous locomotor activity in mice.

The spontaneous locomotor activity, measured as movement counting during 5 min test period, in 2VO- and sham-operated mice was summarized in Figure 13. The spontaneous locomotor activity did not differ significantly between 2VO- and sham-operated mice.

8. Effects of vehicle on spontaneous locomotor activity in 2VO mice.

Vehicle clearly exerted no effect on spontaneous locomotor activity in 2VO- and Sham-operated mice as show in Figure 13.

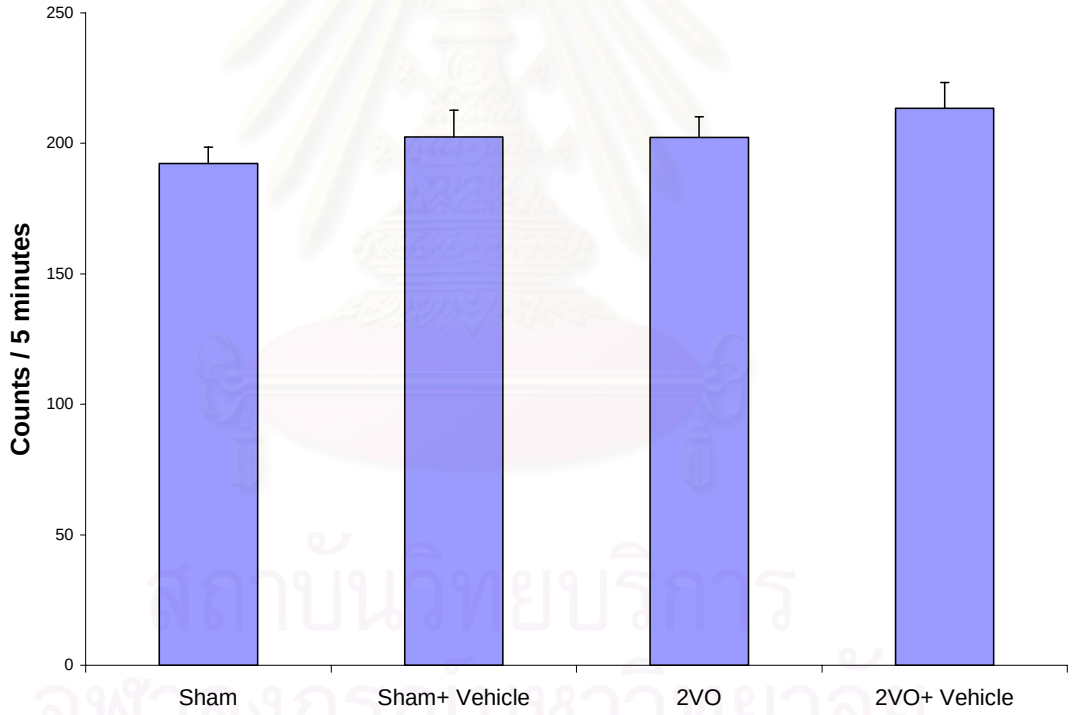


Figure 13 : Effects of vehicle on spontaneous locomotor activity in 2VO- and sham-operated mice. On the ordinate: counts/ 5 min, the values are expressed as the mean $\pm$ S.E.M. (n=8). A significant level of $P < 0.05$ was considered as a significant difference.

9. Effects of *Centella asiatica* on spontaneous locomotor activity in 2VO mice.

Figures 14 shown. Effects of treatment with CE at 100, 300, 1000 or 1500 mg/kg body weight B.W., p.o., on spontaneous locomotor activity. CE administration had no effect on spontaneous locomotor activity in 2VO mice.

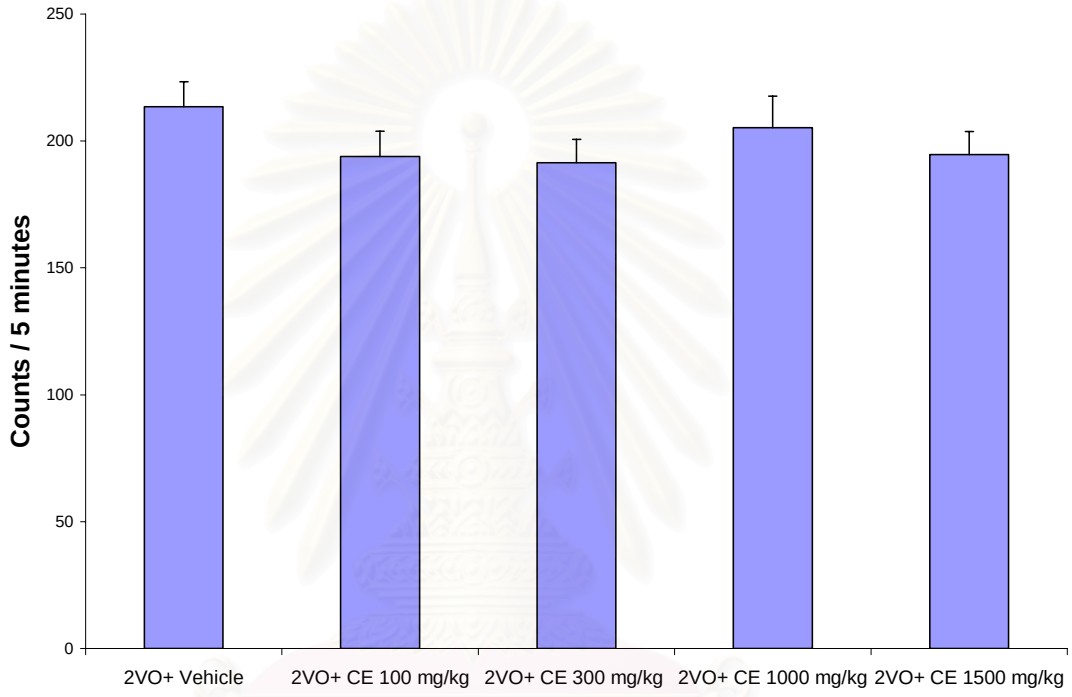


Figure 14 : Effects of CE on 2VO-induced disruption of memory in spontaneous locomotor activity. Mice were orally with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg BW. On the ordinate: counts/ 5 min, the values are expressed as the mean $\pm$ S.E.M. (n=8). A significant level of $P < 0.05$ was considered as a significant difference.

10. Effects of 2VO on lipid peroxidation in mice.

The brain levels of MDA, an indication of lipid peroxidation, in mice 8 days after 2VO- and sham-operated mice procedure were shown in Figure 15. 2VO operated-mice induced a marked significantly higher increased in brain lipid peroxidation than sham operated-mice.

11. Effect of vehicle on brain lipid peroxidation in 2VO mice.

The MDA brain levels of 2VO- and sham-operated mice after 8-day administration of vehicle were shown in Figure 15. There was no significant difference between MDA levels of 2VO as compared vehicle-treated 2VO operated mice.

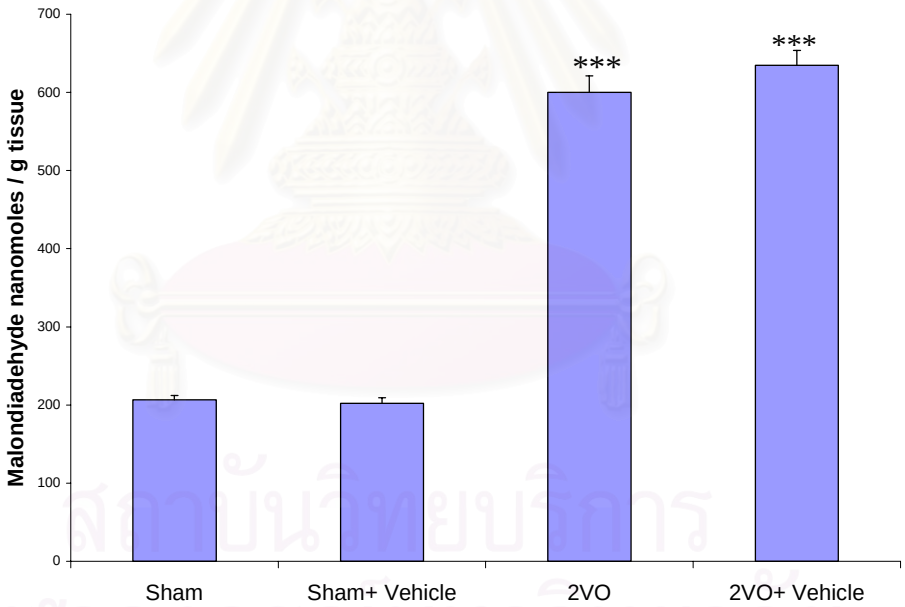


Figure 15 : Effects vehicle administration on brain levels of MDA in 2VO- and sham-operation mice. On the ordinate: MDA nmol/g tissue (mean $\pm$ S.E.M.) (*n*=8) after the 8 day of 2VO.

*** Significance of difference vs. sham-operated mice at $P < 0.001$

12. Effects of *Centella asiatica* administration on brain lipid peroxidation in mice after 2VO.

The protective effect of CE treatment on lipid peroxidation in 2VO mice was shown in Figures 16. MDA levels of the brain homogenates in 2VO- and sham-operated mice increased to 634.63 ± 19.09 nmol/g tissue and 201.89 ± 7.07 nmol/g tissue, respectively, CE administration at dose of 100, 300, 1000 and 1500 mg/kg BW. P.O for 8 days after cerebral ischemia markedly attenuated MDA levels to 520.13 ± 21.92, 507.75 ± 20.52, 403.25 ± 22.13 nmol/g tissue and 383.25 ± 31.38 nmol/g tissue, respectively.

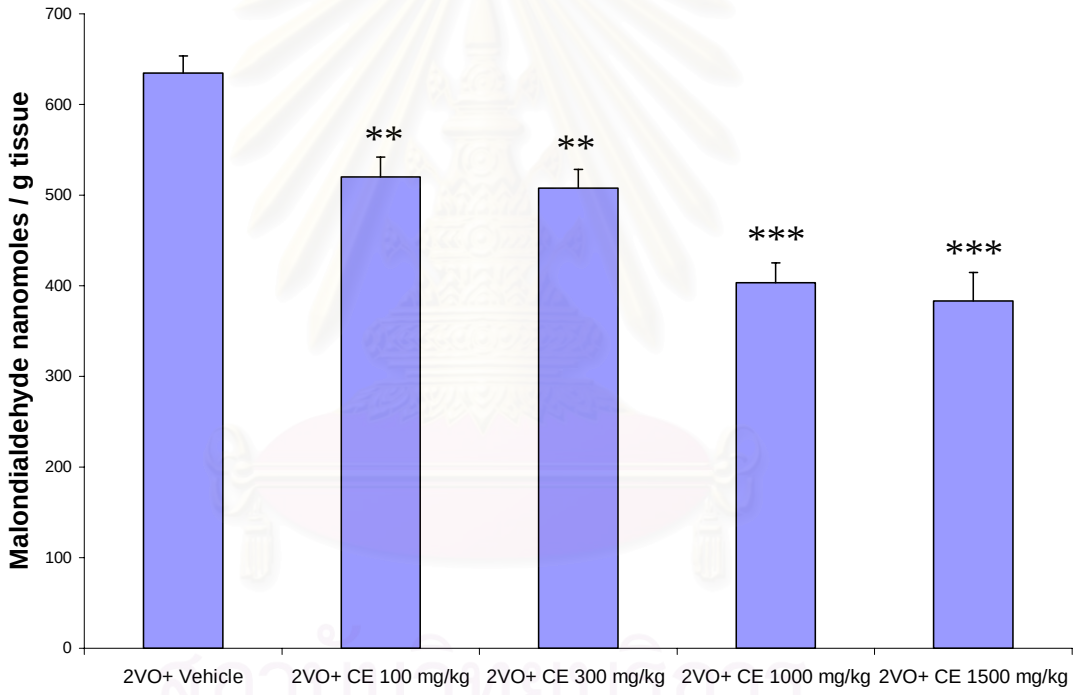


Figure 16 : Effects of CE on MDA levels in nmol/g tissue in 2VO-operted mice. Mice were orally with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg BW. On the ordinate: MDA nmol/g tissue (mean ± S.E.M.) (*n*=8) after the 8 day of 2VO.

, * Significance of difference vs. 2VO-operated mice at $P < 0.01$, $P < 0.001$, respectively.

Effects of *Centella asiatica* ethanol extract on impairment of learning and memory induced by scopolamine

1. Effects of scopolamine on spatial learning and memory performance in mice.

Effects of scopolamine (a muscarinic cholinergic receptor antagonist) administration (0.5 mg/kg, i.p., 30 min before the MWM test) on the MWM performance in mice were summarized in Figure 17. In agreement with previous studies, scopolamine induced a state of amnesia by extending the escape latency to find the hidden platform in spatial memory task when compared to control (normal saline-treated). This effect was seen on the first day of trial and persisted throughout the whole trial schedule. The average escape latency times of the scopolamine-treated mice were significantly higher than those in normal saline-treated mice.

2. Effects of *Centella asiatica* on spatial learning and memory performance in mice induced by scopolamine.

As shown in Figures 18 administration of CE (100, 300, 1000 or 1500 mg/kg BW., p.o. 30-min before the MWM) had no significant effect on scopolamine induced-memory deficit in mice.

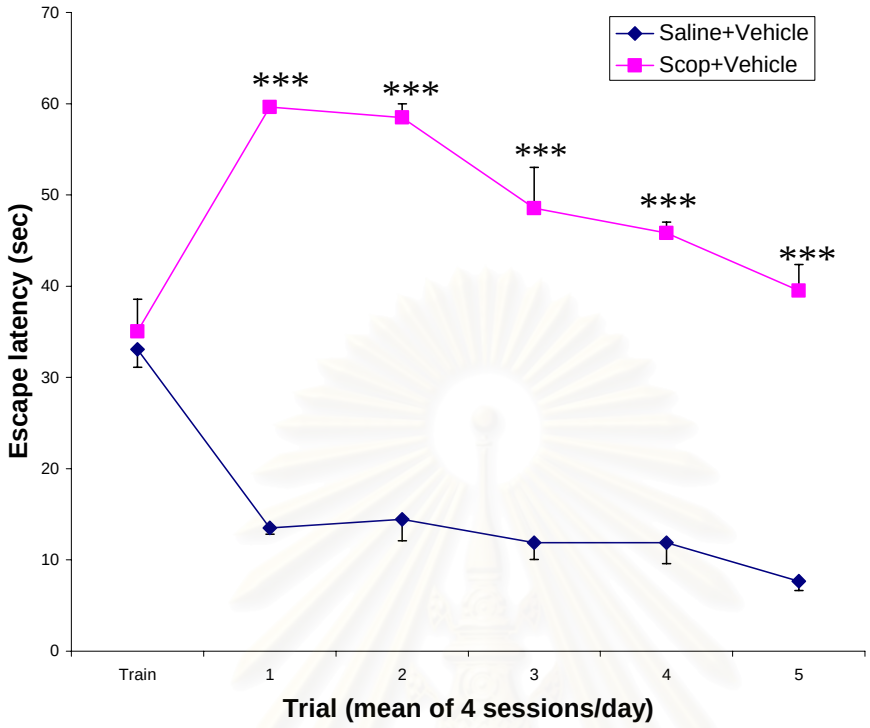


Figure 17 : Effect of scopolamine-induced disruption of memory (0.5 mg/kg) in the MWM performance. The escaping latency onto the platform was measured on 5 consecutive days. Each data point represents the mean± S.E.M. (*n*=8) of four trials.

*** Significance of difference vs. saline-treated mice at $P < 0.001$

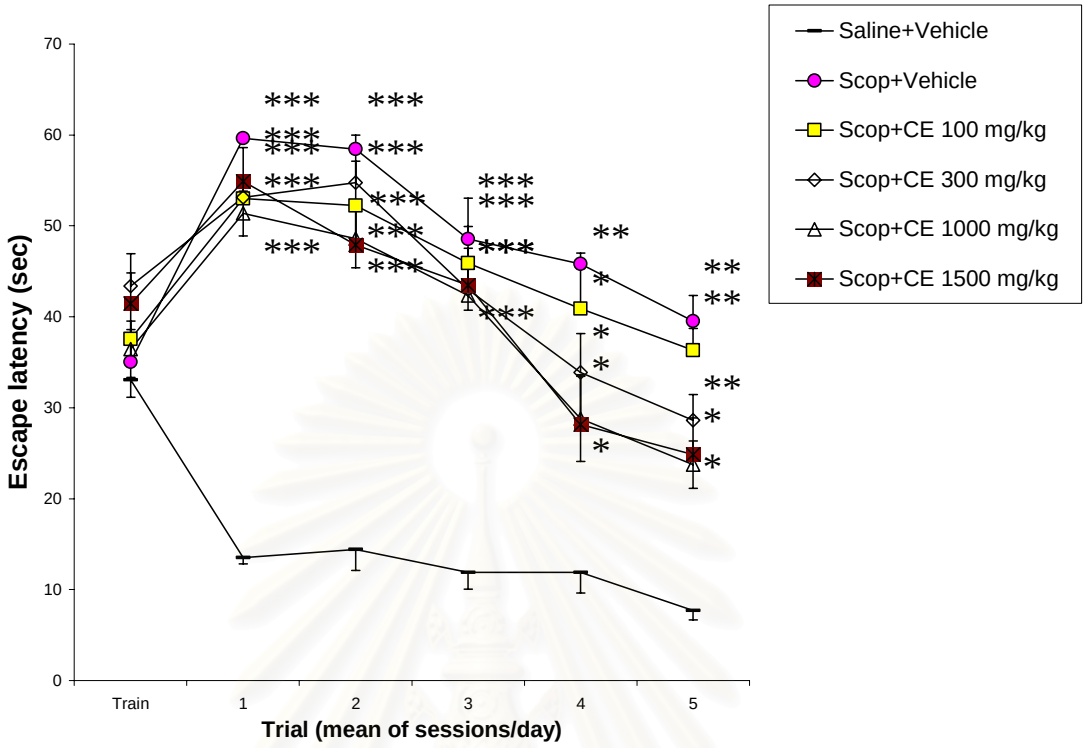


Figure 18 : Effects of CE on scopolamine-induced disruption of memory (0.5 mg/kg) in the MWM performance. Mice were orally given with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg BW. once daily. The escaping latency onto the platform was measured on 5 consecutive days. Each data point represents the mean± S.E.M. (*n*=8) of four trials.

*, **, *** Significance of difference vs. saline-treated mice at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

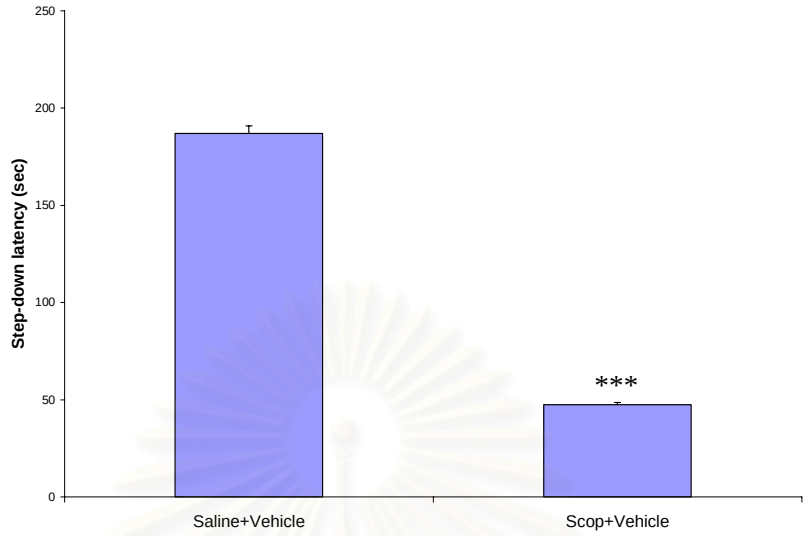
3. **Effects of scopolamine on step-down passive avoidance in mice.**

As shown in Figure 19. Scopolamine (0.5 mg/kg B.W.) significantly shortened the step-down latency and increased the number of errors determined by step-down test.

4. **Effects of *Centella asiatica* on step-down passive avoidance in mice induced by scopolamine.**

Effects of treatment with CE at 100, 300, 1000 or 1500 mg/kg body weight B.W., p.o., in step-down test were shown in Figures 20. CE administration had no effect on step-down latency and number of errors in scopolamine-induced memory deficit in mice.

(A)



(B)

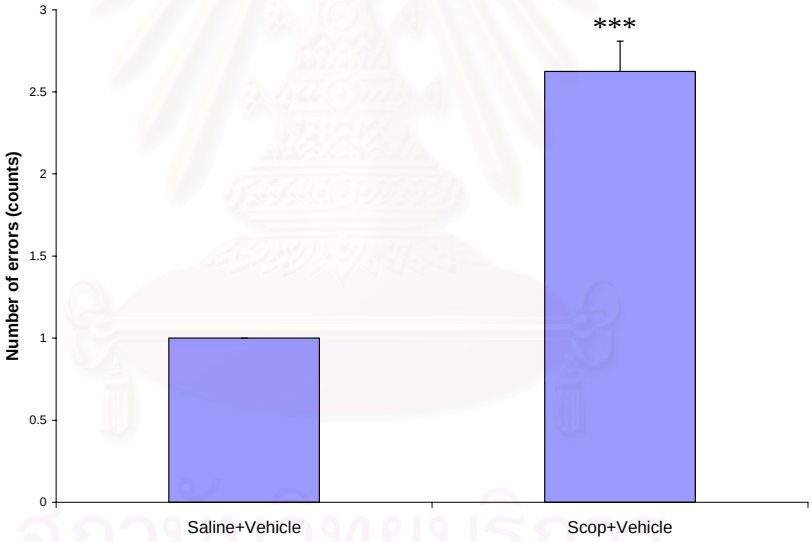
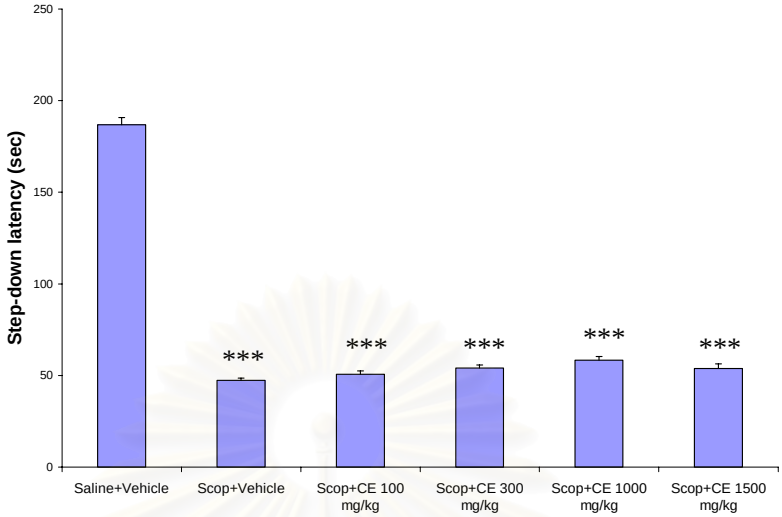


Figure 19 : Effects of scopolamine-induced disruption of memory (0.5 mg/kg) in step-down test. Step-down latency (A) and number of errors (B) were expressed as the mean $\pm$ S.E.M. (n=8).

*** Significance of difference vs. saline-treated mice at $P < 0.001$

(A)



(B)

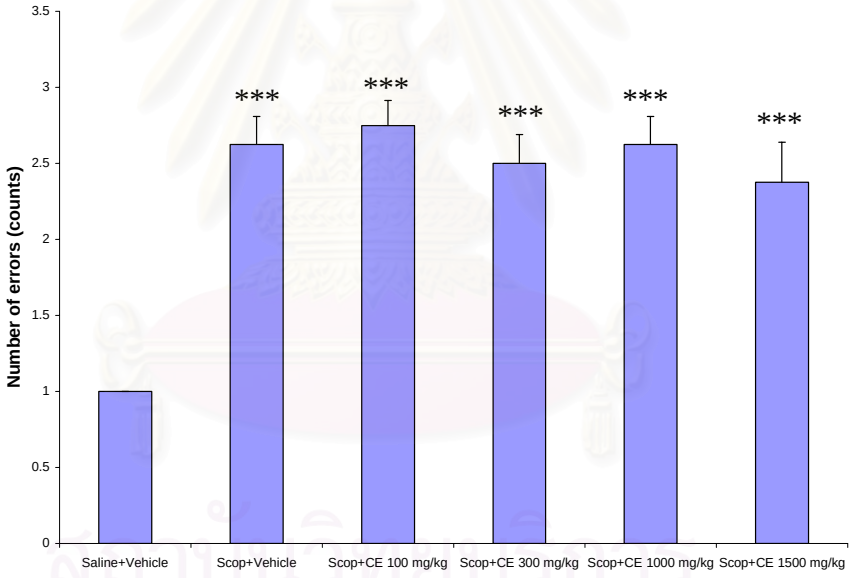


Figure 20 : Effects of CE on scopolamine-induced disruption of memory (0.5 mg/kg) in step-down test. Mice were orally with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg B.W. once daily. Step-down latency (A) and number of errors (B) were expressed as the mean $\pm$ S.E.M. (n=8).

*** Significance of difference vs. saline-treated mice at $P < 0.001$

5. Effects of scopolamine on spontaneous locomotor activity in mice.

The spontaneous locomotor activity, measured as movement counting during 5 min test period, in scopolamine-induced memory deficit and saline-treated mice was summarized in Figure 21. The spontaneous locomotor activity did not differ significantly between scopolamine-induced memory deficit and saline-treated mice.

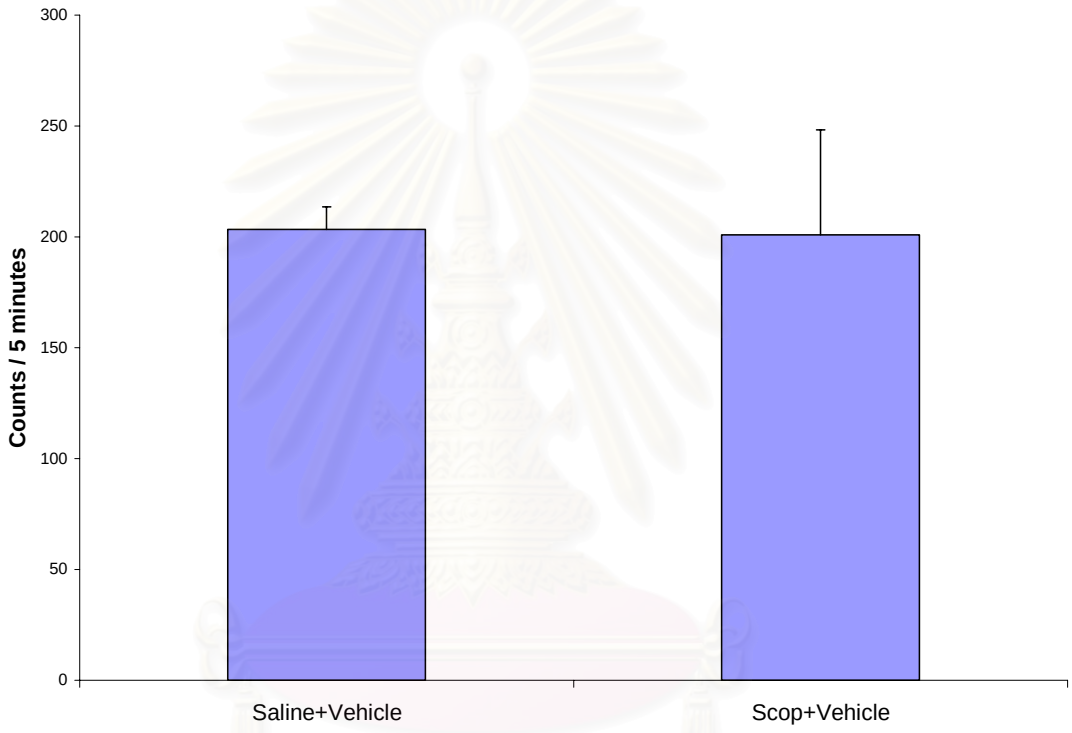


Figure 21 : Effects of scopolamine-induced disruption of memory (0.5 mg/kg) in spontaneous locomotor activity. On the ordinate: counts/ 5 min, the values are expressed as the mean ± S.E.M. (n=8). A significant level of $P < 0.05$ was considered as a significant difference.

6. **Effects of *Centella asiatica* on spontaneous locomotor activity in mice induced by scopolamine.**

Effects of treatment with CE at 100, 300, 1000 or 1500 mg/kg body weight B.W., p.o., on spontaneous locomotor activity. CE administration had no effect on spontaneous locomotor activity in scopolamine-induced memory deficit as shown in Figures 22.

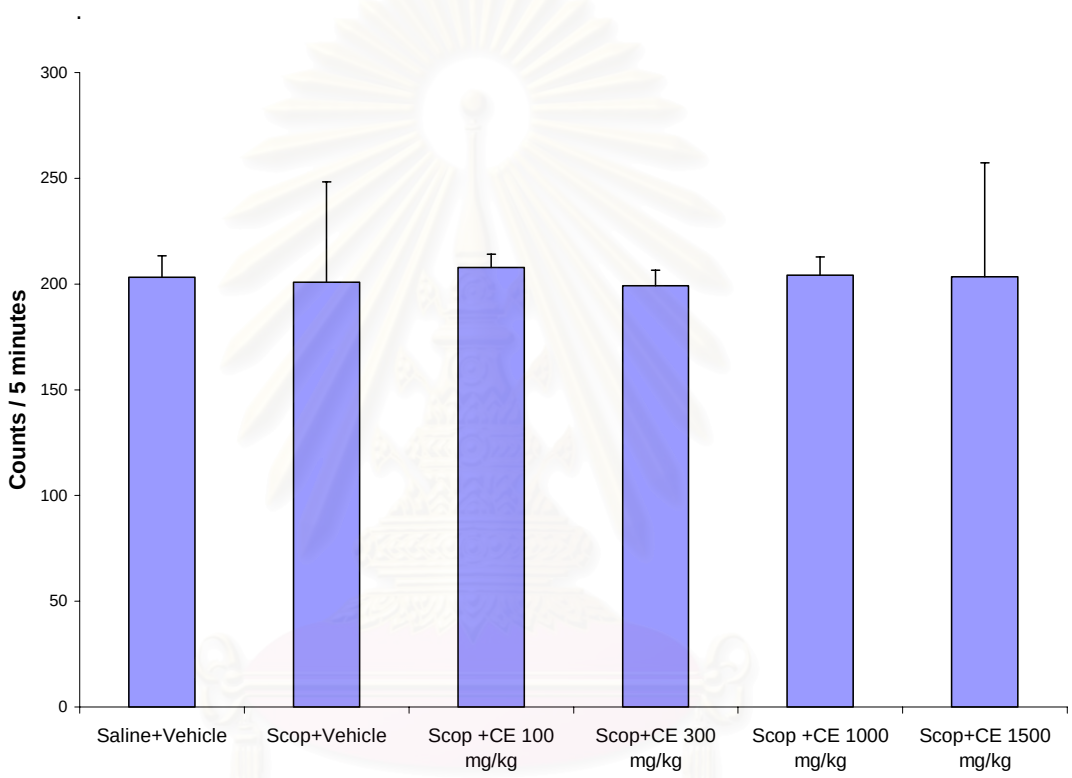


Figure 22 : Effects of CE on scopolamine-induced disruption of memory (0.5 mg/kg) in spontaneous locomotor activity. Mice were orally given with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg B.W. On the ordinate: counts/ 5 min, the values are expressed as the mean $\pm$ S.E.M. (n=8). A significant level of $P < 0.05$ was considered.

7. Effect of scopolamine treated on brain lipid peroxidation in mice.

The MDA brain levels of scopolamine-induced memory deficit and saline-treated mice after 8-day administration of vehicle were shown in Figure 23. There was no significant difference between MDA levels of scopolamine-induced memory and saline-treated mice.

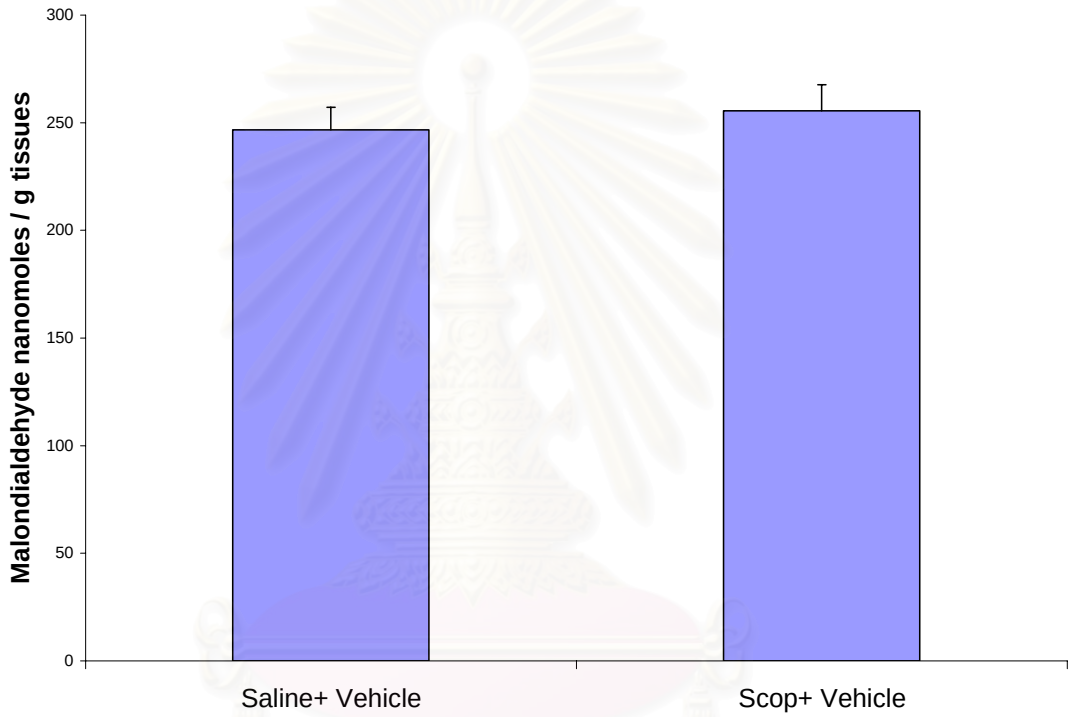


Figure 23 : Effects of scopolamine-induced disruption of memory (0.5 mg/kg) on brain levels of MDA. On the ordinate: MDA nmol/g tissue (mean $\pm$ S.E.M.) (n=8) after the 8 day of 2VO. A significant level of $P < 0.05$ was considered as a significant difference.

8. **Effects of *Centella asiatica* administration on brain lipid peroxidation in mice induced by scopolamine.**

Effects of treatment with CE at 100, 300, 1000 or 1500 mg/kg body weight B.W., p.o., on brain lipid peroxidation were shown in Figures 24. At 8 days following testing; CE administration had no effect on MDA levels of the brain.

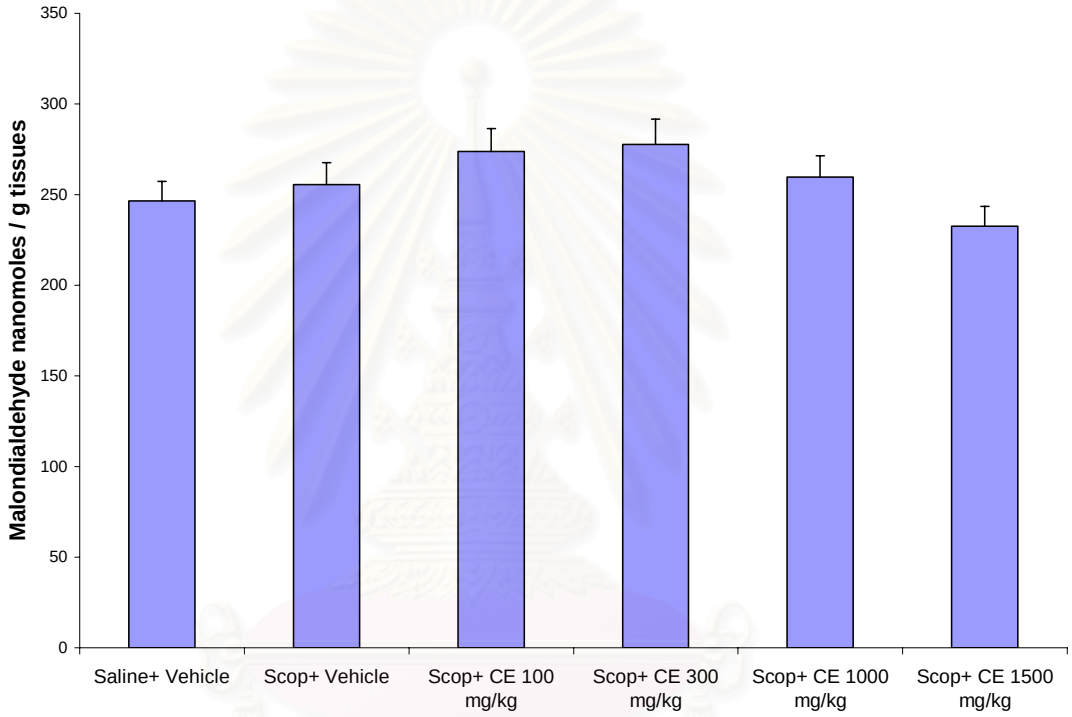


Figure 24 : Effects of CE on MDA levels in nmol/g tissue in scopolamine-induced disruption of memory (0.5 mg/kg). Mice were orally with vehicle and CE at doses of 100, 300, 1000 or 1500 mg/kg B.W. On the ordinate: MDA nmol/g tissue (mean $\pm$ S.E.M.) (n=8) after the 8 day of 2VO. A significant level of $P < 0.05$ was considered as a significant difference.

CHAPTER IV

DISCUSSION AND CONCLUSION

Centella asiatica, a traditional medical plant, has been described for two pronounced effects, i.e. improving learning and memory and, the antioxidant property by decreasing the lipid peroxidation in the brain of normal rats (Veerendra Kumar and Gupta, 2002). In the present study ethanolic extract of *Centella asiatica* was evaluated in mice for its effect on learning and memory impairment induced by cerebral ischemia and by scopolamine as well as its effect on lipid peroxidation, marker of oxidative stress in AD.

Since the hippocampal formation is the primary targets of AD as well as a site of cell loss in ordinary aging, one might suppose that the elderly people with age-related memory loss are in fact manifesting early systems of AD (Squir and Kandel, 1999). Hippocampal region is important for both spatial and nonspatial memory, for both recognition memory and recall, and for both fact and events memory (Block and Schwarz, 1997; Gazzaniga, 1999). Lesions of hippocampus interfere with the formation of new spatial memories-memory for places (Squir and Kandel, 1999). Behavioral studies have been used to screen for changes in learning and memory using Morris water maze (MWM) and passive avoidance test studies due to damage of the hippocampus (Squir and Kandel, 1999). The initiating event or event leading to AD is unknown. Its pathophysiology is complex and likely to involve multiple overlapping and perhaps redundant pathways of neuronal damage. One of the pathways of neuronal damage and death in AD is mediated by lipid peroxidation (Pratico and Delanty, 2000). Animals models of bilateral common carotid occlusion (2VO) exhibited progressive spatial cognitive deficits (De Jong et al., 1999; Sopala and Danysz, 2001) and that this progressive axonal damage in hippocampus (Wakita et al., 2002). In addition hippocampus has shown a sustained elevation of lipid peroxidation markers following cerebral ischemia (Candelario-Jalil, 2001).

In the present study, mice were subject to a 20-min period of cerebral ischemia, produced by 2VO plus removal of 0.3 ml of blood from the tip of the tail to produce

global ischemia, which affected neither general behavior nor spontaneous locomotor activity. However, it produced an increase in escape latencies in MWM and a decrease in step-down latency while increase in number of errors indicating the impaired memory by cerebral ischemia. These observations concur with previous reports that 2VO impaired the behavior performance in learning and memory task in rats (De Jong et al., 1999; Sopola and Danysz, 2001; Wakita et al., 2002) mice (Xu et al., 2002; Watanabe et al., 2003) gerbils (Andersen and Sams-Dodd,1998; Niwa, 1999).

It was found that the 2VO mice receiving oral administration of CE (100, 300, 1000 or 1500 mg/kg B.W.) showed improvement in learning and memory deficits as evidenced by decreased escape latency in MWM, and increased in latencies and decreased in number of errors in passive avoidance test.

The general stimulant or depressant activity of a central nervous system (CNS) active drug may affect the animal response on behavior paradigms. Therefore, the effect of CE on locomotor activity was studied. There was no significant difference between the locomotor activities of sham, 2VO mice receiving vehicle and 2VO mice receiving CE. This makes it unlikely that the changes in MWM and passive avoidance attention observed in 2VO mice receiving CE would have been due to any CNS depressant/stimulant activity of CE.

It has been previously reported that level of brain lipid peroxide in AD is higher than that of normal. (Ramassamy et al., 1999; Smith et al., 2000; Esposito et al., 2002). Markers of brain lipid peroxidation have been the most studied index of oxidative stress in AD (Islekel, 1999; Pratico and Delanty, 2000). A growing number of studies suggest that natural extracts and phytochemical have a positive impact on brain aging (Bastianetto and Quirion, 2002) Moreover, clinical data suggest that nutrition antioxidants might exert some protective effect against AD (Esposito et al., 2002). Oxidative stress is important in pathogenesis of AD, in clinical trials, a potential antioxidant, idebenone, showed a clear dose-related anti-dementia activity in AD (Yamazaki et al., 2002). With regards to natural antioxidant enzymes or agents which are capable of augmenting the functions of these enzymes, several reports have shown that

the natural drugs, possessing antioxidant properly, for example *Ginkgo biloba* (Brailowsky and Montiel, 1997; Winter and Timiners, 1999; Rickard, Kowadlo and Gibbs 2000; Tang et al., 2002 and Maclennan, Darlington and smith, 2002) *Celastrus paniculatus Willd, Clitoria ternates L., Lycoris radiate Herb, Polygala tenuifolia Willd* and *Salvia miltiorrhiza Bung* (Howes and Houghton, 2003) could be relevant to the treatment of AD. Therefore, the effect of CE on lipid peroxidation was further evaluated.

The increase in levels of MDA, a marker of lipid peroxidation in our study, indicates increase oxidative stress generation in 2VO mice receiving vehicle. The significantly lower levels of MDA in the brain of the 2VO mice receiving 100, 300, 1000 or 1500mg/kg B.W. p.o. CE as compared with the 2VO mice receiving vehicle indicate attenuation of lipid peroxidation. The decrease in MDA levels in 2VO mice receiving CE may be due to its antioxidant property and that might be beneficial for AD.

Although pathogenesis of AD remains to be fully defined, several pharmacological strategies for treatment of AD are under active investigation. Cholinergic therapy that is designed to increase cholinergic functions and antioxidant are most attractive approach for treatment of AD (Yamada and Nabeshima, 2000). Tacrine is the first acetylcholine esterase inhibitors that has been approved for treatment of AD (Yamada and Nabeshima, 2000; Holroyd and Shepherd, 2001). It is reported that tacrine could improve impairment of performance of mice in passive avoidance and MWM tests (Xu et al., 2000). Furthermore tacrine produced an ameliorative effect on eight-arm radial maze test deficit caused by 2VO in rats, and raised the levels of extracellular Acetylcholine (Ach) in the brain (Murakami et al., 2000).

Investigation of neuropathological changes associated with AD, has been increasing. An important finding was a significant decrease of choline acetyltransferease (ChAT) activity, a biochemical marker for cholinergic neurons, as well as the discovery that AD was associated with a loss of cholinergic neurons in basal forebrain (Muir, 1997). Accordingly, a large number of pharmacological studies (Palmer, 2002) including our own experiments have shown that acetylcholine receptor antagonists such as scopolamine reduced performance of animals in learning and

memory tasks. However, unlike 2VO, impairment of learning and memory induced by scopolamine was not co concurrent with the increase of brain MDA. CE (100, 300, 1000 or 1500 mg/kg B.W., p.o.) which has been found to improve learning and memory deficit in 2VO, had no effect on impairment of learning and memory (assessed by MWM and step-down test) induced by scopolamine. It has been reported previously that in parallel with the ability inhibit to cholinesterase in cortex and hippocampus, rivastigmine could improve scopolamine induced deficit in MWM and step-down test (Bejar, Wang and Weinstock, 1999). Therefore the lack of beneficial effects of CE on learning and memory function in mice receiving scopolamine suggests that CE might have no effect on cholinergic system.

In conclusion the present study has demonstrated the beneficial effects of CE on learning and memory impairment, measure by MWM and step-down test, exclusively in 2VO mice but not that induced by scopolamine. Based on the finding that 2VO but not scopolamine significantly increased the lipid peroxidation which could be partly ameliorated by CE, Therefore it is suggestive that antioxidative property of CE could, at least partly, contributed to its positive effect on memory deficit in 2VO mice. This may explain also the lack of effect of CE on memory impairment induced by scopolamine in which oxidative stress was not its feature. Thus, CE might be beneficial for memory impairment in AD which oxidative stress is underlying cause. Further study is needed to identity the nature of compound(s) accounted for the positive effect of CE on memory.

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Appendices

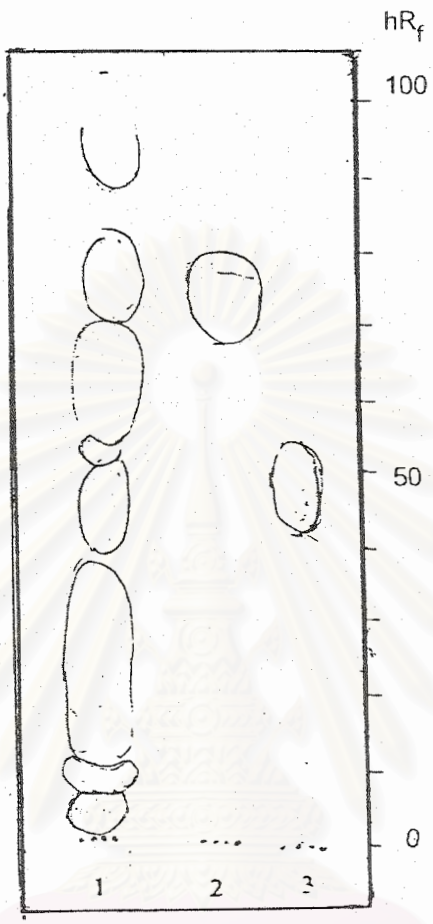

Figure 25 : Thin-layer Chromatogram of Ethanolic Extract of the Aerial Part of *Centella asiatica* (L.) Urban., detected with *anisaldehyde TS*.

1 = CE

2 = Asiaticoside

3 = Asiatic acid

= spots developed in some batches of the samples.

Effects of *Centella asiatica*, Vehicle and Normal saline on performance of 2VO mice in MWM.

Group name	Escape latency in the MWM (sec)						
	n	train	Day1	Day2	Day3	Day4	Day5
Sham	8	44.50 ± 2.92	25.88 ± 1.78	27.72 ± 2.50	18.41 ± 2.70	9.56 ± 1.14	6.00 ± 0.80
2VO	8	39.75 ± 3.76	43.47 ± 1.26***	43.75 ± 2.44***	36.66 ± 3.33***	35.22 ± 1.91***	30.09 ± 2.33***
Sham+Vehicle	8	36.19 ± 3.91	23.91 ± 3.17	18.66 ± 3.16	16.28 ± 3.83	10.50 ± 1.29	9.66 ± 1.81
2VO+Vehicle	8	39.41 ± 2.85	44.88 ± 0.38***	47.97 ± 3.46***	44.66 ± 3.45***	43.53 ± 0.57***	36.38 ± 2.08***
2VO+CE100 mg/kg	8	34.22 ± 4.33	32.06 ± 2.91***###	24.88 ± 1.83***###	22.56 ± 4.09***###	21.56 ± 1.21***###	14.44 ± 0.97***###
2VO+CE300 mg/kg	8	30.63 ± 2.09	30.59 ± 3.50***###	25.19 ± 1.92***###	19.88 ± 2.69***###	15.34 ± 2.64***###	11.88 ± 1.77***###
2VO+CE1000 mg/kg	8	32.91 ± 3.90	22.22 ± 3.87***###	16.47± 3.33***###	13.25 ± 1.83***###	10.34 ± 1.19***###	10.44 ± 0.76***###
2VO+CE1500 mg/kg	8	30.34 ± 3.15	17.63 ± 3.53***###	11.41 ± 1.47***###	11.16 ± 0.75***###	9.28 ± 0.80***###	8.09 ± 0.61***###

Table 2 : Effects of CE, Vehicle and Normal saline on performance of 2VO mice in MWM. The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons.

*** Significance of difference vs. sham operated mice at $P < 0.001$. ### Significance of difference vs. 2VO-operated mice at $P < 0.001$

Effects of *Centella asiatica*, Vehicle and Normal saline on performance of 2VO mice in step-down test.

Group name	Sham	Sham +Vehicle	2VO	2VO +Vehicle	2VO +CE100 mg/kg	2VO +CE300 mg/kg	2VO +CE1000 mg/kg	2VO +1500 mg/kg
n	8	8	8	8	8	8	8	8
Step-down latency	114.61 ± 4.79	195.38 ± 3.78	46.13 ± 1.56***	46.13 ± 1.56***	53.13 ± 3.00***	70.75 ± 8.22***##	160.38 ± 3.78***###	147.88 ± 2.68***###
Number of errors	1.00 ± 0.00	1.00 ± 0.00	2.75 ± 0.17***	2.75 ± 0.16***	2.50 ± 0.19***	1.75 ± 0.31***##	1.38 ± 0.18***###	1.88 ± 0.13***###

Table 3 : Effects of CE, Vehicle and Normal saline on performance of 2VO mice in step-down test. The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons.

* Significance of difference vs. saline-treated mice at $P < 0.001$. ##, ###Significance of difference vs. 2VO-operated mice at $P < 0.01$, $P < 0.001$ respectively.

Effects of *Centella asiatica*, Vehicle and Normal saline on performance of 2VO mice in spontaneous locomotor activity (counts/5 minutes).

Group name	Sham	Sham +Vehicle	2VO	2VO +Vehicle	2VO +CE100 mg/kg	2VO +CE300 mg/kg	2VO +CE1000 mg/kg	2VO +1500 mg/kg
n	8	8	8	8	8	8	8	8
Locomotor activity (counts/5 minutes)	192.25 ± 6.2984	202.38 ± 10.39	202.25 ± 7.88	213.50 ± 9.80	193.88 ± 10.00	191.50 ± 9.19	205.25 ± 12.40	194.63 ± 9.16

Table 4 : Effects of CE, Vehicle and Normal saline on performance of 2VO mice in spontaneous locomotor activity. The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons.

Effects of *Centella asiatica*, Vehicle and Normal saline on performance of 2VO mice in **lipid peroxidation**.

Group name	Sham	Sham +Vehicle	2VO	2VO +Vehicle	2VO +CE100 mg/kg	2VO +CE300 mg/kg	2VO +CE1000 mg/kg	2VO +1500 mg/kg
n	8	8	8	8	8	8	8	8
MDA levels (nmol/g tissue)	206.50 ± 5.79	201.88 ± 7.08	600.25 ± 20.94***	634.63 ± 19.09***	520.13 ± 21.92***##	507.75 ± 20.52***##	403.25 ± 22.13***###	383.25 ± 31.38***###

Table 5 : Effects of CE, Vehicle and Normal saline on performance of 2VO mice in MDA levels. The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons.

* Significance of difference vs. saline-treated mice at $P < 0.001$. ##, ###Significance of difference vs. 2VO-operated mice at $P < 0.01$, $P < 0.001$ respectively.

Effects of *Centella asiatica*, Vehicle and Normal saline on performance of mice induced by scopolamine in MWM.

Group name	Escape latency in Morris water maze (sec)						
	n	Train	Day1	Day2	Day3	Day4	Day5
Saline+Vehicle	8	33.09 ± 1.96	13.50 ± 0.68	14.44 ± 2.34	11.88 ± 1.83	11.91 ± 2.30	7.69 ± 1.03
Scop+Vehicle	8	35.03 ± 3.55	59.64 ± 0.36***	58.47 ± 1.53***	48.56 ± 4.48***	45.81 ± 1.21**	39.53 ± 2.83**
Scop+CE100 mg/kg	8	37.56 ± 3.95	53.00 ± 1.82***	52.25 ± 4.87***	45.91 ± 4.03***	40.91 ± 4.42*	36.34 ± 2.38**
Scop+CE300 mg/kg	8	43.41 ± 3.53	53.16 ± 1.70***	54.75 ± 3.86***	43.00 ± 4.55***	33.88 ± 4.30*	28.63 ± 2.84**
Scop+CE1000 mg/kg	8	36.44 ± 3.90	51.38 ± 2.47***	48.63 ± 3.23***	42.34 ± 1.63***	28.78 ± 4.68*	23.75 ± 2.62*
Scop+CE1500 mg/kg	8	41.47 ± 3.38	54.88 ± 3.73***	47.91 ± 4.82***	43.47 ± 2.40***	28.16 ± 5.48*	24.88 ± 4.03*

Table 6 : Effects of CE, Vehicle and Normal saline on performance of mice induced by scopolamine in MWM. The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons.

*, **, *** Significance of difference vs. saline-treated mice at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

Effects of *Centella asiatica*, Vehicle and Normal saline on performance of mice induced by scopolamine in step-down test.

Group name	Saline+Vehicle	Scop+Vehicle	Scop+CE100 mg/kg	Scop+CE300 mg/kg	Scop+CE1000 mg/kg	Scop+CE1500 mg/kg
n	8	8	8	8	8	8
Step-down latency	186.88 ± 3.92	47.38 ± 1.19***	50.75 ± 1.89***	54.13 ± 1.63***	58.38 ± 2.03***	53.88 ± 2.60***
Number of errors	1.00 ± 0.00	2.63 ± 0.18***	2.75 ± 0.16***	2.50 ± 0.19***	2.66 ± 0.18***	2.38 ± 0.26***

Table 7 : Effects of CE, Vehicle and Normal saline on performance of mice induced by scopolamine in step-down test.

The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons.

*** Significance of difference vs. saline-treated mice at $P < 0.001$

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Effects of *Centella asiatica*, Vehicle and Normal saline on performance of mice induced by scopolamine in spontaneous locomotor activity (counts/5 minutes).

Group name	Saline +Vehicle	Scop +Vehicle	Scop +CE100 mg/kg	Scop +CE300 mg/kg	Scop +CE1000 mg/kg	Scop +CE1500 mg/kg
n	8	8	8	8	8	8
Locomotor activity (counts/5 minutes)	203.25 ± 10.21	200.88 ± 10.74	207.88 ± 6.14	199.25 ± 7.29	204.25 ± 8.50	203.50 ± 9.70

Table 8 : Effects of CE, Vehicle and Normal saline on performance of mice induced by scopolamine in spontaneous locomotor activity. The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons. A significant value of P less than 0.05 ($P < 0.05$) was considered as statistically significant.

Effects of *Centella asiatica*, Vehicle and Normal saline on performance of mice induced by scopolamine **lipid peroxidation**.

Group name	Saline +Vehicle	Scop +Vehicle	Scop +CE100 mg/kg	Scop +CE300 mg/kg	Scop +CE1000 mg/kg	Scop +CE1500 mg/kg
n	8	8	8	8	8	8
MDA levels (nmol/g tissue)	246.63 ± 10.62	255.50 ± 12.13***	273.75 ± 12.72***	277.63 ± 14.02***	259.63 ± 11.73***	232.63 ± 10.85***

Table 9 : Effects of CE, Vehicle and Normal saline on performance of mice induced by scopolamine in MDA levels. The values are expressed as the mean ± S.M.E. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey post-hoc test for comparisons.

*** Significance of difference vs. saline-treated mice at $P < 0.001$

VITAE

Miss Saowalak Doknark was born on March 29th, 1974, in Nakornsawan, Thailand. She was graduated in Bachelor of Science (Physical Therapy) in 1995 from Chiangmai University, Thailand. She has been appointed as physical therapist in Rehabitation Unit, Sawanpracharak Hospital, for 5 years.