ผลของสารสกัดผลมะขามป้อมต่อความบกพร่องของความจำและการตายของเซลล์ประสาท ที่ถูกเหนี่ยวนำด้วยการอุดกั้นหลอดเลือดแดงคอมมอนแคโรติดทั้งสองข้างในหนูถีบจักร

นางสาววิมล นิลยาภรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาสรีรวิทยา ภาควิชาสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

EFFECTS OF *PHYLLANTHUS EMBLICA* FRUITS EXTRACT ON MEMORY IMPAIRMENT AND NEURONAL CELL DEATH INDUCED BY BILATERAL OCCLUSION OF COMMON CAROTID ARTERY IN MICE

Miss Wimon Ninyaporn

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Physiology
Department of Physiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2006

Thesis Title	EFFECTS OF *PHYLLANTHUS EMBLICA* FRUIT EXTRACT ON MEMORY IMPAIRMENT AND NEURONAL CELL DEATH INDUCED BY BILATERAL OCCLUSION OF COMMON CAROTID ARTERY IN MICE
By	Miss Wimon Ninyaporn
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วิมล นิลยาภรณ์: ผลของสารสกัดผลมะขามป้อมต่อความบกพร่องของความจำและการตายของเซลล์ประสาท ที่ถูกเหนี่ยวนำโดยการอุดกั้นหลอดเลือดแดงคอมมอนแคโรติดทั้งสองข้างในหนูถีบจักร EFFECTS OF *PHYLLANTHUS EMBLICA* FRUITS EXTRACT ON MEMORY IMPAIRMENT AND NEURONAL CELL DEATH INDUCED BY BILATERAL OCCLUSION OF COMMON CAROTID ARTERY IN MICE อ.ที่ปรึกษา: รศ.ดร.บุญยงค์ ตันติสิระ, อ.ที่ปรึกษาร่วม: รศ.ดร.มยุรี ตันติสิระ 53 หน้า.

งานวิจัยนี้เป็นการศึกษาผลของสารสกัดมะขามป้อมต่อความบกพร่องของความจำและการตายของเซลล์ประสาท หลังการอุดกั้นหลอดเลือดแดงคอมมอนแคโรติดทั้งสองข้างในหนูถีบจักร พบว่าหนูที่อยู่ในภาวะสมองขาดเลือดใช้เวลาในการหาแท่นพักนานขึ้นเมื่อทดสอบด้วยวิธีมอรีสวอเตอร์เมส เมื่อทดสอบด้วยวิธีสะเต็บดาวพบว่า หนูใช้เวลาอยู่บนแท่นพักลดลงและจำนวนครั้งที่ก้าวลงจากแท่นพักเพิ่มขึ้น แสดงว่าหนูในกลุ่มนี้เกิดความบกพร่องในด้านความจำ แต่เมื่อให้สารสกัดมะขามป้อมทางปากในขนาด 100, 300 และ 1000 มิลลิกรัมต่อกิโลกรัมต่อวัน มีผลทำให้หนูใช้เวลาในการหาแท่นพักลดลงเมื่อทดสอบด้วยวิธีมอรีสวอเตอร์เมส และจากการทดสอบด้วยวิธีสะเต็บดาวพบว่า หนูที่ได้รับสารสกัดมะขามป้อมในขนาด 100, 300 และ 1000 มิลลิกรัมต่อกิโลกรัมต่อวัน ใช้เวลาอยู่บนแท่นพักนานขึ้น และจำนวนครั้งที่ก้าวลงจากแท่นพักลดลงเมื่อเทียบกับหนูในกลุ่มควบคุม แสดงว่าสารสกัดมะขามป้อมสามารถแก้ไขความบกพร่องของความจำที่เกิดจากภาวะสมองขาดเลือดได้ นอกจากนี้ยังพบว่าสารสกัดมะขามป้อมทุกขนาดไม่มีผลต่ออัตราการเคลื่อนไหวของหนูที่อยู่ในภาวะสมองขาดเลือดแต่อย่างใด แต่จะลดระดับเอ็มดีเอในสมองหนูที่ถูกเหนี่ยวนำให้เพิ่มขึ้นจากภาวะสมองขาดเลือดได้

ในการศึกษาทางสัณฐานวิทยาพบว่าหนูที่ถูกเหนี่ยวนำให้สมองขาดเลือด มีการทำลายของเซลล์ประสาทในสมองส่วนฮิปโปแคมปัส และพบว่าสารทดสอบสามารถลดการทำลายของเซลล์ประสาทในสมองส่วนฮิปโปแคมปัสได้

อาจกล่าวได้ว่าความสามารถในการแก้ไขความบกพร่องของความจำของสารสกัดมะขามป้อมน่าจะมีอย่างน้อยส่วนหนึ่งที่เป็นผลจากคุณสมบัติต้านออกซิเดชั่นของสารสกัด ที่สามารถลดการทำลายของเซลล์ประสาทจากสภาวะสมองขาดเลือดได้

ภาควิชา สรีรวิทยา ลายมือชื่อนิสิต.....วิมล นิลยาภรณ์.....

สาขาวิชา สรีรวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4876605133: MAJOR PHYSIOLOGY

KEY WORD:PHYLLANTHUS EMBLICA (Linn.), MEMORY, CEREBRAL ISCHEMIA, MORRIS WATER MAZE, STEP DOWN TEST, LOCOMOTOR ACTIVITY TEST, OXIDATIVE STRESS

WIMON NINYAPORN: EFFECTS OF *PHYLLANTHUS EMBLICA* FRUITS EXTRACT ON MEMORY IMPAIRMENT AND NEURONAL CELL DEATH INDUCED BY BILATERAL OCCLUSION OF COMMON CAROTID ARTERY IN MICE. THESIS ADVISOR: ASSOC. PROF. BOONYONG TANTISIRA, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. MAYUREE TANTISIRA, Ph.D., 53 pages.

The effects of emblic myrobalan (*Phyllanthus emblica*) fruits extract on memory impairment induced by transient cerebral ischemia (bilateral occlusion of common carotid arteries, 2VO) was investigated in mice. The 2VO caused an impairment of memory seen as an increase in the latency to find the platform in Morris Water Maze (MWM) test as well as a reduction of step-down latency and an increment of number of errors in step-down test. Administration of emblic myrobalan extract (p.o.) at doses of 100, 300 and 1000 mg/kg/day significantly reduced the latency to find the platform of 2VO mice in MWM test. Furthermore, in passive avoidance task, emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg/day were found to increase the step-down latency and decrease the number of error of 2VO mice. Thus ameliorating effects of emblic myrobalan on memory deficit induced by cerebral ischemia were demonstrated. In addition, it was found that 2VO had no effects on locomotor activity test. Moreover, emblic myrobalan extract could suppress 2VO-induced increasing of brain malondialdehyde. Therefore it is likely that memory enhancing effect of emblic myrobalan extract might be accounted by its antioxidative effect. In addition, histopathological observation has shown that emblic myrobalan extract could decrease neuronal cell loss in hippocampus.

In conclusion, the present study has demonstrated the beneficial effects of emblic myrobalan extract on memory deficit induced by cerebral ischemia. It is possible that antioxidant property of emblic myrobalan extract which protected neuronal cell loss, at least partly, contribute to its positive effect on memory deficit in 2VO model.

Department	Physiology	Student's Signature	Wimon Ninyaporn
Field of study	Physiology	Advisor's Signature	Boonyong Tantisira
Academic year	2006	Co-Advisor's Signature	Mayuree Tantisira

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my advisor, Assoc. Porf. Dr. Boonyong Tantisira for his excellent advice, constant support and warm encouragement throughout the course of this study.

My deep sincerity extends to my co-advisor, Assoc. Prof. Dr. Mayuree Tantisira for her valuable suggestion and comments. In addition, my sincere appreciation is also extended to the staffs of Department of Physiology and Pharmacology, Faculty of Pharmaceutical Sciences for their encouragement, moral support and friendliness.

Finally, a special gratitude is expressed to all members of my family and my friends for their love, best regards, understanding and moral support.

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LIST OF ABBREVIATIONS

%	Percent
α	Alpha
β	Beta
γ	Gamma
μm	Micrometre
°C	Degree Celsius
AchE	Acetylcholinesterase
AD	Alzheimer's disease
B.W.	Body weight
CA	Cornu ammonis
CAT	Catalase
CBF	Cerebral blood flow
cm	Centrimeter
CNS	Central nervous system
CR	Condition response
CS	Condition stimulus
DC	Direct current
DNA	Deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
EM	Emblic myrobalan
et al.	et alii (and other)
etc.	et cetera
g	Gram
GABA	Gamma-aminobutyric acid
GSH-Px	Glutathione peroxidase
h	Hour
Hz	Hertz
In	Inch
i.p.	Intraperitoneal injection

L.	Linn.
M	Molar
MDA	Malondialdehyde
mg/kg	Milligram per kilogram
min	Minute
ml	Millilitre
mm	Millimetre
mm^2	Square millimeter
MMA	Methylmethacrylate
ms	Milliisecond
MWM	Morris Water Maze
nm	Nanometre
nmol/g	Nanomoles per gram
NO	Nitric oxide
P.	*Phyllanthus*
PMMA	Polymethylmethacrylate
p.o.	Per os
ROS	Reactive oxygen species
rpm	Revolutions per minute
sec	Second
S.E.M.	Standard error of the mean
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reacting substances
UCS	Uncondition stimulus
V	Volt
2VO	Two vessel occlusion
w/v	Weight by volume
w/w	Weight by weight

CHAPTER I

INTRODUCTION

1.1 Background and rational

Plant and plant products are being used as a source of medicine for long. Among the most important constituents of edible plant products, low molecular weight antioxidants are the most important species. It is known that consumption of fruits and vegetables is essential for normal health of human beings. Vegetarian diet can reduce the risk of cancer, atherosclerosis, etc. *Phyllanthus emblica* (emblic myrobalan), also known as amla, has been used in Ayurveda, the ancient Indian system of medicine. It has been used for treatment of several disorders such as common cold, scurvy, cancer and heart disease. It is believed that the major constituent responsible for these activities is vitamin C (ascorbic acid). Ascorbic acid shows antioxidants, anti-inflammatory and antimutagenic properties. It is a very effective free-radical scavenger. However, there are some *in vivo* studies indicating that antioxidant activities of amla cannot be attributed to ascorbic acid alone and that the overall effect is due to other polyphenols such as ellagic acid, gallic acid, tannins, etc (Khopde *et al.*, 2001).

Free radicals are highly reactive molecules implicated in the pathology of traumatic brain injury and cerebral ischemia, through a mechanism known as oxidative stress. After brain injury, reactive oxygen and reactive nitrogen species may be generated through several different cellular pathways, including calcium activation of phospholipases, nitric oxide synthase, xanthine oxidase, by inflammatory cells. If cellular defense systems are weakened, increased production of free radicals will lead to oxidation of lipid, proteins, and nucleic acids, which may alter cellular function in a critical way (Lewen, Matz and Chan, 2000).

Rodents have evolved a CNS highly adapted for navigating complex spatial environments with ease. Indeed, the hippocampus, a brain structure known to be important for spatiai learning, represents a key subcortical structure in the rat and mouse brain. A remarkable feature of the hippocampus is its regularity along the longitudinal axis presenting large arrays of neurons aligned in distinct layers. Indeed, place cells, a subset of hippocampal pyramidal neurons that fire when the animal moves to a specific spatial location, were first identified in the CA1 region of the hippocampus, suggesting that this brain region may contain the cellular substrate for the formation of spatial maps (O'Keefe and Dostrovsky, 1971).

Piracetam (2-oxo-1-pyrrolidine acetamide) is the most well-known nootropic agent. It is a cerebral function regulating drug which is claimed to be able to enhance cognition and memory, slow down brain aging, increase blood flow and oxygen to the brain, aid stroke recovery, and improve Alzheimer's, Down's Syndrome, dementia and dyslexia. Although much progress has been made in understanding the mechanism of action of piracetam since the early research, a unifying hypothesis has yet to receive widespread acceptance. Piracetam has no specific at GABA receptor sites, on dopaminergic, serotenergic, adrenergic transmission, or any other known receptor, enzyme, or transporter system, with the exception of weak interaction at L-glutamate binding sites (Shorvon, 2001; Wischer *et al.*, 2001). Many different mechanisms have been suggested, but this is only further evidence that an exact mechanism is still not well established in the scientific community (Galeotti, Ghelardini and Bartolini, 1996).

In thailand, emblic myrobalan was used in antifebrile, antitussive, expectorant, refreshing, diuretic, laxative, cardiotonic, carminative and antiscorbutic because it has more vitamin C than orange juice by 20 times. The pulp of dried emblic fruits is astringent, antidysenteric and antidiarrheic, and is also used for the treatment of hemorrhoid (Morton, 1987).

Much research has focused on vitamin C, hydrolysable tannin from *P. emblica* fruits. In particular, studies have shown that vitamin C and hydrolysable tannin are associated with antioxidant activities. This activity may be useful to improve memory impairment but studies with particular attention to cognitive disorders are lacking. Therefore, this study attends to the effects of *P. emblica* against the impairment of learning and memory induced by transient cerebral ischemia in mice.

1.2 Objectives

1. To study the effect of emblic myrobalan extract (*Phyllanthus emblica*) on learning and memory impairment induced by cerebral ischemia in mice.
2. To study the effect of emblic myrobalan extract (*Phyllanthus emblica*) on the level of oxidative stress induced by cerebral ischemia in mice.
3. To study the effect of emblic myrobalan extract (*Phyllanthus emblica*) on neuronal cell loss by histological examination.

CHAPTER II

LITERATURE REVIEWS

2.1 *Phyllanthus emblica* Linn.

Phyllanthus emblica Linn. is a member of the plant family Euphorbiaceae (Table 2.1), the synonym *Emblica officinalis* Gaertn., *Phyllanthus glomerata* Wall., *Dichelactina nodicauli* Hance., *Emblica arborea* Raf., *Cicca emblica* Kurz., *Diasperus emblica* Kuntze., *Phyllanthus laxifolius* Don. is the designation most commonly found. The common names of this plant are Amla (Nepal); Amalaci (Hindi); Amlaj (Arab); Adiphala, Dhatri, Amalaka, Shriphala, Vrittophala (Sanskrit); Nelli (Kerala); Emblic, Myrobalan Tree, Indian gooseberry (England); Gebrauchlicher, Amlabaum (Germany); Kyou-rhoo-rah (Tibetan) (Scartezzini et al., 2006). *Phyllanthus emblica* is an evergreen tree. The fruit are yellow berries, 2.5cm in diameter, quite smooth and hard on appearence, with 6 vertical stripes or furrows (Figure 2.1) (Csurhes and Edwards, 1998).

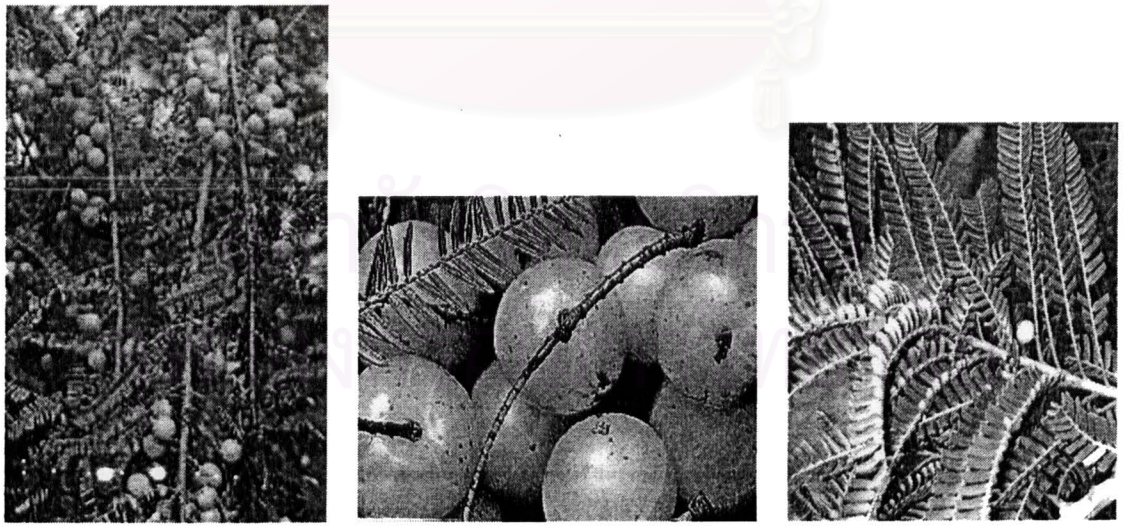


Figure 2.1 *Phyllanthus emblica* Linn.

It grows wildly throughout the tropical and sub-tropical regions, particularly in India and other South East Asian countries. It is commonly cultivated in home gardens throughout India and grown commercially in Uttar Pradesh. Many trees have been planted in southern Malaya, Singapore, and throughout Malaysia. In India, and to a lesser extent in Malaya, the emblic is important and esteemed, raw as well as preserved, and it is prominent in folk medicine (Morton, 1987).

Table 2.1 Scientific classification (Taxonomy) of *Phyllanthus emblica* Linn.

Classification	Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Euphorbiales
Family	Euphorbiaceae
Genus	Phyllanthus L.
Species	Phyllanthus emblica L.

2.1.1 Botany

The tree is a graceful ornamental, normally reaching a height of 60 ft (18 m) and, in rare instances, 100 ft (30 m). Its fairly smooth bark is a pale grayish-brown and peels off in thin flakes like that of the guava. While actually deciduous, shedding its branchlets as well as its leaves, it is seldom entirely bare and is therefore often cited as an evergreen. The miniature, oblong leaves, only 1/8 in (3 mm) wide and 1/2 to 3/4 in (1.25-2 cm) long, distichously disposed on very slender branchlets, give a misleading impression of finely pinnate foliage. Small, inconspicuous, greenish-yellow flowers are

borne in compact clusters in the axils of the lower leaves. Usually, male flowers occur at the lower end of a growing branchlet, with the female flowers above them, but occasional trees are dioecious.

The nearly stemless fruit is round or oblate, indented at the base, and smooth, though 6 to 8 pale lines, sometimes faintly evident as ridges, extending from the base to the apex, give it the appearance of being divided into segments or lobes. Light-green at first, the fruit becomes whitish or a dull, greenish-yellow, or, more rarely, brick-red as it matures. It is hard and unyielding to the touch. The skin is thin, translucent and adherent to the very crisp, juicy, concolorous flesh. Tightly embedded in the center of the flesh is a slightly hexagonal stone containing 6 small seeds. Fruits collected in South Florida vary from 1 to 1 1/4 in (2.5-3.2 cm) in diameter but choice types in India approach 2 in (5 cm) in width. Ripe fruits are astringent, extremely acid, and some are distinctly bitter (Morton, 1987).

2.1.2 Chemical constituents

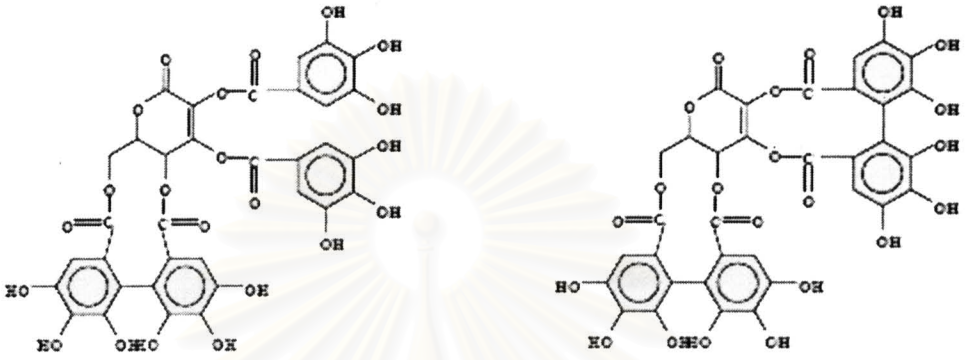
2.1.2.1 Fruits

Moisture 81.2%, protein 0.5%, fat 0.1%, mineral matter 0.7%, fiber 3.4%, carbohydrates 14.1%, Ca (0.05%), K (0.02%), Fe (1.2 mg/100g), nicotinic acid (0.2 mg/100g), phyllemblin, phyllemblic acid, gallic acid, emblicol, ellagic acid, pectin, putranjivain A, two new hydrolysable tannins vitamin C-like called emblicanin A and B (Figure 2.2) and not ascorbic acid as it was believed by mistake until 1996, punigluconin and pedunculagin (Figure 2.3) (Scartezzini and Speroni, 2000).

2.1.2.2 Seeds

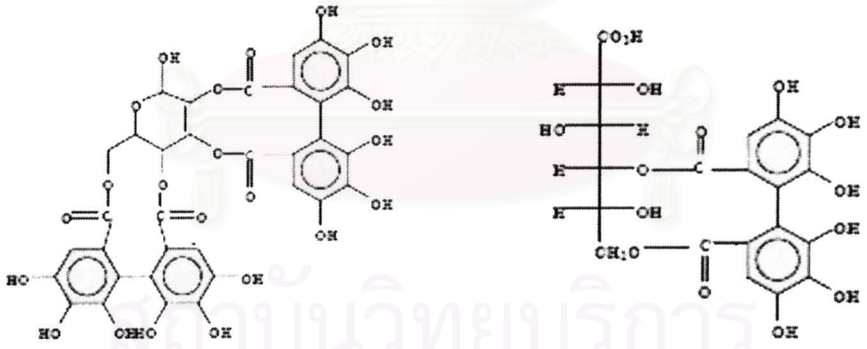
A fixed oil, phosphatides, and a small quantity of essential oil. The fixed oil (yield 16%) has the following physical and chemical characteristics: acid value 12.7; saponification value 185; iodine value 139.5; acetyl value 2.03; unsaponifiable matter

3.81%; sterol 2.70%; saturated fatty acids 7%. Contain linolenic (8.78%), linoleic (44.0%), oleic (28.40%), stearic (2.15%), palmitic (2.99%) and miristic acid (0.95%). Proteolytic and lipolytic substances are present (Scartezzini and Speroni, 2000).



Emblicanin A Emblicanin B

Figure 2.2 Structure of Emblicanin A and Emblicanin B



Pedunculagin Punigluconin

Figure 2.3 Structure of Pedunculagin and Punigluconin

2.1.2.3 Leaves

Gallic, ellagic, chebulic, chebulagic, chebulinic acids, a gallotannin called amlic acid, alkaloids phyllantidine and phyllantine (Scartezzini and Speroni, 2000).

2.1.2.4 Bark

Leukodelphinidin, tannin and proanthocyanidin (Scartezzini and Speroni, 2000).

2.1.2.5 Roots

Ellagic acid and lupeol (Scartezzini and Speroni, 2000).

2.1.3 Traditional applications

Phyllanthus emblica is a medicinal plant described in Ayurveda, the traditional medicinal system of India, passed down through centuries (Gogate, 1982). The fruit (Indian gooseberry) is used as a major constituent in several Ayurvedic preparations such as Chyavanprash and Rasayana, which promote health and longevity. It has been used for anti-inflammatory and antipyretic treatments by rural populations in its growing areas. Malays use a decoction of its leaves to treat fever (Burkill, 1966). In Indonesia, the pulp of the fruit is smeared on the head to dispel headache and dizziness caused by excessive heat (Perry, 1980).

2.1.4 Pharmacological activities

Fruits of *Phyllanthus emblica* have been used for thousands of years in the traditional Indian medicine for the treatment of several diseases. For many years the therapeutic potential of the fruits was attributed to their high content of ascorbic acid: about 1 g of vitamin C per 100 ml of fresh juice (Kapoor, 1990). Because of the presence of certain tannins it did not oxidate even in dried fruit maintaining the antiscorbutic capacity unchanged. Also some studies were published where the high antiscorbutic capacity was exalted. In fact *P. emblica* was used with a great success during the famous famine of Hissar (1939–40) and also in another case of scorbutus which affected the Indian army at Nassirdab in 1837 in Rajputana (Srinivasan, 1944). All

the studies published after that time were based on comparison between ascorbic acid and *P. emblica* the latter was discovered to be more effective than ascorbic acid whether in vitro or in vivo. The fruit extract inhibits micronuclei formation, sister-chromatid exchanges, clastogenicity and mutagenicity metal-induced such as lead, aluminum, cadmium, nickel, caesium (Dhir *et al.*, 1980; Kumar and Shankar, 1986; Roy *et al.*, 1991; Agarwal *et al.*, 1992; Ghosh, Sharma and Talukder, 1992;). All these results were explained through the high content of ascorbic acid. In 1996 professor Shibhnath Ghosal of Banaras Hindu University (Ghosal, Tripathi and Chauhan, 1996) discovered that *Emblica* fruits do not contain ascorbic acid neither in free nor in conjugated form, but it contains two new hydrolysable tannins with low molecular weight (<1000), called emblicanin A (2,3-di-*o*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-δ-lactone) and emblicanin B (2,3,4,6-bis-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-δ-lactone) and other tannins like punigluconino (2,3-di-o-galloyl-4,6-(*S*)-hexahydroxydiphenoylgluconic acid) and pedunclagin (2,3,4,6-*bis*-(*S*)-hexahydroxydiphenoyl-D-glucose) already isolated in other species, *Punica granatum* in particular. These two new tannins have a very strong antioxidant action. The two emblicanins A and B have been found to preserve erythrocytes against oxidative stress induced by asbestos, generator of superoxide radical. Emblicanin A oxidates when put in contact with asbestos becoming emblicanin B and together they have a stronger protective action to erythrocytes than vitamin C. Moreover they improve the efficacy of vitamin C in reducing dihydroascorbic acid to ascorbic acid. The same recycling process has been observed in the rutin-vitamin C combination. The above mentioned tannins prevent the polymerization of vinylic monomers (MMA) in polymers (PMMA) in presence of hydroxyl radicals (Ghosal, Tripathi and Chauhan, 1996). Fruit extract has been shown to have an antimutagenic activity in Ames test (Grover and Kaur, 1989). Recently fruits have been tested for their antiviral activity, particularly for inhibiting reverse transcriptase in the replication of retroviruses like HIV-1. Some active ingredients inhibit the replication of the virus through a non competitive action with respect to the dTTP but competitive action with respect to the template primer (El-Mekkawy *et al.*, 1995). Other studies have shown that the fruit enhances the immunodefence (Suresh and Vasudevan, 1994).

Ginkgo biloba L. has also been used traditionally in Iran to improve memory loss associated with blood circulation abnormalities. Numerous investigations have been conducted regarding the potential of *G. biloba* in cognitive disorders. The *G. biloba* extract Egb 761 has shown biological activities relevant to the treatment of cognitive dysfunction. There is some evidence (electroencephalographic data) to suggest that *G. biloba* extract Egb 761 has a local effect in the CNS; in addition, this extract has shown various biological activities relevant to the treatment of cognitive dysfunction. Favourable effects have been observed on cerebral circulation and neuronal cell metabolism, on the muscarinic cholinergic system, and the extract showed antioxidant activity (Melanie-Jayne and Peter, 2003).

One ancient Ayurvedic remedy is *Centella asiatica* (Umbelliferae), which is reputed to restore youth, memory and longevity. The essential oil (0.1% of the plant) extracted from *C. asiatica* leaf contains monoterpenes, including bornyl acetate, α-pinene, β-pinene and γ-terpinene which are reported to inhibit AchE. The pharmacological basis to explain the reputed antiamnesic effects of *C. asiatica* has been explored experimentally. Studies have shown an alcoholic extract to be tranquillising in rats, an activity that was attributed to a triterpene, brahmoside. Further studies showed the extract of *C. asiatica* leaf to be sedative, antidepressant and potentially cholinomimetic in vivo. These findings suggest that *C. asiatica* may be appropriate to treat symptoms of depression and anxiety in AD, and that it may also influence cholinergic activity, and thus cognitive function. Cognitive-enhancing effects have been observed in rats following oral administration of an aqueous extract of *C. asiatica*; this effect was associated with an antioxidant mechanism in the CNS (Melanie-Jayne and Peter, 2003).

2.2 Learning and memory

2.2.1 Learning

Learning is the acquisition and storage of information as a consequence of experience. It is measure by an increase in the likelihood of a particular behavioral response to a stimulus. Generally, rewards or punishment are crucial ingredients of learning. There are two types of learning: (1) nonassociative learning and (2) associative learning (Rhoades and Pflanzer, 2003)

2.2.1.1 Nonassociative learning

In nonassociative learning, we learn about a single type of stimulus and adapt to it according to its relevance to our desires or survival. This type of behavior, in which we learn to decrease our response to a repeated stimulus, is called habituation. In contrast, we can become more sensitized to certain other sounds. Sensitization is thus an increase in response to a stimulus (Rhoades and Pflanzer, 2003).

2.2.1.2 Associative learning

In associative learning, an animal acquires an understanding about the relationship or association between one stimulus and other. This learning process is called classical conditioning. An example of classical conditioning is the salivation of a conditioned dog in response to a stimulus of light. Normally, the dog salivates only in response to a piece of food. After a light stimulus (the conditioned stimulus, or CS) has

been paired with the presence of a piece of meat (the unconditioned stimulus, or UCS) in a sufficient number of conditioning trials, the light alone is sufficient to cause the dog to salivate (salivation is the conditioned response, or CR). The dog has been conditioned to salivate in response to light (Rhoades and Pflanzer, 2003).

2.2.2 Memory

Memory is the retention of learned information. We learn and remember lots of different things, and it is important to appreciate that these various things might not be processed and stored by the same neural hardware. No single brain structure or cellular mechanism accounts for all learning. Moreover the way in which information of a particular type is stored may change over time (Kupfermann, 1991).

Memory devides into 2 types, based on what kind of information is stored: (1) declarative memory and (2) nondeclarative memory (procedural memory)

2.2.2.1 Declarative memory

Declarative memory refers to the ability to remember names, faces, facts, events. Declarative memory depends on conscious reflection for its acquisition and recall, and it relies on cognitive process such as evaluation, comparison, and inference.

Declarative memory is comprised of semantic and episodic components. Semantic memory includes verbal information as well as pictorial images. Episodic

memory includes personal and autobiographical memories, such as one's home address or activities yesterday.

Declarative memory is often called "explicit memory" because its results from more conscious effort.

2.2.2.2 Nondeclarative memory

Nondeclarative (procedural) memory refers to the ability to learn perceptual-motor tasks such as mirror reading. Nondeclarative memory has an automatic or reflexive quality, and its information or readout is not independent on awareness, consciousness, or cognitive processes such as comparison and evaluation (Kupfermann, 1991).

Nondeclarative memory is also frequently called "implicit memory" because it results from direct experience.

2.2.3 Amnesia

Amnesia is a condition in which memory is disturbed. The causes of amnesia are organic or functional. Organic causes include damage to the brain, through trauma or disease, or use of certain (generally sedative) drugs. Functional causes are psychological factors, such as defense mechanisms. Hysterical post-traumatic amnesia is an example of this.

Amnesia can take 2 forms that are retrograde and anterograde. Retrograde amnesia refers to patients are unable to recall events occurring before the brain insult. Anterograde amnesia refers to new events are not transferred to long-term memory, so the sufferer will not be able to remember anything that occurs after the onset of this type of amnesia for more than a few moments. Both retrograde amnesia and anterograde amnesia can occur together in the same patient, and commonly result from damage to the brain regions most closely associated with episodic/declarative memory: the medial temporal lobes and especially the hippocampus (Devinsky and Arnold, 1992).

2.3 Cerebral ischemia model

The goal of cerebral ischemia model is to reduce oxygen and glucose supply to brain tissue. This process produces brain injury via a variety of cellular and molecular mechanisms that impair the energetics required to maintain ionic gradients. The mechanisms involve a complex series of pathophysiological events that are dependent on the severity, duration, and location of the ischemia within the brain (Traystman, 2003). Disturbances of the cerebral circulation have been associated with the decline of cognitive function in elderly subjects, as well as with the development of several types of dementia. The bulk of this evidence indicates that cerebral hypoperfusion may fail to satisfy the metabolic demands of neuronal tissue because of suboptimal delivery of vital nutrients to the brain. Owing to the inadequate energy supply, cognitive loss and memory deficits may develop (De Jong *et al.*, 1999).

Chronically reduced cerebral blood flow (CBF) can trigger the degeneration of the capillary ultrastructure in the brain. Creating a reduction of CBF in laboratory animals can test such a presumed sequence of events best. The bilateral occlusion of the common carotid arteries (two vessel occlusion, 2VO) is a well characterized method in rodents. The 2VO paradigm is frequently discussed in the context of Alzheimer's disease because of the apparent prevalence of cerebral hypoperfusion in the disease. The 2VO model stands for the visualization of the cerebrovascular and behavioral consequences of the reduced CBF, whatever its trigger may be (Farkas and Luiten,

2001). The functional changes usually observed after 2VO consisted of visuo-spatial memory impairment, hippocampal gliosis, mean hippocampal CBF reduction of 32%, microtubule associated protein-2 loss in the CA1 apical dendrites (a marker of protein synthesis and pre-synaptic activity), cytochrome oxidase decline in CA1 and posterior parietal cortex (a marker of neuronal energy activity), increased hemeoxygenase-1 expression (a marker of oxidative stress), and extracellular deposits of amyloid precursor protein, normally localized to neuronal cell membranes (De la Torre, 2000).

2.4 The Morris Water Maze (MWM)

Morris water maze was devised by Prof. Richard Morris about 20 years ago. It has become a very popular paradigm for the study of spatial learning and memory in rodents. The test apparatus consists of a round pool of water in which a platform is submerged just below the water's surface. Typically, an animal learns to escape from the water by locating the hidden platform with the help of visual cues around the pool. The relative simplicity of the MWM task is undoubtedly one of the reasons for its continuing success. The MWM task has often been used in the validation of rodent models for neurocognitive disorders and the evaluation of possible neurocognitive treatments. Through its many applications, MWM testing gained a position at the very core of contemporary neuroscience research (D' Hooge and De Deyn, 2001).

2.5 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 an elevated position (to a grid floor) or step into a narrow and apparently safer place (with a grid floor). Step-down 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).

2.6 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. Lipid peroxides are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, which is the most abundant malondialdehyde (MDA). Therefore, measurement of malondialdehyde is widely used as an indicator of lipid peroxidation. Markers of brain lipid peroxidation have been the most studied indexes of oxidant stress in AD.

Lipid peroxidation has been quantitatively assessed by measuring malondialdehyde (MDA) levels by thiobarbituric acid-reacting substances (TBARS) assay; lipid peroxides; aldehydes; and isoprostanes. 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).

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental animals

All experiments were performed on male ICR mice, six-weeks old, and weighing 25-30 g. All animals were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpatom, Thailand. Prior to test, mice were housed in the animal of the Faculty of Pharmaceutical Sciences, Chulalongkorn University and maintained on 12:12 light:dark cycle at controlled temperature (25±2^{o}C) for at least one week. Food and water were given *ad libitum*. 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.

3.2 Experimental instruments

1. pH meter (Beckman, U.K.)
2. Stopwatch (SEIKO)
3. Morris water maze set
4. Step down set
5. Locomotor activity apparatus (UGO Basile, Comerico, Italy)
6. Automatic micropipette (Pipet-Lite^{TM}, U.S.A.)
7. Automatic mixer (Vertex, U.S.A.)
8. Homogenizer (Glas-Col, Terre Haute, U.S.A.)
9. Centrifuge (Sorvll, GLC-2B, U.S.A.)
10. Spectrophotometer (Shimadzu, UV1201, Japan)
11. Cryostat (Leica, Germany)

3.3 Drugs and chemicals

1. Emblic myrobalan powder (Supplied by Associate Professor Dr. Ubonthip Nimmannit, Department Pharmacy , Faculty of Pharmaceutical Sciences , Chulalongkorn University)
2. Piracetam (Sigma, U.S.A.)
3. Normal saline solution (Thai Nakorn Patana Co.,ltd., Thailand)
4. Nembutal® (Sanofi-synthelabo, Thailand)
5. Sodium hydrogen phosphate-2-hydrate (Sigma, U.S.A.)
6. Sodium dihydrogen phosphate-2-hydrate (Sigma, U.S.A.)
7. Acetic acid (Sigma, U.S.A.)
8. Sodium dodecyl sulfate (Sigma, U.S.A.)
9. Thiobarbituric acid (Sigma, U.S.A.)
10. N-butanol (Sigma, U.S.A.)
11. Pyridine (Sigma, U.S.A.)
12. 1,1,3,3-Tetraethoxy-propane (Malondialdehyde) (Sigma, U.S.A.)
13. Cresyl violet (Sigma, U.S.A.)
14. Xylene (Sigma, U.S.A.)
15. Permount (Sigma, U.S.A.)
16. Ethanol 95% (GPO, Thailand)
17. Ethanol absolute (Merck, Germany)
18. Diethylether (Labscan Asia, Thailand)
19. Slide and cover glass (China)

3.4 Plant material and preparation of the extract

Emblic myrobalan fresh fruits were procured from Buriram Province, Thailand. The fresh fruits of emblic myrobalan were washed with running tap water and homogenized. The homogenization of the fruits were mixed with distilled water in ratio of 1:1. The whole mixture was crushed with hand and filtered. The filtrate was dried by

spray-dried method. A yellow powder product was stored at 25°C in air-tight container and protected from light.

3.5 Preparation and administration of the test compound

In the present study, emblic myrobalan extract was dissolved in vehicle (distilled water) and given orally to mice once daily at doses of 100, 300 and 1000 mg/kg B.W. Piracetam was dissolved in distilled water and was intraperitoneally injected at a dose of 100 mg/kg B.W. in 2VO model. The control animals in 2VO model were orally administered with vehicle (distilled water).

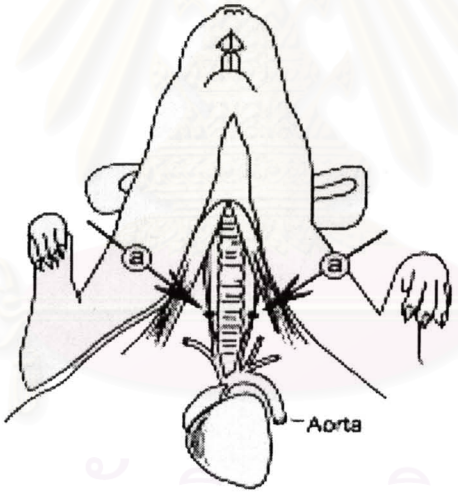
3.6 Experimental methods

3.6.1 Experimental design

Mice were subjected to cerebral ischemia induced by 2VO plus hypotension (Figure 3.1). In brief, the mice were anesthetized with sodium pentobarbital (Nembutal sodium solution, 60 mg/kg B.W., intraperitoneal injection). Under deep anesthesia, the neck skin of mice was vertically incised. The common carotid arteries were exposed and carefully separated from the adjacent veins and vagus nerves, and then occluded by arterial 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°C. Sham-operated mice were subjected to the same procedure without carotid artery clamping and bleeding. After 24 h, the following MWM were carried out (Xu *et al.*,2000; Watanabe *et al.*,2003).

To study the effects of emblic myrobalan extract (*Phyllanthus emblica*) on impairment of learning and memory induced by cerebral ischemia, six groups of animals were used. One group of sham-operated animals (n=13) and one group of 2VO animals (n=13) were administered with vehicle. Three groups of 2VO animals (n=13 per group)

were administered with the extracts of emblic myrobalan at the dose of 100, 300 and 1000 mg/kg B.W. The last group of 2V0 animals (n=8) were administered with piracetam at the dose of 100 mg/kg B.W. (positive control). Vehicle and emblic myrobalan extract were orally administered while piracetam was intraperitoneal injection for 8 consecutive days. In each group of animals three behavioral tests were performed, MWM test, step-down test and spontaneous locomotor activity test. MWM test was tested for 5 consecutive days after 2VO. Six days after 2VO the step-down test was performed for 2 days and followed with spontaneous locomotor activity test in the last day. Following three behavioral tests, the animals were sacrificed for an estimation of lipid peroxidation (n=8 in each group of animals) and histology study (n=5 in each group of animals) (Bejar, Wang and Weinstock, 1999).



2VO

Figure 3.1 Experimental mice model of cerebral ischemia (bilateral common carotid artery occlusion, two vessel occlusion: 2VO); (a) the common carotid arteries

3.6.2 Behavioral tests

3.6.2.1 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 3.2), painting with black color, which was 70 cm in diameter and depth of 13 cm of water with temperature maintained of 25±1°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. The pool was placed in a large test room, and surrounded by various visual cues in a fix position. Daily swimming consisted of four trials in which the mice were placed in the water from 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 for 15 sec (Watanabe *et al*, 2003).

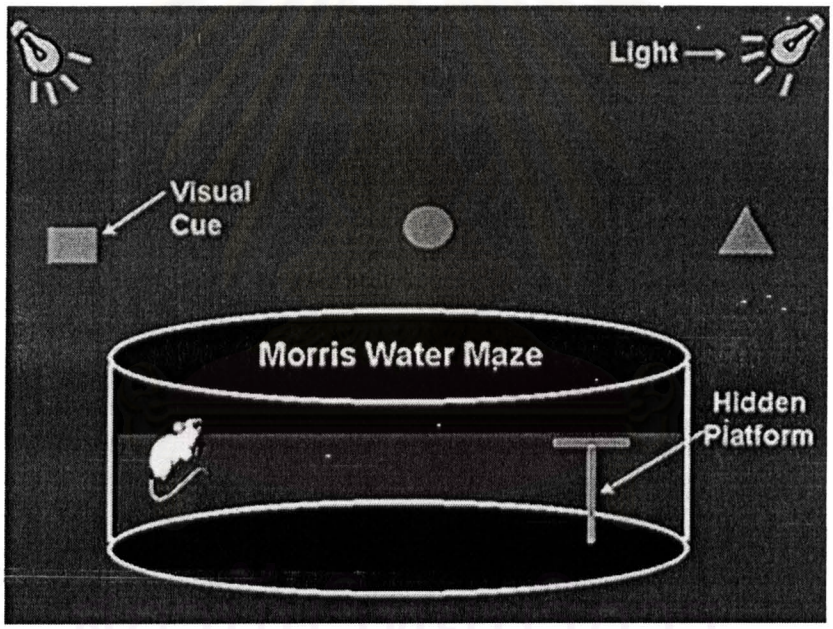
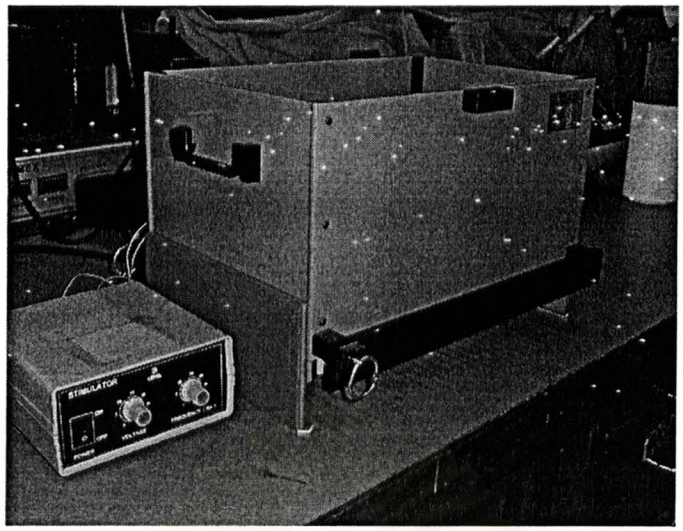


Figure 3.2 Equipment for Morris water maze test

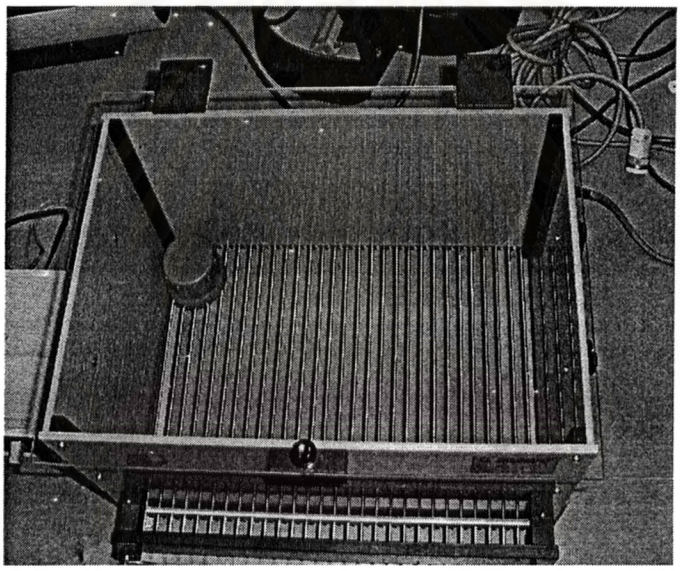
3.6.2.2 Step-down test

A step-down passive avoidance was examined using apparatus consisted of plexiglass chamber (Figure 3.3). 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 plastic platform (5 cm diameter, 4 cm height) set on the 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. Mouse was placed on the platform to get adapted to environment for 3 min without electric shock. Then electric shocks were delivered to the grid when the mouse stepped down from the platform. The cut-off time was 5 min. After 24 h of training, mouse was placed on the platform for retention test. The electric shocks were still delivered for 5 min. Step-down latency and number of errors were recorded. The time (step-down latency) that elapsed until the mouse stepped down from the platform was recorded. If the mouse did not step down from the platform within 300 sec, the retention test was terminated and the maximal step-down latency of 300 sec was recorded. An error was counted whenever the mouse stepped down from the platform and the number of errors made in 5 min was recorded (Luo, Yin and Wei, 2003).



a. side view



b. top view

Figure 3.3 Equipment for Step-down test

3.6.2.3 Spontaneous locomotor activity test

Each animal was placed in an activity cage consisting of plexiglass chamber and counting device (Figure 3.4). 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).

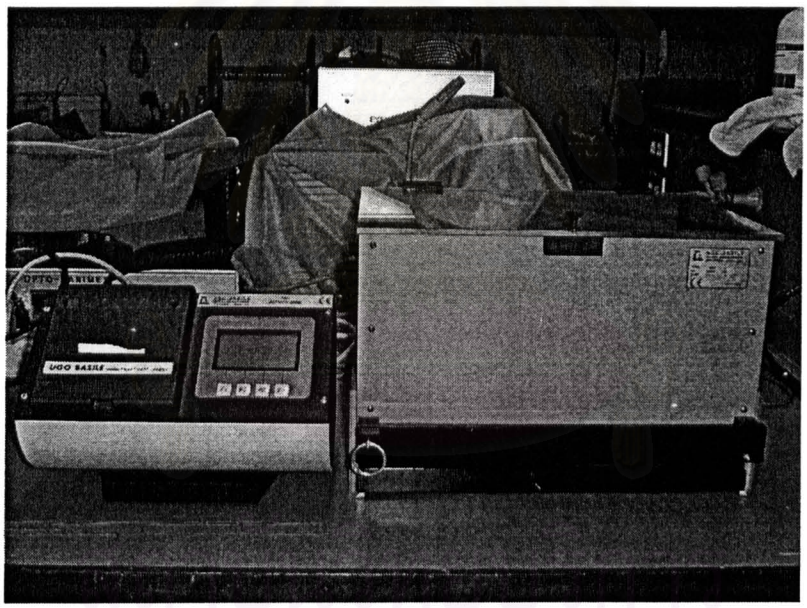


Figure 3.4 Activity meter

3.6.3 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°C.

3.6.3.1 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 from mice brain were separated and used to determine the marker of oxidative stress (malondialdehyde).

3.6.3.2 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^{o}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 were 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 nmol/g tissue (Gupta *et al.*, 2003).

3.6.4 Histological examination

After the behavioral testing, five animals per each group were used to investigate neuronal damage in hippocampal formation (CA1 and CA3) with Cresyl violet staining method. Animals were decapitated. The whole brains were removed and quickly frozen in dry ice. Coronal sections (10 μm thick) were taken at the level of hippocampus (approximately 1.5 mm caudal to the bregma) by using a cryostat and stained with 1% Cresyl violet for the microscopic observation. Photograph (x40) of the CA1 and CA3 subfields of the hippocampus were taken and then the numbers of surviving pyramidal neurons (the neuron with a distinct nucleus) per 0.068 mm^2 in CA1 and CA3 subfields were counted in a selective manner. Average surviving cell numbers were counted over consistent fields, over both hemispheres and over three sections in each brain. The degree of surviving pyramidal neurons at the hippocampal CA1 or CA3

areas (Figure 3.5) were expressed as the density of surviving CA1 or CA3 pyramidal cells / the area of CA1 or CA3 region (0.068mm^2) (Ni *et al.*, 1995;Nanri *et al.*, 1998).

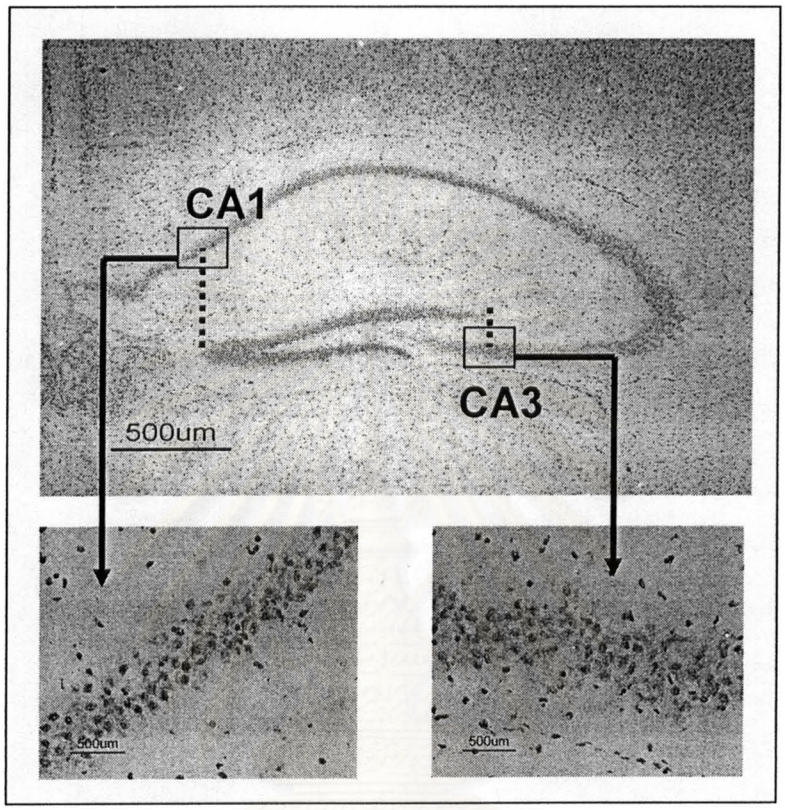


Figure 3.5 Selection area of CA1 and CA3 in hippocampus

3.7 Data analysis

All data are expressed as the mean value for the group $\pm$ standard error of mean (SEM). Statistical analyses were performed by one-way ANOVA and Duncan post-hoc test for planned comparisons between control versus different treatment groups. A significance value of $P < 0.05$ was considered as statistically significant.

CHAPTER IV

RESULTS

4.1 Effects of emblic myrobalan extract on impairment of learning and memory induced by cerebral ischemia.

4.1.1 Effects of 2VO on spatial learning and memory performance.

The MWM performance in 2VO and sham-operated mice as measured by latency to reach the hidden platform during 5 consecutive days was summarized in Figure 4.1. The mean search time to find the platform on the training day between those of 2VO (33 ± 3.03sec) and sham-operated mice (35 ± 5.28sec) did not differ. During days 1-5, mice subjected to 2VO required a longer time to locate the hidden platform than sham-operated mice. The escape latency was significantly delayed in 2VO mice as compared to the sham-operated mice. The escape latencies of 2VO and sham-operated mice on day 5 were 30 ± 1.42 and 5 ± 0.66 sec, respectively.

4.1.2 Effects of emblic myrobalan extract on spatial learning and memory performance in 2VO mice.

After 24 h of cerebral ischemia, the MWM test was performed. emblic myrobalan extract was orally given to animals 1 h before testing. Administration of emblic myrobalan extract at doses 100, 300 and 1000 mg/kg B.W. and piracetam at dose 100 mg/kg B.W. attenuated the memory deficits in 2VO mice. The escape latency of emblic myrobalan extract-treated mice was shorter than that of the vehicle-treated mice. All dose of emblic myrobalan extract significantly improved learning and memory performance on day 4 and day 5. On day 5, the escape latencies of emblic myrobalan extract-treated mice at doses of 100, 300 and 1000 mg/kg B.W. were 9 ± 1.33, 14 ± 2.59 and 18 ± 4.54 sec, respectively, whereas it was 7 ± 0.90 sec in piracetam-treated group (Figure 4.2).

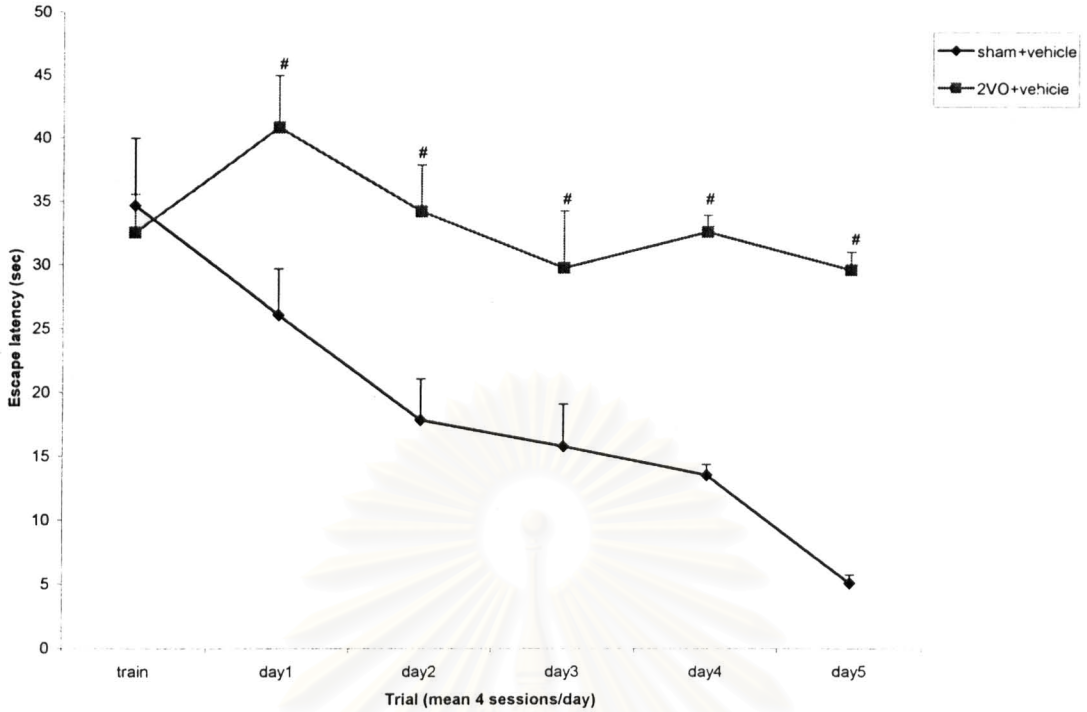


Figure 4.1 : The MWM performance of 2VO and sham-operated mice (subset of data from Figure 4.2). The escaping latency onto the platform was measured during 5 consecutive days. Each data point represents the mean ± S.E.M. (n=13) of four trials. A significant level of $P < 0.05$ was considered as a significant difference.

[#] Significantly different from values in sham-operated mice

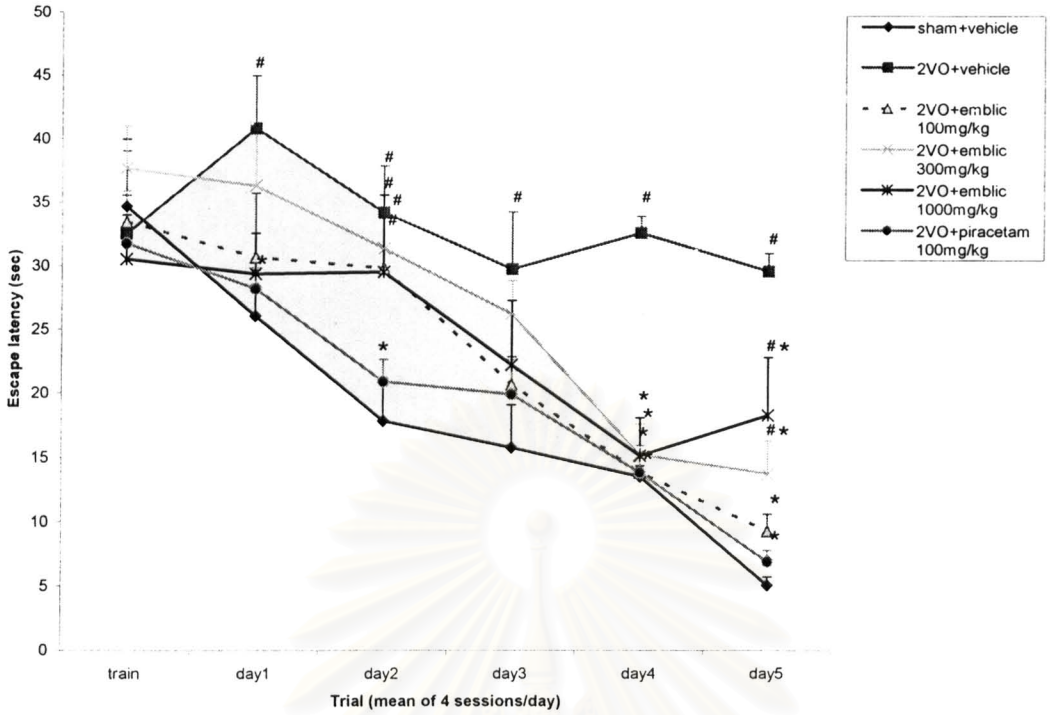


Figure 4.2 : Effects of emblic myrobalan extract on spatial learning and memory performance in 2VO mice. Mice were orally given with vehicle or emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg B.W. or piracetam at dose of 100 mg/kg B.W., once daily. The escaping latency onto the platform was measured during 5 consecutive days. Each data point represents the mean ± S.E.M. (n=13) of four trials. A significant level of $P < 0.05$ was considered as a significant difference.

Significantly different from values in sham-operated mice

* Significantly different from values in 2VO mice

4.1.3 Effect of 2VO on step-down passive avoidance.

Impairment of learning and memory in step-down was observed after cerebral ischemia. As shown in Figure 4.3, 2VO caused a significant reduction in step-down latency and increased the step-down errors on retention Trial. Step-down latencies were 181 ± 18.21 and 29 ± 3.48 sec in sham- and 2VO- operated mice, respectively. Numbers of errors were 2 ± 0.36 and 6 ± 0.73 sec in sham- and 2VO- operated mice, respectively.

4.1.4 Effects of emblic myrobalan extract on step-down passive avoidance in 2VO mice.

Similar to piracetam (100 mg/kg B.W.), treatment with emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg B.W., significantly reversed the reduction in step-down latency and decreased the step-down errors of 2VO mice (Figure 4.4). Step-down latencies of emblic myrobalan-treated mice at doses of 100, 300 and 1000 mg/kg B.W. were 150 ± 7.77, 148 ± 12.66 and 141 ± 9.19 sec, respectively. Numbers of errors of emblic myrobalan-treated mice at doses of 100, 300 and 1000 mg/kg B.W. were 3 ± 0.29, 2 ± 0.35 and 3 ± 0.38 sec, respectively. In piracetam-treated group, step-down latency and number of errors were 174 ± 9.28 and 2 ± 0.35 sec, respectively.

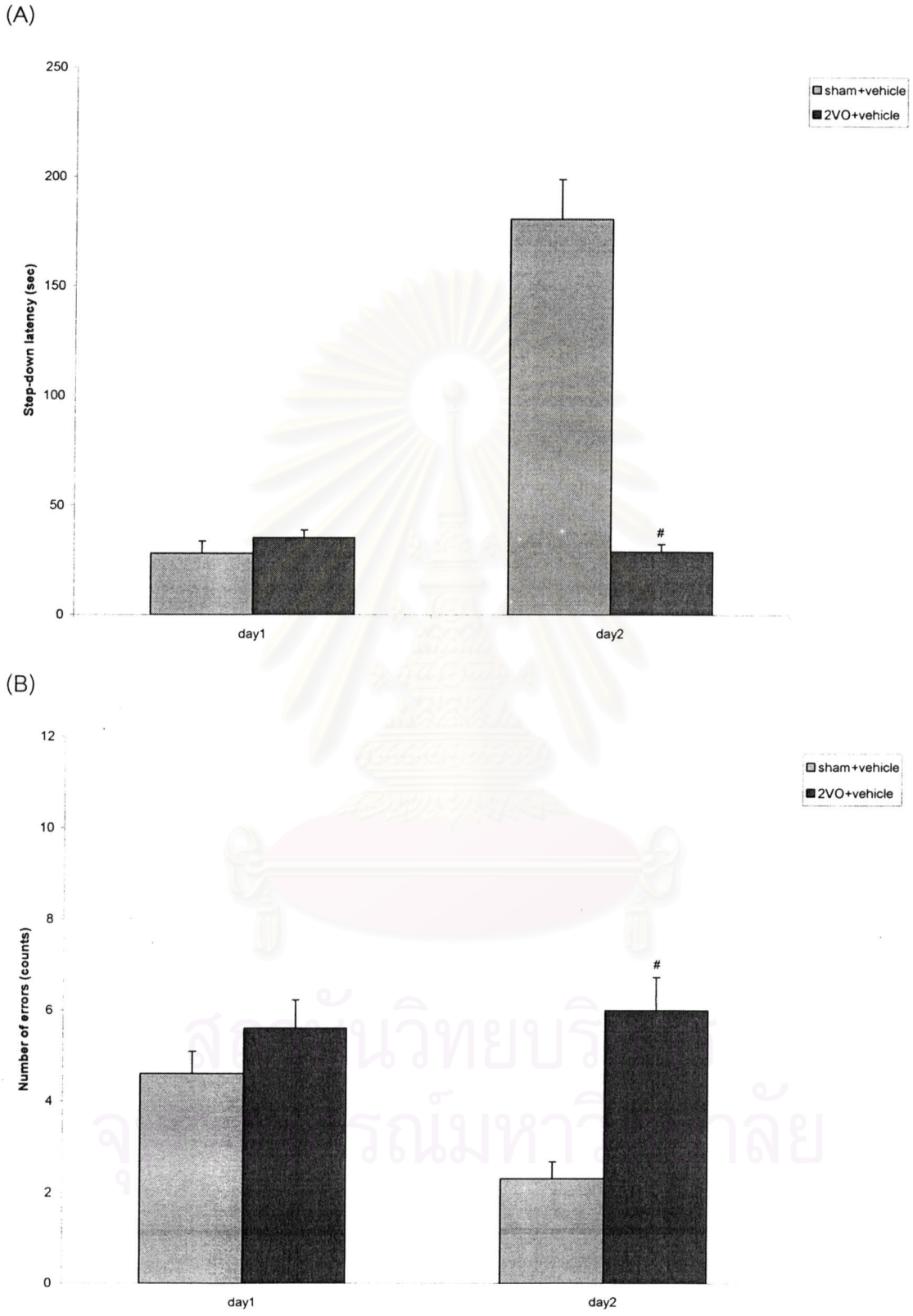


Figure 4.3 : Step-down latency and number of errors in 2VO and sham-operated mice on (subset of data from Figure 4.4). Step-down latency (A) and number of errors (B) were expressed as the mean ± S.E.M. (n=13). A significant level of $P < 0.05$ was considered as a significant difference. # Significantly different from values in sham-operated mice

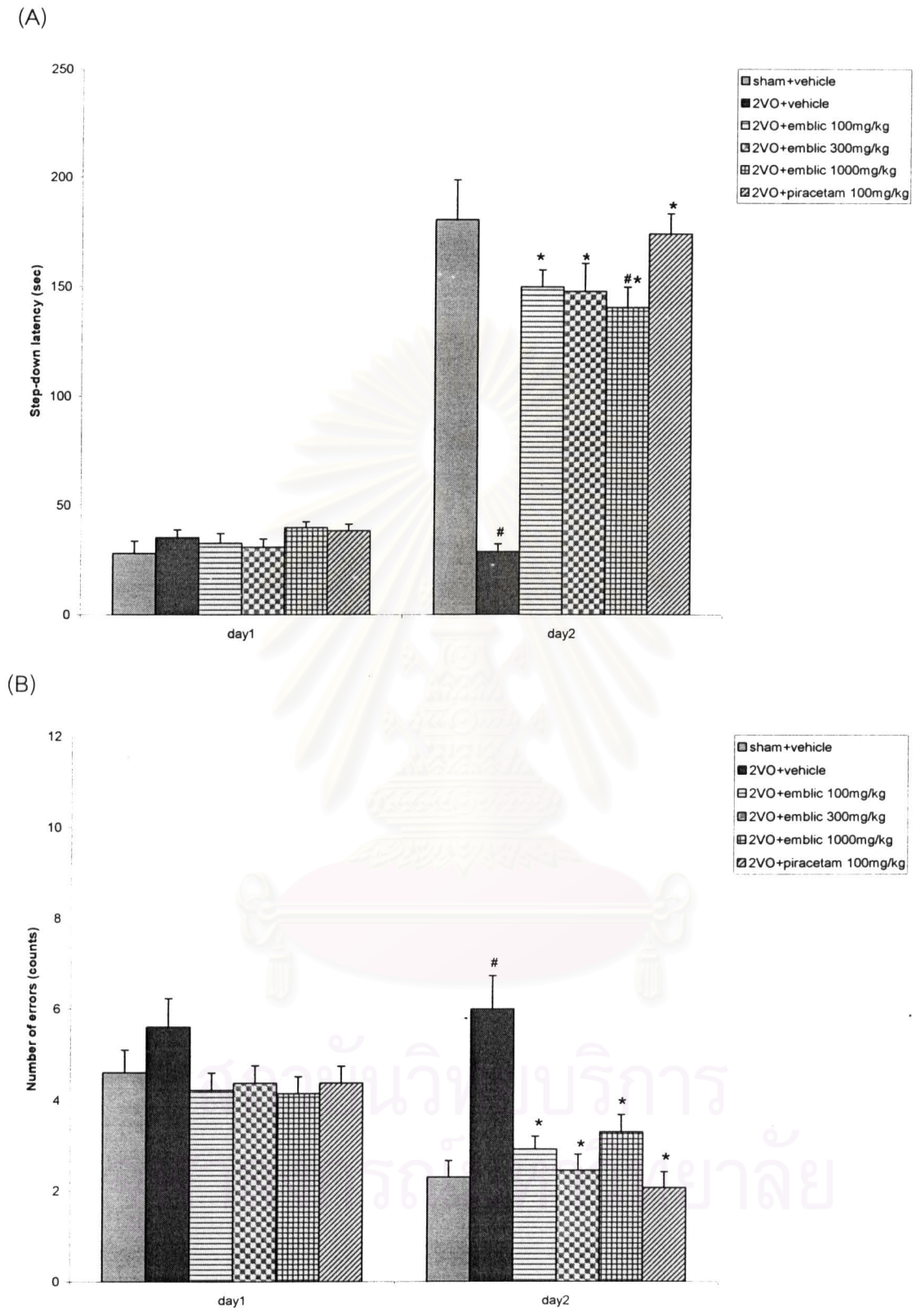


Figure 4.4 : Effects of emblic myrobalan extract on step-down passive avoidance in 2VO mice. Mice were orally given with vehicle and emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg B.W. or piracetam at dose of 100 mg/kg B.W., once daily. Step-down latency (A) and number of errors (B) were expressed as the mean ± S.E.M. (n=13). A significant level of $P < 0.05$ was considered as a significant difference.

Significantly different from values in sham-operated mice * Significantly different from values in 2VO mice

4.1.5 Effects of 2VO on spontaneous locomotor activity.

The spontaneous locomotor activity, measured as movement counting during 5 minutes test period, in 2VO and sham-operated mice was shown in Figure 4.5. The spontaneous locomotor activity did not differ between 2VO and sham-operated mice.

4.1.6 Effects of emblic myrobalan extract on spontaneous locomotor activity in 2VO mice.

Oral administration of emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg B.W as well as admistration of piracetam had no effect on spontaneous locomotor activity in 2VO mice. The results were shown in Figure 4.5.

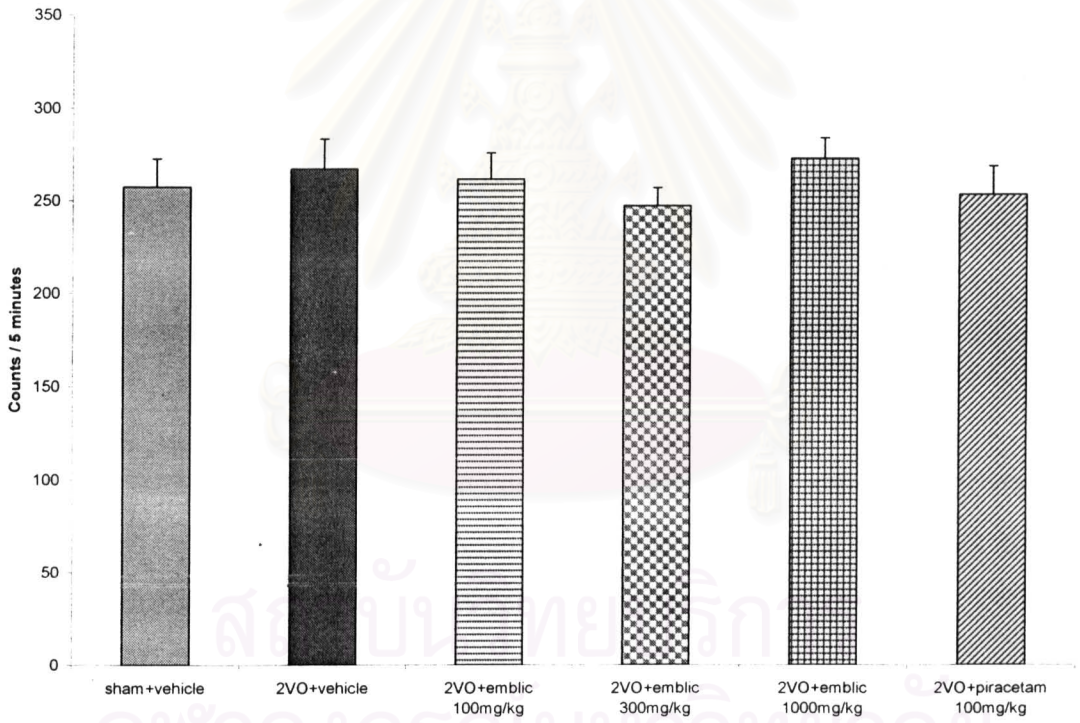


Figure 4.5 : Effects of emblic myrobalan extract on spontaneous locomotor activity in 2VO mice. Mice were orally administered with vehicle, emblic myrobalan extract at doses 100, 300 and 1000 mg/kg B.W. or piracetam at dose of 100 mg/kg B.W. The registered numbers or counts of movements were recorded at 5 min intervals and expressed as the mean ± S.E.M. (n=13). A significant level of $P < 0.05$ was considered as a significant difference.

4.1.7 Effects of 2VO on lipid peroxidation.

In the 2VO mice was observed significant increase in MDA levels as compared to the sham-operated mice. MDA levels of 2VO and sham-operated mice were 70 ± 3.92 and 24 ± 2.7 nmol/g tissue, respectively (Figure 4.6).

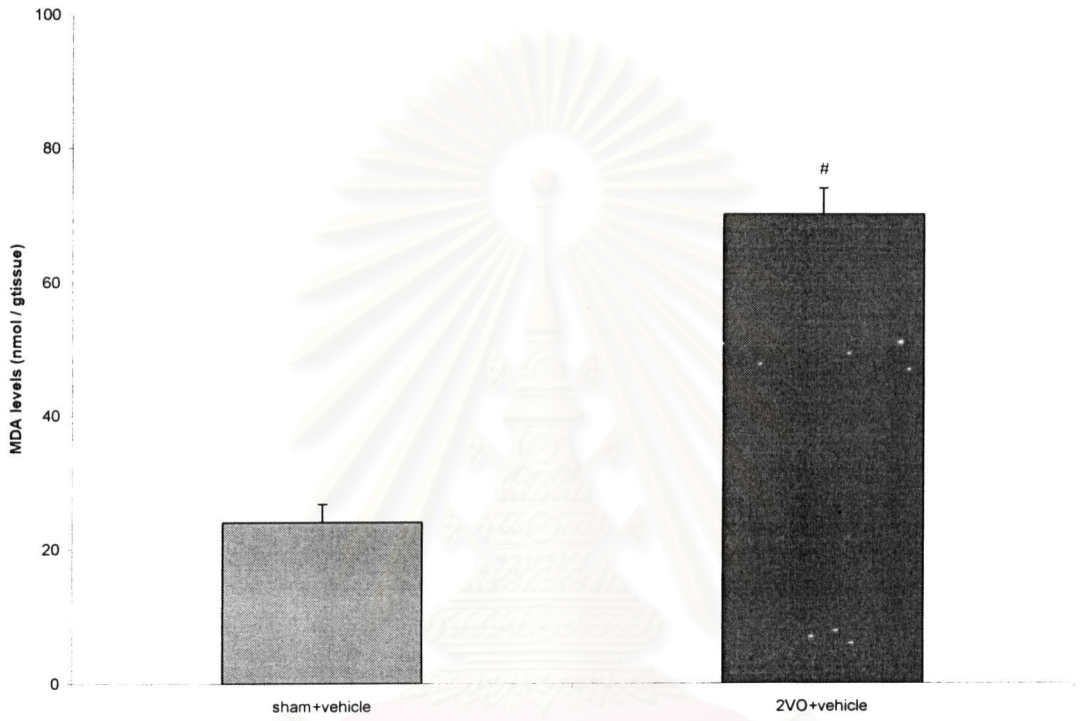


Figure 4.6 : MDA levels in brain of 2VO and sham-operated mice (subset of data from Figure 4.7). The concentration of MDA is expressed as nmol/g tissue (mean ± S.E.M : n=8). A significant level of $P < 0.05$ was considered as a significant difference.

Significantly different from values in sham-operated mice

4.1.8 Effects of emblic myrobalan extract on lipid peroxidation in 2VO mice.

The effects of emblic myrobalan extract on lipid peroxidation in 2VO mice were shown in Figure 4.7. There was a significant decrease in 2VO induced-increment of MDA levels in both emblic myrobalan- and piracetam-treated group when compared to the 2VO mice. Administration of emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg B.W. markedly reduced MDA levels to 36 ± 2.87, 54 ± 3.41 and 50 ± 4.91 nmol/g tissue, respectively. In piracetam-treated mice, MDA level was reduced to 22± 2.37 nmol/g tissue.

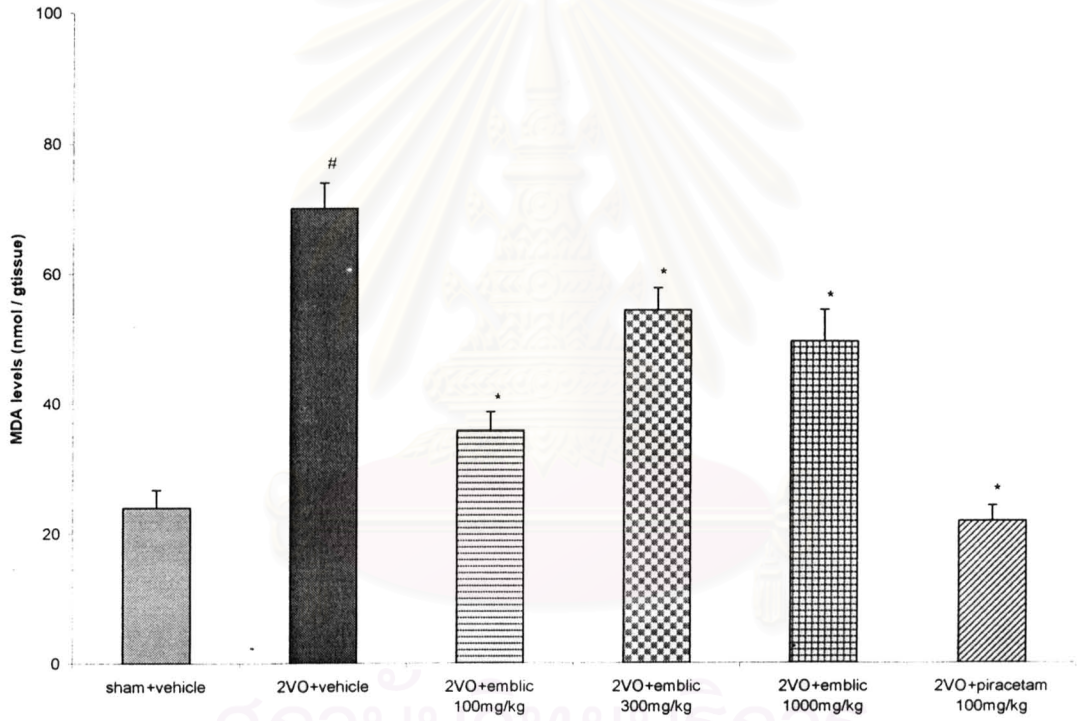


Figure 4.7 : Effects of emblic myrobalan extract on MDA levels in brains of 2VO mice. Mice were orally administered with vehicle, emblic myrobalan extract at doses 100, 300 and 1000 mg/kg B.W. or piracetam at dose of 100 mg/kg B.W. The concentration of MDA is expressed as nmol/g tissue (mean ± S.E.M : n=8). A significant level of $P < 0.05$ was considered as a significant difference.

Significantly different from values in sham-operated mice

* Significantly different from values in 2VO mice

4.2 Effect of emblic myrobalan extract on survival CA1 and CA3 pyramidal neurons induced by cerebral ischemia.

4.2.1 Effect of 2VO on survival CA1 and CA3 pyramidal neurons in mice.

To determine the relative survival of pyramidal neurons in the CA1 and CA3 regions after cerebral ischemia, an average surviving cell numbers were counted over consistent fields, over both hemisphere, and over three sections in each brain.

Obvious cell loss was noted in 2VO mice (Figure 4.8). Histological examination of the specimen changes observed in CA1 and CA3 regions of the hippocampus. No histological lesion was shown in sham-operated mice. Neuronal loss, shrinkage and dark staining of neurons were observed in both CA1 and CA3 regions of the hippocampus in 2VO mice. In figure 4.10, the number of pyramidal neurons in both regions of both sides was gradually decreased in 2VO mice.

4.2.2 Effect of emblic myrobalan extract on survival CA1 and CA3 pyramidal neurons in 2VO mice.

As seen in Figure 4.9, similar to piracetam (100mg/kg B.W.), treatment with emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg B.W., p.o., attenuated neuronal damage cause by cerebral ischemia. All dose of emblic myrobalan extract significantly attenuated neuronal damage in both side of CA1 and CA3, number of neuronal survival cells in CA1 at doses of 100,300 and 1000 mg/kg B.W. were 73 ± 2.88, 69 ± 2.58, 71 ± 3.71 in left side, 73 ± 3.44, 69 ± 2.44, 71 ± 2.71 in right side, respectively. Number of neuronal survival cells in CA3 at doses of 100,300 and 1000 mg/kg B.W. were 71 ± 1.76, 67 ± 1.57, 67 ± 2.62 in left side, 71 ± 1.29, 66 ± 1.66, 67 ± 2.07 in right side, respectively. Whereas piracetam treated group attenuated neuronal damage in both side of CA1 and CA3, number of neuronal survival cells in CA1 were 80 ± 0.99 in left side and 75 ± 2.33 in right side, in CA3 were 80 ± 1.24 in left side and 75 ± 1.39 in right side.

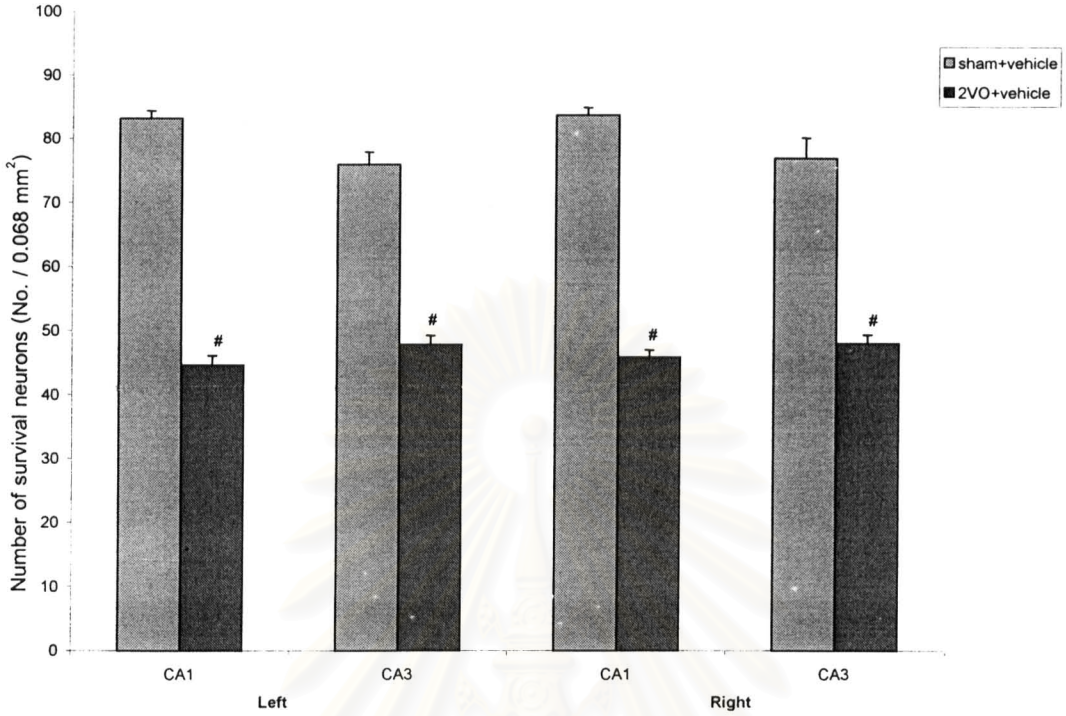


Figure 4.8 : Number of survival CA1 and CA3 pyramidal neurons in 2VO and sham-operated mice (subset of data from Figure 4.9). The number of survival CA1 and CA3 hippocampal pyramidal neurons were expressed as the mean ± S.E.M. (n=5). A significant level of $P < 0.05$ was considered as a significant difference.

Significantly different from values in sham-operated mice

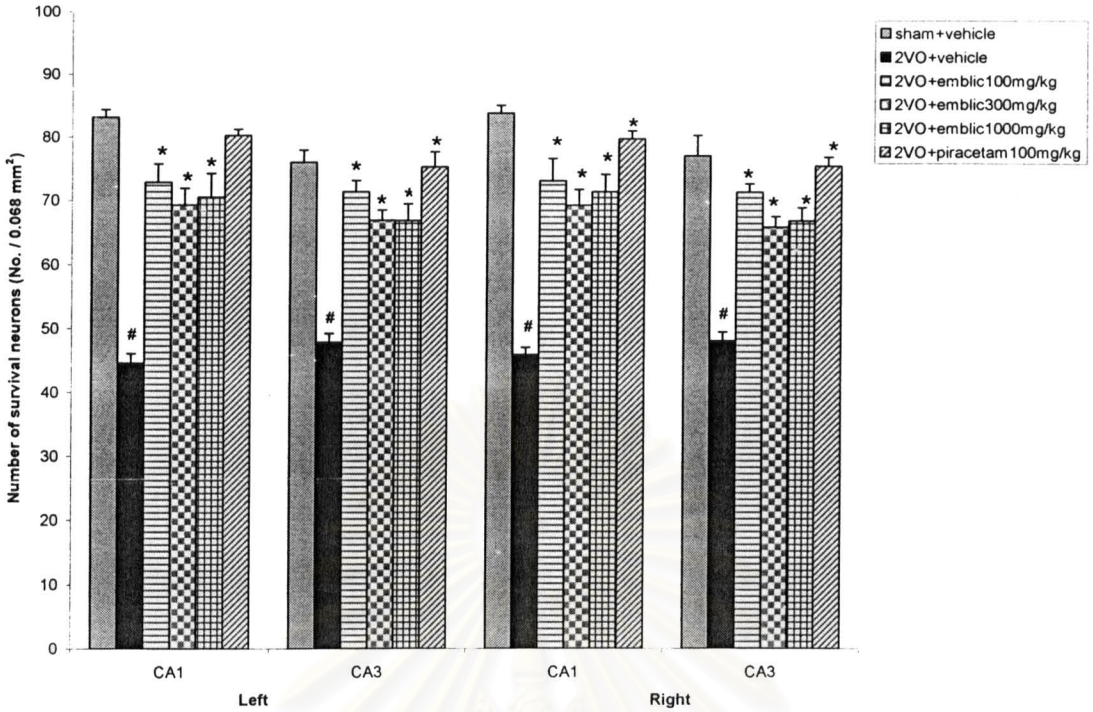


Figure 4.9 : Effects of emblic myrobalan extract on survival CA1 and CA3 pyramidal neurons in 2VO mice. Mice received vehicle, emblic myrobalan extract at doses of 100, 300 and 1000 mg/kg B.W. or piracetam at dose 100 mg/kg B.W. The number of survival CA1 and CA3 hippocampal pyramidal neurons were expressed as the mean ± S.E.M. (n=5). A significant level of $P < 0.05$ was considered as a significant difference.

Significantly different from values in sham-operated mice

* Significantly different from values in 2VO mice

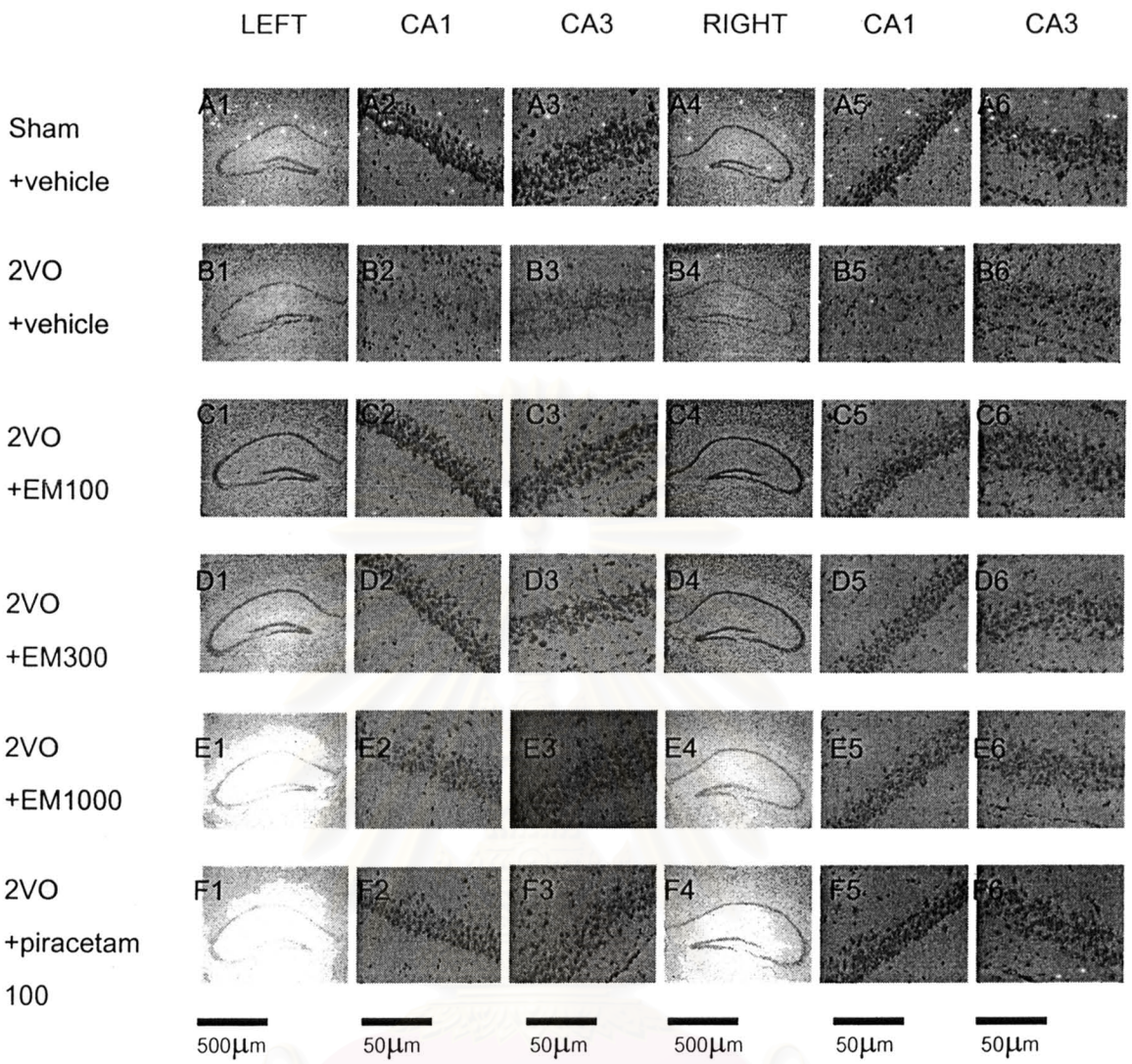


Figure 4.10 : Nissl staining with cresyl violet of CA1 and CA3 neurons in transverse left and right hippocampal slices. A1-A6 in sham+vehicle, B1-B6 in 2VO+vehicle, C1-C6 in 2VO+EM 100mg/kg B.W., D1-D6 in 2VO+EM 300mg/kg B.W., E1-E6 in 2VO+EM 1000mg/kg B.W. and F1-F6 in 2VO+piracetam treated group. Scale bar are 500 μm for colume1 and 4 and 50 μm for colume 2,3,5 and 6.

CHAPTER V

DISCUSSION AND CONCLUSION

In traditional practices of Ayurvedic and Chinese medicine, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases. An ethnopharmacological approach has provided leads to identifying potential new drugs from plant sources, including those for cognitive disorders. Many drugs currently available in western medicine were originally isolated from plants, or are derived from templates of compounds isolated from plants.

There are various pharmacological activities of *Phyllanthus emblica*. One of these is an antioxidant activity. This activity may associate with improving learning and memory. In the present study, emblic myrobalan (*Phyllanthus emblica*) extract was evaluated in mice for its effect on learning and memory impairment induced by cerebral ischemia. In addition, the effect of *Phyllanthus emblica* on lipid peroxidation and histology examination were also performed.

The development of animal models of ischemia induced amnesia is vital to the analysis of the functional consequences of ischemic damage and to test the behavioral efficacy of potentially therapeutic drugs (Xu *et al.*, 2000). Transient cerebral ischemia provoked by bilateral common carotid artery occlusion or two-vessel occlusion (2VO) is a well known procedure to induce global and extensive brain injury and neuronal damage (Pulsinelli and Brierley, 1979).

In this study, mice were subjected 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. After 2VO procedure, we found that mice with transient cerebral ischemia showed an increasing in escape latencies in MWM but a decreasing in step-down latency while the number of errors was increased. These observations indicate that transient cerebral ischemia impaired spatial memory (MWM task) and passive avoidance task which concur with

previous report (Itoh, Ukai, and Kameyama, 1993; Yamamoto *et al.*, 1993; Hirakawa *et al.*, 1994; Olsen *et al.*, 1994; Li *et al.*, 1999). In addition we found that 2VO had no effect on locomotor activity test indicating the memory deficit induced by 2VO did not involve motor system.

Previous report found that pirecetam significantly improved the impaired learning performance of 2VO mice in the radial maze task, indicating the ameliorative effect of piracetam on the spatial working memory impairment induced by chronic cerebral hypoperfusion (Kumar *et al.*, 2000). In the present study, we used piracetam as a positive control and it was shown to improve the memory deficit induced by transient cerebral ischemia in MWM and passive avoidance tasks.

Like piracetam, oral administration of emblic myrobalan extract at the dose of 100, 300 and 1000 mg/kg B.W. significantly and similarly improved 2VO-induced deficit in learning and memory observed in MWM and step-down tests while no alteration in motor activity was noted. This finding implied that beneficial effects of emblic myrobalan extract on impairment of learning and memory were unlikely to involve the improvement of motor function.

Free radicals are highly reactive molecules implicated in the pathology of traumatic brain injury and cerebral ischemia, through a mechanism known as oxidative stress. After brain injury, reactive oxygen and reactive nitrogen species may be generated through several different cellular pathways by inflammatory cells. If cellular defense systems are weakened, increased production of free radicals will lead to oxidation of lipids which may alter cellular function in a critical way (Lewen, Matz and Chan, 2000). Potential antioxidant therapy should therefore, include either natural antioxidant enzymes or agents, which are capable of augmenting the functions of these enzymes (Kumar and Gupta, 2002). Earlier reports have shown that the natural drugs like *Ginkgo biloba* (Sastre *et al.*, 1998; Christen *et al.*, 2000) and *Withania somnifera* (Bhatacharya *et al.*, 1997) which improves cognition also shown to have antioxidant properties. Therefore, the different doses of emblic myrobalan extract (100, 300 and

1000 mg/kg B.W.), which showed improvement in learning and memory paradigms were further tested on the oxidative stress parameter (levels of MDA) in brain of mice.

In the present study, we found that the levels of MDA in 2VO mice were significantly increased when compared to those of sham-operated mice. This finding is in line with the previous report that bilateral common carotid artery occlusion followed by reperfusion generated reactive oxygen species (ROS) (Sorrenti *et al.*, 1994; Nakashima *et al.*, 1999). Excessive generation of ROS results in the lipid peroxidation of the cell membrane and subsequent damage is reflected by accumulation of MDA, a byproduct of lipid peroxidation (Halliwell, 1991). Oral administration of emblic myrobalan extract at dose of 100, 300 and 1000 mg/kg B.W. showed a significant decrease in the MDA levels of the brain indicating attenuation of lipid peroxidation. These results could probably be explained by the finding that *Emblica officinalis* (emblic myrobalan) may increase the activities of superoxide dismutase, catalase and glutathione peroxidase (endogenous antioxidant defense enzymes), with a concomitant decrease in lipid peroxidation in different animal models (Bhattacharya *et al.*, 2002). As an increase in MDA was noted in 2VO mice which exhibited impairment of spatial learning and memory in MWM and passive avoidance tasks which could be improved by the administration of emblic myrobalan extract, it is suggestive that antioxidant property exerted by emblic myrobalan extract could contribute to its beneficial effect on impairment of learning and memory.

In experimental animals, this global cerebral ischemia may be induced by permanent occlusion of both vertebral arteries and transient occlusion of both carotid arteries (in rats) or by transient occlusion of both carotid arteries (in gerbils), which in both cases led to selective cell death of the CA1 pyramidal hippocampal neurons (Block and Schwarz, 1997). In addition, the pyramidal CA1 neurons of the hippocampus are known to be essential for the formation of spatial memory. Hence, deletion of these neurons in rodents leads to severe deficits in spatial learning and memory. The CA1 neurons are especially vulnerable to global cerebral ischemic insults, and undergo selective delayed neurodegeneration in various models inducing transient global

ischemia (Nelson *et al.*, 1997; Euler *et al.*, 2005). Because of the brain's large amount of oxidizable unsaturated fatty acids, which are especially sensitive to free radical-induced lipid peroxidation, and relatively low activities of antioxidative enzymes including SOD, GSH-Px and CAT, it is very vulnerable to ROS induced by ischemia–reperfusion, which cause oxidative damage to brain lipids, proteins, and DNA, leading to brain dysfunction and cell death (Zhan and Yang, 2006). The present study investigated the brain pathology after cerebral ischemia. We found that the survival cell in CA1 and CA3 regions in 2VO mice were significantly decreased when compared to those of sham-operated mice. Furthermore, number of survival neuron in CA1 and CA3 of hippocampus seems to be higher than those observed in 2VO mice receiving emblic myrobalan extract at the doses of 100, 300 and 1000 mg/kg B.W. Similar profile of responses seen on both the number of survival neurons CA1 and CA3 of hippocampus and lipid peroxidation suggest a strong relationship.

In conclusion the present study has demonstrated the beneficial effects of emblic myrobalan extract on learning and memory impairment in 2VO model. Based on the finding that 2VO significantly increased the lipid peroxidation which could be ameliorated by emblic myrobalan extract, it is possible that antioxidant property of emblic myrobalan extract could, at least partly, contribute to its positive effect on memory deficit in 2VO mice. Protective effect of emblic myrobalan on neurons in CA1 and CA3, which were responsible for spatial memory, from oxidative stress induced by 2VO could subsequently attributed to an improvement of memory deficit assessed by MWM and step-down test. Though further investigations are needed to clarify the mechanism of action of emblic myrobalan extract in details, the present study has clearly demonstrated the potential of emblic myrobalan extract to be further developed for dementia.

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