ผลของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ต่อความบกพร่องของการเรียนรู้และความจำที่ถูกเหนี่ยวนำโดยเปปไทด์เบตาอะมัยลอยด์ 25-35 ในหนูเมาส์

นางสาวอัญชลี คำเอก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON COGNITIVE DEFICITS INDUCED BY β-AMYLOID PEPTIDE (25-35) IN MOUSE

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Thesis Title	EFFECTS OF THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON COGNITIVE DEFICITS INDUCED BY β-AMYLOID PEPTIDE (25-35) IN MOUSE
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อัญชลี คำเอก : ผลของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ต่อความบกพร่องของการเรียนรู้และความจำที่ถูกเหนี่ยวนำโดยเปปไทด์เบตาอะมัยลอยด์ 25-35 ในหนูเมาส์. (EFFECTS OF THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON COGNITIVE DEFICITS INDUCED BY β-AMYLOID PEPTIDE (25-35) IN MOUSE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. มยุรี ตันติสิระ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. บุญยงค์ ตันติสิระ, 90 หน้า.

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ต่อความบกพร่องของการเรียนรู้และความจำ ที่ถูกเหนี่ยวนำด้วยการฉีดเปปไทด์เบตาอะมัยลอยด์ ($A\beta_{25-35}$) ขนาด 9 นาโนโมล เข้าสู่ช่องว่างในสมองของหนูเมาส์ เพศผู้ น้ำหนัก 25-35 กรัม หลังจากฉีดเปปไทด์เบตาอะมัยลอยด์ 7 วัน จะทำการทดสอบพฤติกรรมด้านสติปัญญา ได้แก่ Morris water maze, step-down และ locomotor activity นอกจากนั้นในวันที่ 15 หลังจากฉีดเปปไทด์เบตาอะมัยลอยด์ จะวิเคราะห์หาระดับ MDA ในสมองและประเมินดูความหนาแน่นของเซลล์บริเวณ CA1 และ CA3 ของสมองส่วนฮิปโปแคมปัส จากการวิจัย พบว่าหนูที่ฉีดเปปไทด์เบตาอะมัยลอยด์ จะเกิดความบกพร่องของการเรียนรู้และความจำ มีระดับ MDA ในสมองเพิ่มขึ้นและเซลล์ประสาทบริเวณ CA1 และ CA3 ของสมองส่วนฮิปโปแคมปัสถูกทำลาย อย่างมีนัยสำคัญ ($p < 0.05$) เมื่อเทียบกับกลุ่มควบคุม ได้แบ่งการทดลองออกเป็น 2 แบบ ในแบบแรกจะเริ่มต้นให้สารสกัดมาตรฐานบัวบก อีซีเอ 233 ในขนาด 10, 30 มก. ต่อ กก. น้ำหนักตัว ทางปากติดต่อกันทุกวัน วันละ 2 ครั้ง โดยเริ่มให้ 7 วัน ก่อนฉีดเปปไทด์เบตาอะมัยลอยด์และให้ต่อเนื่องตลอดการทดลอง ส่วนการทดลองแบบที่สองเริ่มให้สารสกัดมาตรฐานบัวบก อีซีเอ 233 ในขนาดเดียวกับการทดลองแรก หลังจากฉีดเปปไทด์เบตาอะมัยลอยด์ เป็นเวลา 15 วัน ซึ่งพบว่าหนูที่ได้รับสารสกัดมาตรฐานบัวบก อีซีเอ 233 ทั้งก่อนและหลังจากฉีดเปปไทด์เบตาอะมัยลอยด์นั้น สามารถแก้ไขภาวะบกพร่องของการเรียนรู้และความจำ จากการทดสอบด้วยวิธี Morris water maze, step-down และสามารถลดระดับ MDA ในสมองและลดการตายของเซลล์ประสาทในบริเวณ CA1 และ CA3 ของสมองส่วนฮิปโปแคมปัส ของหนูที่ได้รับการฉีดเปปไทด์เบตาอะมัยลอยด์ โดยไม่มีผลต่ออัตราการเคลื่อนไหว (locomotor activity) แต่อย่างใด

การศึกษาครั้งนี้แสดงให้เห็นว่า สารสกัดมาตรฐานบัวบก อีซีเอ 233 สามารถแก้ไขภาวะบกพร่องของการเรียนรู้และความจำจากการฉีดเปปไทด์เบตาอะมัยลอยด์เข้าช่องว่างในสมองของหนูเมาส์ ซึ่งเป็นโมเดลจำลองของภาวะการเกิดโรคอัลไซเมอร์ได้ โดยอาจมีกลไกส่วนหนึ่งผ่านคุณสมบัติในการต้านออกซิเดชัน ควรมีการศึกษาต่อไปถึงกลไกที่เกี่ยวข้องอื่น ๆ ของสารสกัดมาตรฐานบัวบก อีซีเอ 233

สาขาวิชา.....เภสัชวิทยา.... ลายมือชื่อนิสิต……..

ปีการศึกษา...2552..... ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก……...

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม……...

5087238920 : MAJOR PHARMACOLOGY
KEYWORDS : ALZHEIMER' S DISEASE / β-AMYLOID PEPTIDE / *CENTELLA ASIATICA* / COGNITIVE DEFICIT

ANCHALEE KAM-EG : EFFECTS OF THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON COGNITIVE DEFICITS INDUCED BY β-AMYLOID PEPTIDE (25-35) IN MOUSE. THESIS ADVISOR : ASSOC. PROF. MAYUREE TANTISIRA, Ph.D., THESIS CO-ADVISOR : ASSOC. PROF. BOONYONG TANTISIRA, Ph.D., 90 pp.

The present study aimed to investigate the effects of a standardized extract of *Centella asiatica* ECa 233 on cognitive deficits induced by an intracerebroventricular (i.c.v.) injection of β-amyloid peptide 25-35 ($A\beta_{25-35}$) 9 nmol/mouse into male ICR mice weighing 25-35 g. Seven days after $A\beta_{25-35}$ injection, we examined their behavioral changes by Morris water maze test, step-down test and locomotor activity test. In addition, the level of lipid peroxidation in the cerebral cortex and the neuronal cell density was assessed using a cresyl violet staining. In comparison to sham-operated group, significant impairment of learning and memory, increased levels of brain lipid peroxidation and the loss of neuronal cell in CA1 and CA3 of hippocampus were observed in mice treated with $A\beta_{25-35}$. The experiment was divided into two protocols. For the 1st protocol, pre-treatment by ECa 233 (10 and 30 mg/kg B.W., p. o., twice daily) was performed for 7 days before the injection of $A\beta_{25-35}$ and throughout experiment. In the 2nd protocol of post-treatment ECa 233 (10 and 30 mg/kg B.W., p. o., twice daily) was orally given for 15 days after the injection of $A\beta_{25-35}$. It has been shown that ECa 233 giving either pre-treatment or post-treatment could significantly improve deficits in learning and memory, assessed by Morris water maze test and step-down passive avoidance task, however, ECa 233 did not alter locomotor activity. In addition, ECa 233 could significantly improve increased levels of brain lipid peroxidation and decreased neuronal cell loss of hippocampus in mice receiving $A\beta_{25-35}$.

The results obtained suggested that pre-treatment and post-treatment of a standardized extract of *Centella asiatica* ECa 233 is effective in ameliorating cognitive deficits induced by an intracerebroventricular injection of $A\beta_{25-35}$ in mice which is an animal model of Alzheimer's disease. Anti-oxidation property is likely to be at least, in part an underlying mechanism of the results observed. Further investigation should be conducted to determine the other possible mechanisms.

Field of study :...Pharmacology... Student's signature……...

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CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

Aβ	β – Amyloid peptide
$A\beta_{1-40}$	β – Amyloid peptide fragment 1-40
$A\beta_{1-42}$	β – Amyloid peptide fragment 1-42
$A\beta_{25-35}$	β – Amyloid peptide fragment 25-35
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
AGE	Advanced glycation end products
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
bid	bis in die (twice a day)
^{o}C	Degree Celsius
CA	Cornu Ammonis
Ca^{2+}	Calcium ion
C. asiatica	*Centella asiatica*
cm	Centimeter
CMC	Carboxymethylcellulose
CNS	Central nervous system
CR	Condition response
CS	Condition stimulus
CTF	C-terminal fragment of APP
Cu^{2+}	Copper (II) ion
DNA	Deoxyribonucleic acid
ECa 233	Standardized extract of *Centella asiatica*
et al.	et alii (and other)
Fe^{2+}	Ferrous ion
Fig	Figure
g	Gram

GSH	Glutathione
H_2O_2	Hydrogen peroxide
4HNE	4-hydroxynonenal
h	Hour
Hz	Hertz
ICR mice	Imprinting Control Region mice
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
kg	Kilogram
LOOH	Lipid hydroperoxide
MAPK	Mitogen- activated protein kinase
MDA	Malondialdehyde
mg	Milligram
mg/kg B.W.	Milligram per Kilogram body weight
mg/ml	Milligram per Milliliter
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mV	Millivolt
MWM	Morris water maze
NF-kB	Nuclear transcription factor kB
NFT	Neurofibrillary tangles
nm	Nanometer
nmol	Nanomole
NO	Nitric oxide
NSS	Normal saline solution
$O_2^{-\bullet}$	Superoxide anion
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PHF	Paired helical filament
p.o.	Per oral

PS	Presenilin
PS-1	Presenilin-1
PS-2	Presenilin-2
PTZ	Pentylene-tetrazole
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Round per Minute
SDAT	Sporadic dementia of Alzheimer's type
sec	Second
S.E.M.	Standard error of mean
SOD	Superoxide dismutase
SPSS	Statistical package for social sciences
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substance
US	Uncondition stimulus
V	Volt
w/v	Weight per Volume
µl	Microliter
µm	Micrometer
μm^2	Square micrometer

CHAPTER I

INTRODUCTION

1. Background and rationale

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common type of dementia in the age population. Clinically, it is a chronic illness which characterized by loss of memory, inability to think or to learn new things, loss of language function, inability to do calculations, indifference, depression, hallucinations and finally inability to perform daily activity. The patient will be fatal within 5 to 10 years (Yamada and Nabeshima, 2000). This chronic disease has an impact on health and quality of daily life. It has become a problem of public health and economy which could be more important as numbers of age population continued to grow. Until now, the cause of AD is not yet clears (Limon et al., 2009).

The pathological hallmarks of AD are numerous including memory loss and decline in cognitive function, high level of brain oxidative stress as well as a deposit of senile plaques containing β-amyloid peptide (Aβ) and neurofibrillary tangle in brain region (Yankner, 1996). Aβ, a 39-43 amino acid hydrophobic peptide cleaved from amyloid precursor protein (APP) were related to oxidative stress and subsequently neuropathology observed in AD (Behl, 1997). Previous studies showed that intracerebral injection of Aβ to rodents can induce learning and memory impairments as well as neurodegeneration in brain area related to cognitive functions. In addition, intracerebroventricular (i.c.v.) injection of $A\beta_{25-35}$ could induce histological and biochemical changes, oxidative stress and impairment of learning and memory assessed by a Y-maze, passive avoidance, Morris water maze and novel object recognition in mice and rats (Maurice et al., 1996; Yamada and Nabeshima, 2000; Kim et al., 2008; Tsunekawa et al., 2008). Similarly, an intracerebroventricular (i.c.v.) injection of $A\beta_{25-35}$ has a critical role to induce oxidative stress as well as neurotoxicity resemble to those found in AD in human being (Trubetskaya et al., 2003). Thus, an intracerebroventricular (i.c.v) injection of $A\beta_{25-35}$ are frequently used as a model for understanding the pathogenesis of AD and its treatment.

Centella asiatica (*C. asiatica* Linn. Urban) belongs to family Umbellifferae. This plant is widely used in many countries as a traditional herbal medicine. *C. asiatica* is a small herbaceous, perennial creeping herb. The therapeutic uses of *C. asiatica* are extremely wide. *C. asiatica* has shown many beneficial effects such as spasmolytic, diuretic, antihypertensive, anti-asthma, treatment of leprosy, anemia, infectious disease, venous disease, wound healing and neurological disease (Brinkhaus et al., 2000). In addition, in many pharmacological studies of *C. asiatica*, it was shown that the extract of this plant inhibited gastric ulceration by reducing myeloperoxidase enzyme and increasing the expression of basic fibroblast growth factor of wound (Cheng and Koo, 2000). *C. asiatica* possessed wound healing effect (Hussin et al., 2007), anti-inflammatory effect (Somchit et al., 2004), antimicrobial and antiviral effect (Ahmand, Mehmood and Mohammand, 1998) and was able to inhibit epidermal cell distributions (Sampson et al., 2001) and protect blood vessels (Montecchio et al., 1991). Furthermore, *C. asiatica* could also exert antidepressant, sedative and cholinomimetic effects (Wijeweera et al., 2006). It could improve learning and memory by reducing oxidative stress. In animal treated with streptozotocin, *C. asiatica* decreased malondialdehyde (MDA) and increased the level of glutathione peroxidase (GSH-Px) (Veerendra Kumar and Gupta, 2003). Many researchers reported that *C. asiatica* was effective in improving learning and memory. Treatments of *C. asiatica* during postnatal period enhance learning and memory in mice (Rao, Chetana and Devi, 2005). An aqueous extract of *C. asiatica* was used as adjuvant to antiepileptic drugs with advantage of preventing cognitive impairment (Gupta, Veerendra Kumar and Srivastava, 2003). Recently the orally given standardized extract of *C. asiatica* ECa 233 was demonstrated to possess an ameliorating effects on learning and memory deficit in a transient bilateral common carotid artery occlusion (2VO) model in mice (Tantisira, 2009). By definition, ECa 233 is a white to off-white standardized extract of *C. asiatica* containing triterpenoids not less than 80% and the ratio of madecassoside and asiaticoside would be kept at 1.5 ± 0.5. In terms of acute toxicity and sub-chronic toxicity, ECa 233 was found to exhibit very favorable toxicological profiles. No death was observed in mice acutely treated with ECa 233 in the dose up to 10 g/kg and oral administration of ECa 233 in the doses of 10,100 and 1,000 mg/kg for 90 days did not cause any significant changes in all parameters observed (Tantisira, 2009). Therefore, it is of our interest to

investigate further the effects of ECa 233 on cognitive deficits induced by the i.c.v. injection of $A\beta_{25-35}$ in mice.

2. Objectives of this study

The present experiment aimed to investigate the effects of ECa 233 on cognitive deficits induced by the i.c.v. injection of $A\beta_{25-35}$ in mice. In addition, effects of ECa 233 on lipid peroxidation and density of neuronal cell loss in hippocampus (CA1 and CA3 regions) were also determined.

3. Hypothesis

ECa 233 can improve cognitive deficits and decrease neuronal cell loss induced by an i.c.v. injection of $A\beta_{25-35}$ in mice.

CHAPTER II

LITERATURE REVIEW

Alzheimer's Disease (AD)

AD is the most prevalent of dementia, approximately 60-70 % of all dementia cases and it is the most common cause of the syndrome of senile dementia in the aged population (Mathis, Brian and William, 2007). The main clinical features of Alzheimer's disease are cognitive impairments and mental deterioration. The characterized symptoms were memory loss, inability to learn new things, language deterioration, poor judgment, confusion, restlessness and mood swings (Behl, 1997). AD is the most comprehensively studied degenerative disease of the central nervous system. In the initial stages of AD, the personality, the ability to think and the memory will be eliminated. Patients may be unable to carry out daily living such as managing, driving and disturbance of reasoning. All cognitive deficits were worsening in overtime. It is estimated that AD currently affected 17-25 million people worldwide. Furthermore, AD represents the fourth leading cause of death after heart disease, cancer, and stroke in Western countries. On average the patient has survival of 7 years after AD diagnosis. Number of AD rises rapidly among elderly despite preventions and treatments available. The number of people with the disease doubles every 5 years beyond age 65 (De Felice and Ferreira, 2002). AD may represent a significant problem for patient as well as an economic burden for the family and society. There are many different theories suggesting that the overproduction and aggregation of β-amyloid peptide and the aggregation of hyperphosphorylated tau to tangles are cause of the degenerative processes (Selkoe, 1994; Mandelkow et al., 2003; Holscher et al., 2007). Furthermore, various neurotransmitter systems, especially cholinergic neurons which are initially reduced in the course of AD could be responsible for cognitive deficits observed (Maurice et al., 1996). Unfortunately, at present, the exact cause of AD remains unknown. Scientists are trying hard to find therapeutic approaches which could prevent or delay the progression of Alzheimer's disease.

Risk Factors for Alzheimer's Disease

The risk factors of AD are many such as gene, age, environment, food and state of health, however, age is considered as one of the most important risk factors of the disease. About 13% of people older than age 65 have the AD and the disease was found in women more often than men. In addition, traumatic head injuries, high blood pressure, cholesterol and poorly controlled diabetes are also some of the contributing risk factors to Alzheimer's disease (Yamada and Nabeshima, 2000).

Genetics Risks of Alzheimer's disease

It is believed that many genetics might play a role in Alzheimer's disease and that a large majority of AD cases have underlying genetic determinants. AD was divided into familial and sporadic AD (Selkoe, 2001). Sporadic AD was the most common type of AD which occurs in the people age over 65 years. In familial type, it is resulted from mutations in related gene such as APP or presenilin and uncommon form of AD. It can occur in the age before 65 years old. The recognition of polymorphic alleles of apolipoprotein E predisposes strongly to develop an AD (Yamada and Nabeshima, 2000). The other polymorphic genes that could predispose to the disorder are difficult to detect in genetic epidemiological studies, because they do not penetrance and always produce the disease. Clinically, typical AD can cluster in families and can specifically be inherited in an autosomal dominant. Furthermore, an autosomal dominant form caused by mutations in the APP and presenilin genes is likely to be directly relevant to the early-onset familial AD (Sherrington et al., 1995). The genes that related to Alzheimer's disease were shown in Table 1.

A.) APP gene

The first specific gene identified to cause AD was the missense mutations in APP (Mullan et al., 1992). The location of the mutations and the subsequent delineation of their genotype-to-phenotype relationships provided critical insights to the mechanism of AD. These mutations are strategically located before the β-secretase cleavage site, shortly after the α-secretase site, or shortly COOH-terminal to the γ-

secretase cleavage site. The missense mutations of APP lead to AD by altering proteolytic processing at the three secretase sites in different pathways. Generally, the APP gene is mapped on chromosome 21 and the mutations leading to abnormal processing of APP and Aβ production which were found in early-onset of AD (Goate et al., 1991; Mullan et al., 1992). The chromosomal area were overlap between Down's syndrome (DS) and AD thus, the disease will develop to AD after a certain age. However, AD is not caused by an overproduction of APP, but could be accounted by other mechanism, such as APP molecule abnormality or a defect of normal APP processing that is amyloidogenic.

B.) Presenilin 1 and 2 gene

Presenilin 1 and presenilin 2 are encoded by genes on chromosomes 14 and 1, respectively and are predominantly present in neurons, similarly to APP (Chapman et al., 2001). Presenilin were found to undergo constitutive endoproteolysis in many cell types including brain and exist mostly as stable heterodimers (Borchelt et al., 1996). The intensive genetic study identified that there are 75 missense mutations in presenilin 1 and 2. The molecular causes of early-onset AD were missense mutation in presenilin 1and 2 (Hardy, 1997). Furthermore, the fact those mutations of conserved residues in PS1 as well as PS2 can elevate $A\beta_{42}$ production which linked to familial AD.

C.) Apolipoprotein E

Apolipoprotein E (ApoE), one risk factor for Alzheimer's disease, is a protein that carries lipids in and out of the cell. ApoE gene is located on chromosome 19 (Yankner, 1996). It has three isoforms: ApoE2, ApoE3 and ApoE4. The ApoE4 molecule has a greater ability than other human ApoE to facilitated Aβ aggregation and toxicity. Furthermore, allele of ApoE4 is over expression in subjects with AD (Holtzman, 2001). Clinical symptoms of AD demonstrated that ApoE4 genotype linked with amounts of diffuse $A\beta_{1\text{-}42}$ plaques in the brain, suggesting the relationship between Aβ and ApoE4. Thus, the inheritance of ApoE4 is one of the first genetic risk factors for a common late-onset of Alzheimer's disease (Yamada and Nabeshima,

2000). In addition ApoE4 has been found in NFTs and in Aβ suggesting that ApoE may involve in the processing of APP perhaps by modulating APP secretase or due to the assembly of the neuronal cytoskeleton.

Table 1 Genetic factors associated with Alzheimer's disease

Table 1. Known Genetic Associations with Alzheimer's Disease

Gene	Chromosome	Typical Age of Onset (years)
Presenilin 2	1	30–40
Presenilin 1	14	50–65
Apolipoprotein E	19	Over 60
Amyloid precursor protein	21	40–50

(Modified from Pratico and Delanty, 2000)

Pathological Hallmarks of Alzheimer's Disease

The pathological hallmarks of AD were amyloid plaques and neurofibrillary tangles (NFTs) (Figure 1). These hallmarks were first reported 90 years ago by Alois Alzheimer (Mathis, Brian and William, 2007). Herein, pathological hallmarks and mechanism of β-amyloid peptides in relation to Alzheimer's disease will be described.

1. Neurofibrillary Tangles (NFTs)

Neurofibrillary tangles (NFTs) are intracellular structures composed of an abnormally aggregated, hyperphosphorylated form of the microtubule binding protein (tau). Tau plays a role in stabilizing axonal microtubules, which essential for axonal transport (Yamada and Nabeshima, 2000). Normally, tau is synthesized and found in all neuron. It aggregates in a form of NFTs with high β-sheet content and appears as paired helical filaments (PHF) which is associated with detachment of tau from their binding site and re-localization to the cell bodies and dendrites (Holtzman, 2002). Neurons with neurofibrillary tangles (NFTs) will degenerate and die (Yamada and

Nabeshima, 2000). Many researchers suggested that neurofibrillary pathology was likely to contribute to neuronal dysfunction. In addition, neurofibrillary tangles (NFTs) occur in many neurodegenerative diseases such as Pick's disease, Boxer's disease and also in Alzheimer's disease (Giasson et al., 2003). It is possible that β-amyloid could be triggered by neurofibrillary tangles (NFTs).

2. Amyloid Plaques

Plaques are extracellular accumulations of protein molecules surrounded by dystrophic neuritis leading to a disrupted neuronal connectivity and the sprouting of intact neighbouring axons in the brain (Burbach, 2005). These plaques were found in cortex and hippocampus, the structures related to learning and memory (Holtzman, 2002). The principal component of senile plaque is the amyloid-β peptides (Aβ), which consisted of a 39-43 amino acid peptide that were cleaved from amyloid precursor protein (APP). In normal brain, these protein fragments are eliminated whereas, in Alzheimer's brain, the fragments accumulate to form hard and insoluble plaques (Uversky et al., 1999).

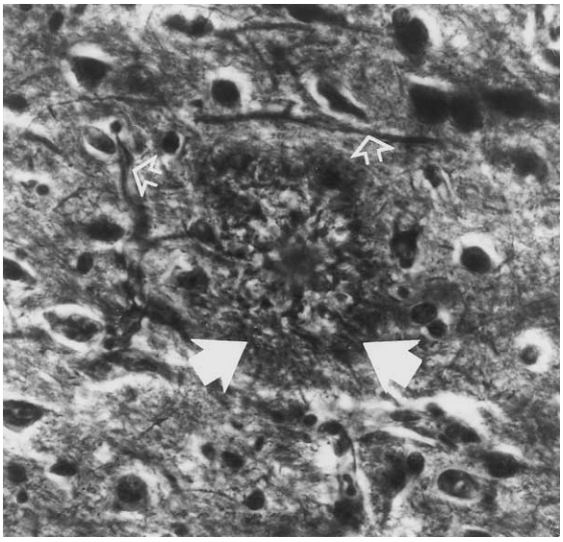


Figure 1 Histopathology of AD: senile plaques (big arrows) and neurofibrillary tangles (small arrows) in the frontal cortex of an AD brain

(Modified from Behl, 1999)

The β-Amyloid Peptide

The principal component of amyloid plaque (senile plaque) is the β-amyloid peptide (Aβ) which composed of 39-43 amino acids in a portion of the transmembrane domain and the extracellular domain of the amyloid precursor protein (APP). The processing of APP proteolysis and Aβ production were shown in Figure 2. In AD pathology, APP proteolysis is the fundamental procedure for the production of Aβ implicated. APP proteolytic products arise from the coordinated action of β-, α-, and γ-secretase. APP can be cleaved by two different pathways (Behl, 1997; Yan et al., 2004). The first pathway is nonamyloidogenic or α-pathway, by α-secretase that generates a soluble N-terminal APP fragments (α-APPs) which secreted from the cell. The C-terminal fragment of APP (αCTF) can additionally be cleavage by γ-secretase, leading to the formation of nonamyloidogenic (P3) which is non toxic and being secreted from the cell body. Conversely, in the amyloidogenic pathway, Aβ are produced by the β-secretase complex at the N-terminus end of the Aβ domain that released to a truncated soluble APP fragments (β-APPs) which secreted from the cell. Subsequently, the C-terminal fragment of APP (βCTF) was cut again by γ -secretase, release the Aβ (Yamada and Nabeshima, 2000). The most common isoforms of Aβ are $A\beta_{1-40}$ (90%) and $A\beta_{1-42}$ (10%). The shorter form is typically produced by cleavage that occurs in the endoplasmic reticulum, while the longer form is produced by cleavage in the trans-Golgi network. However, the most prevalence of AD was $A\beta_{1-42}$ because it is more capable to aggregating into fibril (Iwatsubo et al., 1994; Mathis, Brian and William, 2007).

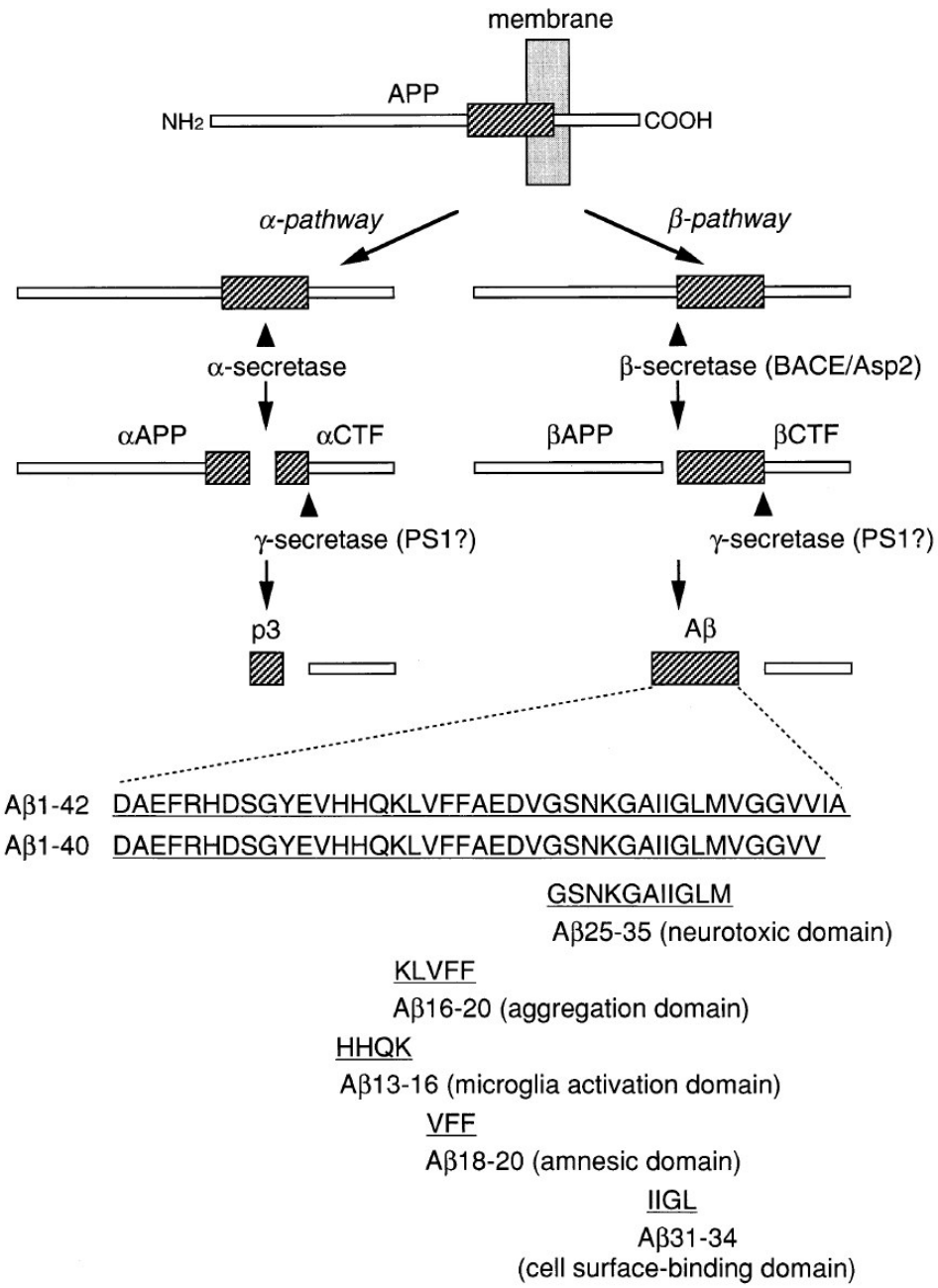


Figure 2 APP proteolysis and Aβ production. Processing of APP proteolytic is divided into two pathways: the non-amyloidogenic α-pathway involves the cleavage of APP by α-secretase, and thereby precludes the formation of Aβ. In the amyloidogenic β-pathway, APP is cleaved by β-secretase, and then cut by γ–secretase and generate insoluble Aβ. CTF, C-terminal fragment of APP; p3, the shorter APP fragment derived by α-secretase cleavage followed by γ–secretase cleavage

(Modified from Yamada and Nabeshima, 2000)

In the brain, there are both soluble and insoluble peptides formed in a random coil and α-helix folded peptide with aggregation states from monomer to oligomer. An insoluble Aβ is β-sheeted and forms either fibrillar or amorphous deposits. Briefly, in initial it is monomers which subsequently oligomerise into larger forms such as oligomers and fibrils (Figure 3). The deposit of these fibrils lead to the senile plaques. It has been reported that the various forms of Aβ in any form such as oligomers, fibrils and senile plaques are all neurotoxic (Dahlgren et al., 2002). In addition, Haass and Selkoe (2007) also found that soluble Aβ oligomer intermediates in the Aβ amyloidogenic pathway are key contributors to mediate neurodegeneration.

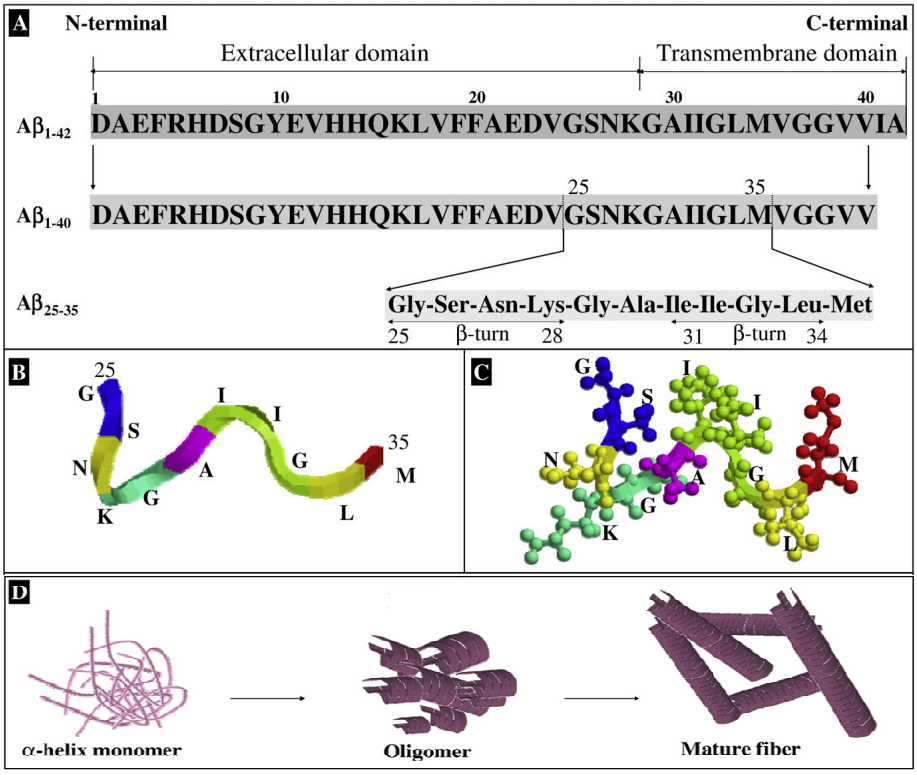


Figure 3 (A) Short sequence of $A\beta_{25–35}$ (and its parent $A\beta_{1–42}$ or $A\beta_{1–40}$) with the probable localization of the β turn that facilitates its transformation from α-helix into Aβ fibrils. (B) The schema $A\beta_{25–35}$ with its β turn. (C) Ball-stick configuration of the peptide skeleton. (D) Schematic diagram of fibrillation: the random coiled α-helix Aβ is transformed into β sheets that association to form the oligomer, which ultimately extends to mature fibers.

(Modified from Hashimoto et al., 2009)

The hypothesis of amyloid-cascade in the etiology and pathogenesis of AD are;

1.) The mutation of genetic of APP and PS may associate directly to AD

2.) The highly fibrillogenic of Aβ via the amyloidogenic processing of APP, triggers a neurotoxic, thereby causing neurodegeneration and AD

3.) The over expression of gene on chromosome 21 which, associated with Down's syndrome, were found in typical AD (Munoz and Feldman, 2000)

Based on genetic evidence supporting amyloid-cascade-hypothesis, Aβ can directly act as neurotoxic (Yankner, 1996) and Aβ is widely used to the development of AD for underlying and developing therapeutic strategies.

β-Amyloid Peptide Neurotoxicity

The neurotoxicity of Aβ was determined in various studies. Neurotoxic effects of Aβ involve various mechanisms (Figure 4) including ROS production leading to oxidative stress, disruption of cellular calcium homeostasis, alteration of the cytoskeleton or nucleus and inflammatory process (Mattson, 2004). Aβ can destabilize Ca^{2+} homeostasis and induce neurons vulnerable to excitotoxicity (Koh, Yang and Cotman, 1990; Mattson et al., 1992). Furthermore, there is evidence that Aβ alters cellular ionic activities, either through interaction with existing ion channel or by de novo channel formation (Fraser, Suh and Djamgoz, 1997). The alteration in ionic homeostasis pathway induced by Aβ may be related with its neurotoxicity. It also found that the mitogen-activated protein (MAP) kinase mediates multiple aspects of Aβ-induced neurotoxicity (accumulation of intracellular Ca^{2+}, reactive oxygen species, phospho-tau immunoreactivity and apoptosis) (Yamada and Nabeshima, 2000). In addition, soluble Aβ at low concentrations has been shown to compromise cholinergic neuronal functions independently of apparent neurotoxicity. It is shown that Aβ inhibits high-affinity choline uptake and ACh release in rat hippocampal slices. ApoE which is accumulate in the senile plaque and NFTs bind to soluble Aβ *in vitro* and promotes amyloid fibril formation in an isoform-specific manner. It has been found that ApoE4 could promote Aβ fibrillogenesis more potently than ApoE3. Furthermore, neurotoxicity of Aβ might involve activation of microglia to produce

neurotoxin, such as proteolytic enzymes, cytokines, free radicals and NO. The two microglia receptors for Aβ were scavenger receptors for fibrils (Paresce, Ghosh and Maxfield, 1996) and the receptor for advanced glycation end products (RAGE). All evidences mentioned above demonstrate the involvement of Aβ in energy depletion, excitotoxicity, oxidative stress, inflammatory and apoptotic cell death (Yamada and Nabeshima, 2000).

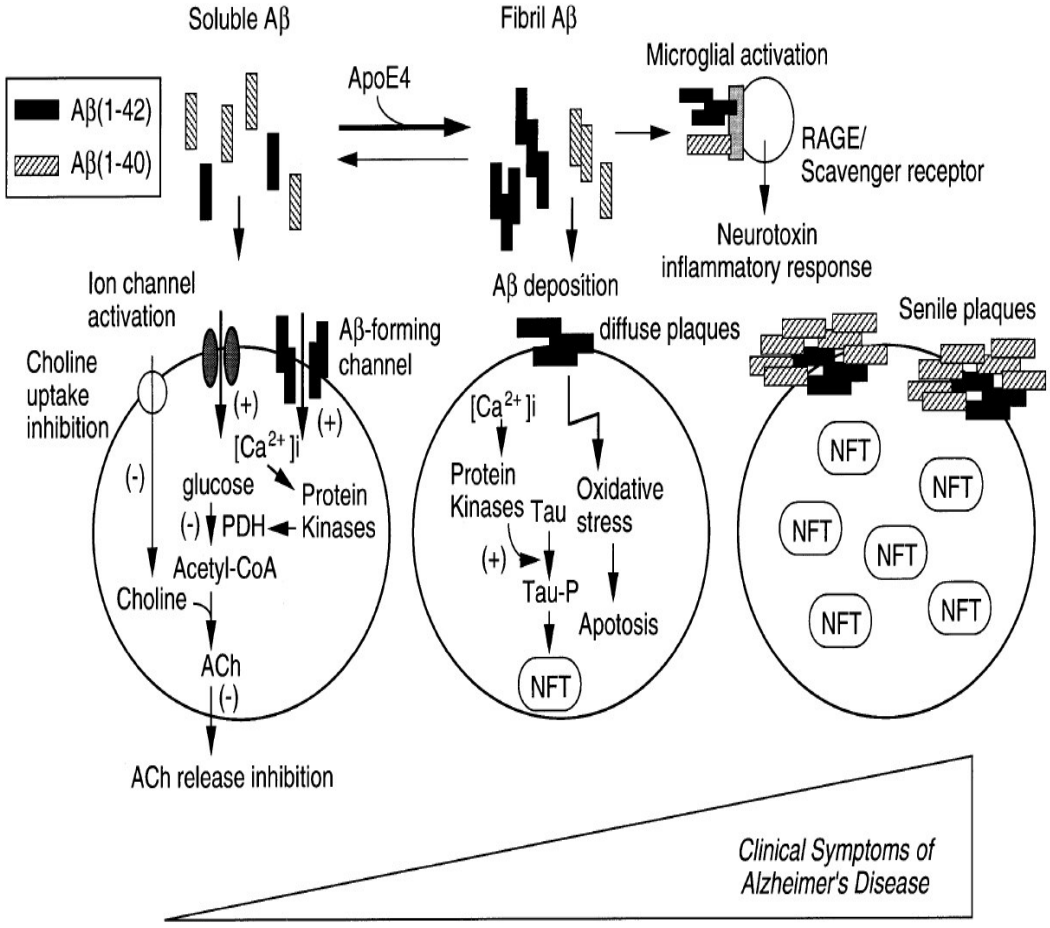


Figure 4 Potential mechanisms of Aβ neurotoxicity. $Aβ_{1-42}$ is highly fibrillogenic, readily aggregated and deposited early and selectively in amyloid plaques

(Modified from Yamada and Nabeshima, 2000)

Oxidative Stress

Oxidative stress is the result of generation of reactive oxygen or nitrogen species and the imbalanced of antioxidant defenses. A free radical is any chemical species that contains one or more unpaired electron. Unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical, because they act as electrons acceptors and steal electron from other molecules. The loss of electron also called "oxidation" and the free radical may be referring to an oxidizing agent because they tend to cause other molecules to donate their electrons. The most common free radicals are hydroxyl radical ($OH^{\bullet}$), superoxide radical ($O_2^{-\bullet}$) and nitric oxide (NO). While, hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are not free radicals, but could generate free radical through various chemical reactions. Free radicals and related molecules are able to promote oxidative intracellular changes. Cells exposed to severe oxidative stress may suffer from degeneration of DNA, membrane lipids, protein and enzymes, leading to various pathological conditions. Therefore, the oxidative damage is considered as one of the mechanisms leading to chronic diseases, such as atherosclerosis, aging, cancer, rheumatoid arthritis, neurodegenerative disease including Alzheimer's disease, Parkinson's disease and amyotrophic lateral (Brown, Lockwood and Sonawane, 2005; Hussin et al., 2007). Normally, cell has two types of mechanisms against free radicals; non-enzymatic antioxidants encompassing vitamin E, vitamin C, beta-carotene and a trace element such as selenium, manganese and zinc as well as enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase (Arbona et al., 2003). However, when production of ROS exceeds and the antioxidant system decreases then the cell damage occurs. The imbalance between cellular production of ROS and antioxidant is called "oxidative stress" (Ebadic, Srinivasan and Baxi, 1996). Oxidative stress can cause neuronal damage and cell death mainly in apoptosis pathway because the ROS could oxidize or interact with the cellular components such as lipid, protein and DNA.

Oxidative Stress in Alzheimer's Disease

Oxidative stress has been implicated as a major cause of neurotoxicity in a number of neurodegenerative disorders including AD. Oxidative stress may cause DNA and RNA damage, fragmentation or modification the level of protein such as carbonyl and nitration of tyrosine residues and lipid peroxidation which eliminate cell membrane (Zhu et al., 2004). During oxidative stress, the free radicals produced namely "reactive oxygen species" (ROS) can potentially damage the brain for the reason that;

(1) The brain has high glucose-driven metabolic rate
(2) The brain has low levels of antioxidant defense enzymes compared with other cell
(3) The brain contains high concentrations of polyunsaturated fatty acids, which are potential substrates for lipid peroxidation
(4) The brain is rich in enzymatically active transition metals which can catalyze radical formation (Behl, 1997)

Previously, increased levels of oxidation end products such as advanced glycation end products (AGEs) could lead to DNA oxidation and membrane lipids peroxidation mostly found in the brain of AD (Good et al., 1996; Crouch et al., 2008). The oxidation of membrane lipids can disturb membrane integrity and affect membrane functions immediately (Mamelak, 2007). In addition, the reaction product of nitric oxide and superoxide radicals could lead to alter phosphorylation of neurofilaments and subsequently damaging neuronal cells (Behl, 1997).

Oxidative Stress Induced by β-Amyloid Peptide

It is suggestive that β-amyloid peptide induced oxidative stress could play an important role in AD. There is high correlation between amyloid plaques and lipid peroxidation (Lovell et al., 1995). The mechanism of Aβ inducing oxidative stress could be direct or indirect. Mitochondrial dysfunction represents an indirect source of oxidative stress whereas Aβ induced neurotoxicity represents direct source of oxidative stress (Crouch et al., 2008). Recently, several different amyloid peptides have been reported to be able to aggregate into β-pleated sheets which was cytotoxic

and caused oxidative stress by a common oxidative mechanism as a basic pathway of peptide toxicity (Yan et al., 1996). In the study of membrane damage caused by Aβ, using pheochromocytoma PC12 cells, it was found that Aβ can induce rapid membrane disintegration and breakdown of membraneous structure (Behl, 1997). Consistent with these findings, an increased peroxidation of membrane lipids can be found in AD brain *in vivo*. H_2O_2 is hypothesized to be one mediator of Aβ toxicity as well as an inducer of the transcription factor NF-kB, which mediate immune response and found to be increased by Aβ and by other fibril forming peptides. Interestingly, $OH^{\bullet}$ can also react with Aβ itself and promote Aβ aggregation (Atwood et al., 2004). Interestingly, it has been found that several different amyloid peptides could aggregate into cross β-pleated sheet which can promote cytotoxic, sharing a common oxidative mechanism. Thus, it is suggested that the oxidative mechanism might be a basic pathway of peptide toxicity. In addition, interactions between Cu and Aβ also result in oxidative damage, as Aβ has the ability to reduce bound Cu (II) to Cu (I) and this reaction produces H_2O_2. The secondary interaction of H_2O_2 with Aβ-bound Cu (I) generates $OH^{\bullet}$ which will react with lipids, proteins and nucleic acids. Also peroxidations of lipids in fatty acids lead to radical chain reaction. The degenerative lipid peroxidation is the parameter for radical damage (Opazo et al., 2002). Furthermore, interaction of Aβ with iron (Fe^{2+}) or copper (Cu^{2+}) produced hydrogen peroxide (H_2O_2) which then interact with superoxide anion ($O_2^{-\bullet}$) which is a highly reactive oxyradical. Aβ aggregations at the cell membrane induced oxidative stress causing lipid peroxidation and consequent generation of 4-hydroxynonetal (4HNE). 4HNE promotes toxicity via tau protein aggregation and the formation of NFTs. Moreover, Aβ can cause mitochondrial dysfunction and Ca^{2+} homeostasis dysregulation leading to an increased production of $O_2^{-\bullet}$ and oxidative stress (Mattson, 2004). In conclusion, Aβ toxicity is associated with various oxidative processes such as direct aggregation of Aβ to neuronal membrane or generation of other radicals through microglial activation or by radicalization of Aβ itself (Behl, 1997).

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. The peroxyl radical can react with other nereby lipids and form a lipid hydroperoxide (LOOH) (Pratico and Delanty, 2000). Lipid hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. Their formations occur in enzymatic or non-enzymatic reactions involving activated chemical species known as "reactive oxygen species" (ROS) which are responsible for various tissues damaging in the body. Amplified lipid peroxidation has been described in several neurodegenerative diseases including AD (Butterfield and Lauderback, 2002). Enzymatically, metabolism of catecholamines and indolamines by monoamine oxidase can produce H_2O_2. On the other hand, H_2O_2 can be produced non-enzymatically from autooxidation of catecholamines, lipooxygenases, cyclooxygenases and various flavin oxidases (Behl, 1997). In the central nervous systems (CNS) which derive the energy from oxidative phosphorylation in the respiratory chain of the mitochondria, during this process, a leakage of high energy electrons can potentially generate superoxide radicals and through the action of superoxide dismutase (SOD), hydrogen peroxide (H_2O_2) is finally produced. Aβ can directly react with H_2O_2 and cause neuronal damage (Mattson, 2004).

Lipid peroxidation could induce various molecular events such as membrane structural damage, loss of Ca^{2+} homeostasis, loss of protein transport function, disruption of signaling pathway and induced nuclear transcription factors in apoptotic pathways. As a consequent of this cellular dysfunction, cell shrinkage, nucleus morphology changes, increment of DNA fragmentation and neuronal cell death occur. The process of apoptotic which occur rapidly has been suggested to be a fundamental mechanism of cognitive deficit in AD (Butterfield, 1997). The compound widely used as an indicator of lipid peroxidation was malondialdehyde (MDA) which was a breakdown product of peroxidized lipids (Pratico and Delanty, 2000). Generally, MDA was determined by thiobarbituric acid-reacting substances (TBARS) assay which was easy to perform and less expensive (Moore and Roberts, 1998).

Learning and Memory

Hippocampus and cerebral cortex play an important role in learning and memory (Martin, Grimwood and Morris, 2000). Hippocampus was divide in to three part as CA1, CA2 and CA3 (CA stands for Cornu Ammonis, a Latin name for the Ammon's horn) (Brodal, 1998). The hippocampal formation has an essential role in long term consolidation of memory and the consolidation occur by a continuous interaction between the hippocampus and some part of the cortex. The combination of information procedure and memory in cerebral cortex was build up in the neural network and became the structure of knowledge (Martin, Grimwood and Morris, 2000). Learning and memory are related mechanism of the brain essential for daily life. Term of learning associated with behavioral changes that result from observation and storage of new information.

A.) Learning

Learning is the process by which experiences change our nervous system and hence our behavior. Generally, there are two types of learning : nonassociative learning and associative learning.

Nonassociative learning : related with type of stimulus and the ability to adap to desires or survival. There are two types : habituation and sensitization. Habituation is a decrease in response to benign stimulus when the stimulus is happen repeatedly while sensitization enhanced response to a wide variety of stimuli after the presentation of noxious stimulus (Kandel, Kupfermann and Iversen, 2000).

Associative learning : associated between events. There are two types of associative learning : classical conditioning and instrumental conditioning. Classical conditioning involving a stimulus that evokes a measurable response with a second stimulus that normally does not evoke this response. Example the salivation of a condition dog which response to a stimulus of sound. Generally, the dog salivates when they receive food. After a sound of bell stimulus (the conditioned stimulus or CS) has been paired with the presence of food (the unconditioned stimulus or US) in

enough number of conditioning trials, the sound alone is enough to cause the dog to salivate (salivation is the conditioned response or CR). In classical conditioning, the subject learns that one stimulus (CS) predicts another stimulus. In instrumental conditioning an individual learns to associate response a motor act with a meaningful or reward stimuli, typically reward such as food, example, placed a hungry rat in a box with lever that dispenses food. In the course of exploring the box, the rat bumps the lever and out pops a piece of food. After this accident occurs a few more times, the rat learns that pressing the lever lead to food reward. In instrumental conditioning, the subject learns a particular behavior is associated with a particular consequence. Also like classical condition, timing is important (Bear, Connors and Paradiso, 2007).

B.) Memory

Memory is the outcome of learning which were divided into two types, (1) declarative memory, (2) nondeclarative memory (procedural memory).

Declarative or explicit memory : refer to the ability to stores facts and event, such as ability to remember names, faces, event, facts and this memory is unconscious. This type of memory tends to form easily and be forgotten easily. They are two types of declarative memory; Episodic memory refer to episodic events that contains what, when, where information. This is major types of memory in our daily life. Semantic memory refers to facts and knowledge which happen through either single or repeated experience.

Nondeclarative memory or implicit memory : refer to various form of memory and it is conscious awareness. This type of memory does not depend on the structural integrity of the temporal lobe system. Nondeclarative memory is an automatic or reflex quality sometime it also called “implicit memory” because it results from direct experience (Tsien, 2006).

Process of the Memory

Encoding : refer to processing of newly learn information into the memory process when first encountered. The natures of encoding are importance for how to remember in later time. This process is accomplished by attending to the information and associating it meaningfully and systematically with knowledge that is already well establish in memory so as to allow one to integrate the new information with that one already known memory.

Consolidation : refer to the process that alters the newly stored and still labile information so as to make it more stable for long term storage. The consolidation involved the expression of the gene and synthesis of new proteins, giving rise to structural changes that store memory stably over time.

Storage : refer to the mechanism and site by which memory is retained overtime. One remarkable feature about long-term storage is that it seems to have an almost unlimited capacity but short-term working memory is very limited.

Retrieval : refer to the process that permit to recall and use of the storage information. Retrieval is an important aspect of memory processing because it is the only way that can be measured. Retrieval involves bringing together different kinds of information that are stored separately in different storage sites. Retrieval of information is most effective when it occurs in the same context in which the information was acquired and in the presence of same cues that were available to the subject during learning. Retrieval, particularly of explicit memories, is critically dependent on short-term working memory (Kandel, Kupfermann and Iversen, 2000).

Method of Memory Assessment

Among features found in AD, the loss of memory is the earliest and most prominent feature. Because there are similarities in the type of learning and memory displayed by rodent and human behavioral test focusing on learning and memory is conducted in mouse AD model (Chapman et al., 2001).

Morris water maze : This method measures spatial learning memory which critically dependent on hippocampal function. The procedure of the MWM must use accurate localization and platform locating. MWM has several advantages than other maze such as rodent is natural swimmers and they want to get out of the water. Output occur is based on the natural tendencies of the rodent therefore pre-trained rodent can be used and the assessment of this test does not require motivating agents or any condition like food or water restriction. Furthermore, the MWM is easy to build up and operate with low cost than other types of behavior. The task has been given various names such as Morris swimming pool, Morris maze, water maze, spatial navigation maze task, etc. The most common name is Morris water maze as it was developed by Richard Morris. The traditional MWM paradigm consists of training trials and probe test. In the training trials, the platform remains at a fixed location in the pool. Animals use three-dimension cues in the testing room to learn and find a hidden platform in a swimming pool. The test involved repeated trials in which the animal is placed in different part of pool then the time to find platform were measured. Calculating the time to find platform on successive trials generates a learning curve that can be used to compare the acquisition of the spatial learning test between animals. In order to determine that the animal is using a mapping strategy as an index of memory, the probe trial was given, the hidden platform were removed and the time that animal swim in the area where the platform used to be is record (Terr, 2001; Baldi, Lorenzini and Corrado, 2003; Choi et al., 2007).

Step-down passive avoidance : This method has been used to assess the animal's learning and memory associated with emotionally salient events. In general, it is an avoidance paradigms that require the subject to initiate a specific type behavior, usually escape or avoidance, in order to preclude the administration of an

aversive event, or negative reinforcement. Inhibitory avoidance, also frequently referred to as passive avoidance, requires that the subjects (typically rodents) to behave in a manner contrary to their normal inclination or preference. Step-down test is one type of passive avoidance task that animal learns to avoid a noxious event. Fear-motivated avoidance test are usually based on electric current as source of punishment. The animal has to refrain from executing a previous response by step-down from an elevated position to a grid floor or step in to safer place. Step-down 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).

Model of Alzheimer's Disease

The development of model for AD is a critical step for understanding the disease pathology and searching for therapeutic drugs. Many models resemble all of the cognitive, behavioral and biochemical abnormalities found in AD have been used in the study of pathology of AD. The cause of AD is closely related to the accumulation of Aβ in the neuritic plaques. The use of animal model systems represents a good strategy to elucidate the molecular mechanism behind the development of this pathology. Related animal models for AD are divided into two groups; transgenic and nontransgenic animal models.

Transgenic animal model demonstrated the mutation of APP which link to familial AD. Amyloid deposits in the animal model resemble to AD brain. Accordingly, this model is most suitable for assessing the effects of drugs that inhibit Aβ synthesis, fibril formation and deposition in brain. However, the developed of Aβ deposit was slowly and the animal do not have a substantial amount of intracerebral amyloid until old 11-13 months of age. Whereas, in nontransgenic model, acute or continuous injection of Aβ are efficient as quick screening tools for an anti-Aβ compound that can inhibit both the formation of Aβ fibrils and deposition (Yamada and Nabeshima, 2000). Acute or continuous i.c.v infusion of Aβ into nontransgenic animal models for AD was a useful animal model for drug screening. Various Aβ fragments such as $A\beta_{1\text{-}40,}$ $A\beta_{1\text{-}42,}$ $A\beta_{1\text{-}28}$ and $A\beta_{25\text{-}35}$ have been found to exhibit toxicity to neurons (Frautschy, Baird and Cole, 1991; Sigurdsson et al., 1997; Yamada and Nabeshima, 2000). Kowall et al. (1991) demonstrated that injection of $A\beta_{1\text{-}40}$ could

induce neuronal cell loss. Similarly, Flood, Morley and Roberts (1991) found that injection of $A\beta_{1-28}$ also caused memory loss in animal model. Moreover, it has been shown that acute or continuous i.c.v infusion of Aβ into hippocampus, cortex, basal forebrain nuclei and intracerebroventricular cause neurodegeneration and impairment of learning and memory in behavioural test with Y-maze, step-down, novel object recognition and Morris water maze test.

$A\beta_{25–35}$ is considered one of the neurotoxic domains of full-length $A\beta_{1-40/42}$ which composed of 11 amino acids, it is potent as a full-length peptide that produces toxic effect (Hashimoto et al., 2009; Limon et al., 2009). Previous study showed that acute exposure of hippocampal cultures with aged $A\beta_{25-35}$ induced apoptotic-mediated neuronal toxicity during 6 days incubation. After that, the researcher also found that $A\beta_{25–35}$ injected into the hippocampus of rats impair spatial memory and enhances oxidative stress (Perez-Severiano et al., 2004). In pathological study they found plaque, which leading to the deposit of Aβ in hippocampus and cortex (McDonal et al., 1994; Giovannelli et al., 1995; Chen, Wright and Barnes, 1996; Maurice, Lockhart and Privat, 1996; Sweeny et al., 1997; Harkany et al., 1998; Trubetskaya et al., 2003; Tsunekawa et al., 2008). Indeed, the intracerebroventricular (i.c.v.) administration of $A\beta_{25–35}$ into rodent brain also induced histological and biochemical changes, memory deficits and oxidative stress (Kim et al., 2008). In addition, $A\beta_{25-35}$ contains the residues essential for aggregation and toxicity that is methionine 35 likes $A\beta_{1-42.}$ Furthermore, $A\beta_{25-35}$ is shorter and more toxic than full length peptide. $A\beta_{25-35}$ is also less expensive and more easily to manipulate and often causes more oxidative damage than $A\beta_{1-42}$ (Varadarajan et al., 2001). Thus, an i.c.v injection of $A\beta_{25-35}$ models is useful to clarify the pathogenic mechanism and evaluate new treatment strategies for AD.

Treatment of Alzheimer's Disease

Alzheimer's disease affects millions of people and has become a major medical and social problem for developing societies. Many studies found that the pathology of AD involve in Aβ which neurotoxicity via various mechanisms (Behl, 1997). Nowadays, the most effective available pharmacotherapy for AD is the use of acetylcholinesterase inhibitors (AChE-Is) (e.g. donepezil, rivastigmine and

galantamines) thereby, enhancing cholinergic function but it also causes side effects such as nausea, anorexia, vomiting and diarrhea. In addition, other drugs such as selegiline, vitamin E, estrogen and anti-inflammatory drugs have been studied, but their use remains controversial (Akhondzadeh and Abbasi, 2006). Thus, it has been interested in the biochemical functions of natural product like vegetables, fruits, herbals or medicinal plants, which can be a candidate for the prevention and improving memory. Herbal remedies for Alzheimer's disease have become more and more popular in the recent years, by possibility to slow down the brain's degeneration. Many natural herbal treatments have been researched and may hold the key to cure this devastating disease. In addition, these herbs are inexpensive and can be easily obtained. There are several studies and documents that indicate a unique role of herbal medicines in the treatment of AD.

- *Ginkgo biloba*, which is native to China and now cultivated in Europe and America, is the oldest tree on earth. The study shown that the standardized extract EGb761 (extract of *Ginkgo biloba* 761) exerts multiple cellular and molecular neuroprotective mechanisms, including the attenuation of apoptosis, the inhibition of membrane lipid peroxidation, anti-inflammatory effects and the direct inhibition of Aβ aggregation (Izzo and Capasso, 2007).

- *Salvia officinalis* (sage), might be relevant to the treatment of cognitive disorders. The pharmacological activities of sage that are relevant to AD include antioxidant activity, anti-inflammatory effects and weak AChE inhibition. Moreover, rosmarinic acid (the active ingredient of sage) reduces several deleterious events induced by Aβ, including the formation of reactive oxygen species, lipid peroxidation, DNA fragmentation, caspase-3 activation and tau protein hyperphosphorylation (Izzo and Capasso, 2007).

- *Angelica gigas* Nakai (Umbelliferae) (EAG), has been used in traditional Korean folk medicine. The pharmacological activities of EAG can be protected against the $A\beta_{1-42}$ induced memory impairment in part by antioxidative activity in brain (Yan et al., 2004).

- *Polygonum multiflorum* Thunb, the root of a Korean medicinal herb, has been used for a long time as an anti-aging agent. These finding suggested that *Polygonum multiflorum* Thunb water extract (PWE) exerts a preventive effect against

cognitive deficits induces by $A\beta_{25-35}$ accumulation in Alzheimer's disease and this effect is mediate by the antioxidant properties (Um et al., 2006).

- Ginseng saponin (GS) has been used in Far East to treat ailments associated with aging. Pre-clinical and clinical studies have shown that the extract of ginseng leaves or roots has promising therapeutic potential as a cognitive enhancing drug. GS can functionally prevent the β-amyloid induced memory loss possibly by minimizing the inhibitory effect of β-amyloid on hippocampal cholinergic transmission (Wang et al., 2006).

- Huperzine A is a chemical derived from a particular type of club moss (*Huperzia serrata*), this substance is really more a drug than an herb. This drug inhibits the breakdown of the neurotransmitter acetylcholine, allowing more of it available for brain functions, including memory (Izzo and Capasso, 2007).

- *Curcuma longa* Linn., has a long history as a traditional Indian food preservative and herbal medicine and possesses a number of pharmacotherapeutic effects. Moreover, it is effective in preventing cognitive deficits and might be beneficial for the treatment of sporadic dementia of Alzheimer's type (SDAT) (Isharat et al., 2009).

- *Tabernaemontana divaricata* (TDE), the Thai medicinal herb, could prevent the memory loss and decrease AChE activity, lipid peroxidation and neuronal loss (Nakdook, 2009).

- Green tea (GT) and green tea byproduct (GTB) are CNS stimulants. A long term administration of GT or GTB may help to restore the cognitive deficits caused by $A\beta_{25-35.}$ The mechanism of GT or GTB may involve up-regulated anti-oxidant enzyme activity in the brain (Preedapirom, 2009).

Thus, in the present studies aim to establish the effect of *Centella asiatica* cultivated in Thailand on learning and memory, which is an alternative treatment choice for AD.

***Centella asiatica* (Linn.) Urban**

Figure 5 Herbs of *C. asiatica*

Centella asiatica (Linn.) Urban is a tropical herb, belongs to family Umbelliferae and was found in wet places of tropical and subtropical regions. It has been widely used as a traditional herbal medicine in many cultures. It has a long history of use in India as a memory enhancing drug in Ayuvedic literature and still being used in folk medicine. Nowadays *C. asiatica* has many names and synonyms depend on the country where they grow. In English they were called “Indian Pennywort, “Hydrocotyle Asiatigue” in French “Idrocotyle” in Italian Tsubo-kusa in Japanese, “Tungchain and Luei Gong Gen” in Chinese and Bua-Bok in Thai (Brinkhaus et al., 2000).

Morphological Description

C. asiatica is a perennial herb with long stem, the leaves have long petioles arising in a rosette-like form a common base. The leaves are thin and soft and about 2-5 cm in diameter. The leaves are fleshy, orbicular-reniform and base cordate (Figure 5). The flowers are red, pink or white and looks like umbella. The flowers are seen between August and September. *C. asiatica* can be grown in many habitats preferably moist and shady. It can be harvested at any time of the year (Brinkhaus et al., 2000).

Chemical Components

Saponin containing triterpene acids and their sugar ester namely asiaticoside, asiatic acid, madecassoside and madecassic acid are found in *C. asiatica* (Figure 6). Moreover the leaves were found to contain 90 % water, 7 % carbohydrate and 2 % organic matter (Randriamampionona et al., 2007).

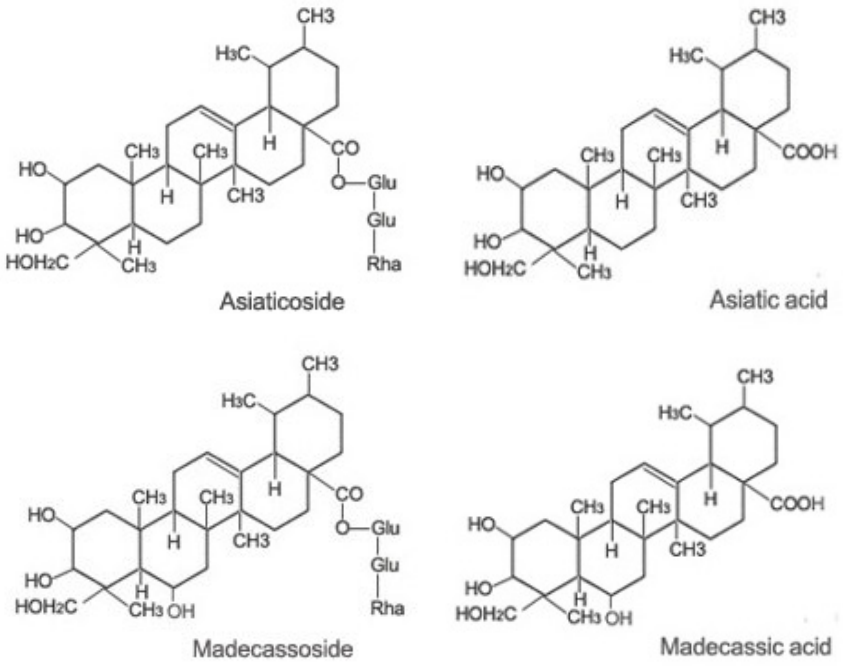


Figure 6 The chemicals structure of active compound; asiaticoside, asiatic acid, madecassoside and madecassic acid

Pharmacological Effects of *Centella asiatica*

C. asiatica is widely used in the field of traditional medicine for many purposes such as wound healing effect (Suguna, Sivakumar and Chandrakasan, 1996), antibacterial activity (Ahmand, Mehmood and Mohammand, 1998), antifungal activity (Minija and Thoppil, 2003), anti-inflammatory (Dunstan et al., 1997), antinociceptive effects (Somchit et al., 2004), antiproliferant effects (Sampson et al., 2001), antiulcer activity (Cheng and Koo, 2000), cytotoxic activity (Babu, Juttan and Padikkala, 1995), antioxidant (Gupta, Veerenda Kumar and Srivastava, 2003; Veerenda Kumar and Gupta, 2003) and other diseases. It's chemical

constituents, asiatic and madecassic acid are known to possess neuroprotective properties (Grimaldi et al., 1990). The beneficial of *C. asiatica* on the central nervous system was frequently reported. In the model of Pentylenetetrazole (PTZ) induced convulsion, administration of *C. asiatica* (300 mg/kg orally) into mice decreased the PTZ induced seizures and showed improvement in the learning deficit induced by PTZ seen as decreased seizure score and increased latencies in passive avoidance behavior (Gupta, Veerenda Kumar and Srivastava, 2003).

- Administration of *C. asiatica* at dose of 300 mg/kg/day for 60 days was able to reduce regional brain lipid peroxidation and protein carbonyl levels which were comparatively increased in age rats (Subathra et al., 2005).

- Administration of aqueous extract of *C. asiatica* (200 mg/kg body weight) for 15 days from post partum increased dendritic arborization of hippocampal CA3 neurons in young adult mice both at one month and six months. Significant improved learning and memory in radial arm maze and hole board test were noted (Rao, Chetana and Uma, 2005).

- In 2VO model, administration of *C. asiatica* at doses of 300, 1000 and 1500 mg/kg/day by oral route for 8 days significantly attenuated levels of MDA in brain. Accordingly, improved learning and memory in Morris water maze, step-down test of the 2VO mice were also reported (Doknark, 2003).

- Intracerebroventricular injection of steptozotocin (STZ) in rats has been associated with free radical generation. Administration of aqueous extract of *C. asiatica* at dose 200 and 300 mg/kg for 21 days significantly decreased oxidative stress parameters, MDA and increased level of antioxidant enzyme such as glutathione and catalase. Deficit in cognitive behavior in passive avoidance and plus-maze paradigms were improved in dose dependent manner (Veerenda and Gupta, 2003).

- In AD model of intracerebroventricular injection of $A\beta_{25-35}$ associated with free radical generation leading to oxidative stress, oral administration of asiaticoside at dose 5-25 mg/kg/day prevent learning and memory deficits in MWM and significantly decreased MDA and increased antioxidant activity of GSH and SOD levels suggesting a strong antioxidant property (Salaoh, 2003).

Pharmacokinetics of *Centella asiatica*

Previous study on pharmacokinetics of single or repeated administration of *C. asiatica* (30 or 60 mg orally) in twelve healthy human volunteers was carried out. It was found that the elimination half-life was 2 to 3 hours irrespective of the dose used. The peak plasma concentrations of asiatic acid, madecassic acid and asiaticoside, was reached in 2 to 4 hours. The peak plasma concentration, area under the curve (0 to 24 hours) and the plasma half life were significantly increased following repeated administration of *C. asiatica*. The triterpine constituents were minimally excreted via the kidney but can be excreted primarily in the feces in period of 24 to 76 hours (Grimaldi et al., 1990).

Standardized Extract of *Centella asiatica* ECa 233

A standardized extract of *C. asiatica* ECa 233 is a white to off-white titrated extract of *C. asiatica* containing triterpenoids not less than 80% and the ratio of madecassocide and asiaticoside is 1.5 ± 0.5 (Tantisira, 2009).

Pharmacological and Toxicological Evaluation of the Standardized Extract of *Centella asiatica* ECa 233

Ameliorating effects of orally given ECa 233 on learning and memory deficit in a transient bilateral common carotid artery occlusion (2VO) model was demonstrated in mice. Mice receiving ECa 233 at doses 10 and 30 mg/kg B.W. improved memory and learning impairments in MWM and step-down tests. MDA level in brain was reduced whereas no change in locomotor activities was observed (Tantisira, 2009).

ECa 233 was found to exhibit very favorable toxicological profiles in terms of acute toxicity and sub-chronic toxicity. In acute toxicity testing, 20 rats receiving ECa 233 up to 10 g/kg by oral route did not exhibit any toxic sign or death within the observation period of 14 days. Sub-chronic toxicity study in Wistar rats demonstrated that ECa 233 treated rat showed no difference in body weight, food consumption and health in comparison with control group. Furthermore, histopathological examination

of internal organ did not show any lesion in a dose-dependent manner. Thus, it was concluded that mice acutely treated with ECa 233 in the dose up to 10 g/kg and oral administration of ECa 233 in the doses of 10, 100 and 1,000 mg/kg for 90 days did not cause any significant changes in all parameters observed (Tantisira, 2009).

CHAPTER III

MATERIALS AND METHODS

1. Animals, Chemicals and Reagents

1.1 Animals

Male ICR weighing 25-35 g obtained from National Laboratory Animal Center at Salaya campus, Mahidol University, Nakornpathom, Thailand were housed in groups for four to five under controlled environmental conditions of a 12- h light/ dark cycle at 25±2 ^{o}C and allowed free access to standard food and tap water were provided *ad libitum*.

All experiments reported herein were conducted with an approval of Ethical committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

1.2 Chemicals and Reagents

-The standardized extract of *Centella asiatica* ECa 233 was kindly supplied by Assistant Professor Chamnan Patarapanich and co-workers, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

- Aβ fragment 25-35 (Sigma)
- Double distilled water
- Pentobarbital sodium (Nembutal®)
- Normal saline solution (NSS)
- Carboxymethylcellulose (CMC) (Fluka, Finland)
- Sodium hydrogen phosphate-2-hydrate (Sigma, U. S. A.)
- Sodium dihydrogen phosphate-2-hydrate (Sigma, U. S. A.)
- Thiobarbituric acid (Sigma, U. S. A.)
- N-butanol (Sigma, U. S. A.)
- Pyridine (Sigma, U. S. A.)
- Acetic acid (Sigma, U. S. A.)
- Sodium dodycyl sulfate (Sigma, U. S. A.)
- 1, 1, 3, 3-Tetraethoxy-propane (Malondialdehyde) (Sigma, U. S. A.)
- Cresyl violet (Sigma, U. S. A.)

- Xylene (TJ Baker, U. S. A.)
- Ethanol absolute (Merk, Germany)
- Permount (Sigma, U. S. A.)

2. Equipments and Instruments

- Stainless steel spoon
- Eppendoph plastic tubes
- Automatic pipettes
- Halmilton microsyringes 10 µl
- Stainless needle No. 27 length 3 mm
- Glass cylenders
- Erlenmayer flasks
- Syringes 1 cc.
- Needle No. 20 and 26
- Beaker
- Forceps
- Feeding tube
- Stop watches (Seiko)
- Slide and cover glass (China)
- pH meter (Sevenmuti, Switzerland)
- Incubators
- Water bath
- Centrifugeter (Sorvoll, GLC-2B, U. S. A.)
- Spectrophotometer (Shimadzu, UV1201, Japan)
- Cryostat (Leica, Germany)
- Refrigerators
- Electronic balance
- Vortex mixer
- Homogenizer (Glas-Col, Terre Haute,U. S. A.)
- Morris water maze set
- Step-down set
- Locomotor activity set (UGO Basile, Comerico, Italy)

- Nikon eclipse E 200 (Hollywood international LTD)

3. Experimental Designs

After acclimatization, mice were randomly divided into four groups of 10 animals each. One sham-operated group received 0.5% CMC orally at a dose of 0.3 ml/ 30 mg B.W., twice a day. Animals in groups 2-4 were treated with intracerebroventricular (i.c.v.) injection of β-amyloid peptide (25-35). Groups 2 received 0.5% CMC and groups 3-4 were administered orally by ECa 233 at the dose of 10 and 30 mg/kg B.W., twice a day (Figure 7).

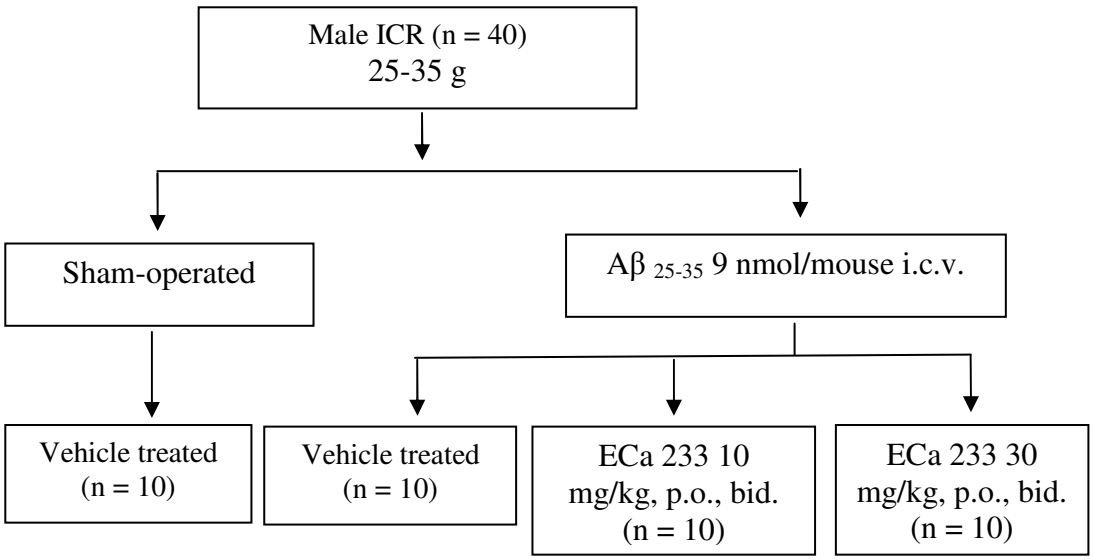


Figure 7 Experimental designs

Vehicle = 0.5% CMC (Carboxymethylcellulose)

ECa 233 = Standardized extract of *Centella asiatica*

4. Preparation of the Test Compound

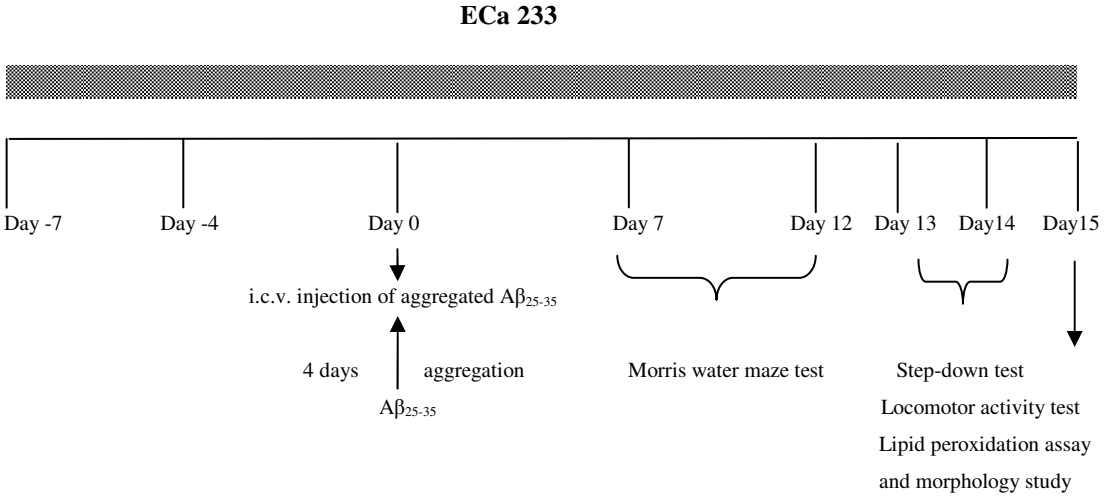
ECa 233 was suspended in 0.5% carboxymethylcellulose (0.5% CMC) solution. The test substance, ECa 233 at dose of 10 and 30 mg/kg, B.W. or 0.5% CMC was orally administered by gavage tube twice daily.

5. Experimental Protocol

As shown in Figure 8. ECa 233 at dose 10 and 30 mg/kg, B.W. were suspended in 0.5% CMC and were administered orally two times a day. $A\beta_{25-35}$ (9 nmol/mouse) dissolved in sterile double distilled water was prepared and being injected intracerebroventricularly into mice at day 0. The experiment was carried out in two different protocols. The first protocol, ECa 233 was administered orally for 23 consecutive days starting 7 days before injection of $A\beta_{25-35}$ and continued throughout experiment. The second protocol, ECa 233 was administered orally after an injection of $A\beta_{25-35}$ for 15 consecutive days.

Seven days after an injection of $A\beta_{25-35}$, we examined their behavioral change by Morris water maze (MWM) test followed by step-down test. In addition, locomotor activity tests were also performed. At the end of experiment, mice were decapitated and brain was removed for biochemical and histopathological analysis.

Protocol I (Pre-treatment)



Protocol II (Post-treatment)

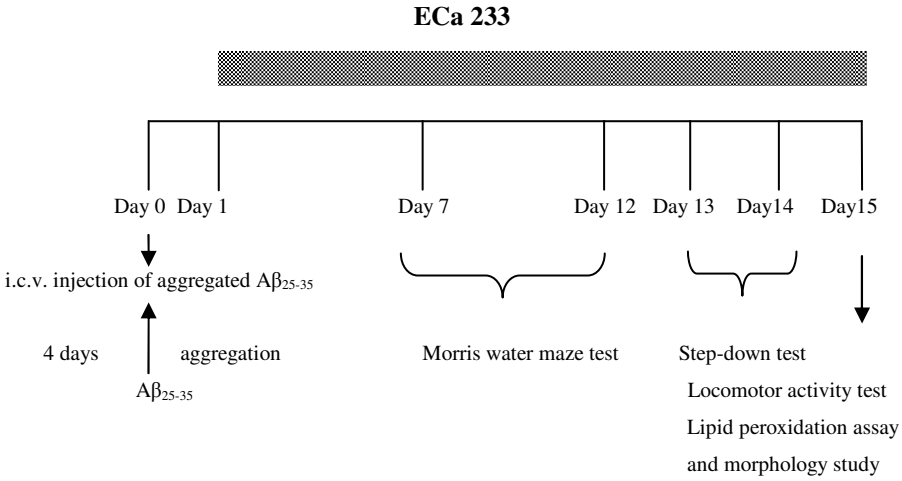


Figure 8 Experimental schedules of two experimental protocols. Upper panel shows the pre-treatment schedule and lower panel shows the post-treatment schedule.

6. Intracerebroventricular (i.c.v.) Injection of β-Amyloid Peptide

The $A\beta_{25-35}$ (Sigma) was dissolved in sterile double distilled water at a concentration of 1 mg/ml. They were incubated at 37 ^{o}C for 4 days (for aggregated or 'aged') and subjected to an examination by light microscopy to assess the formations of birefringent fibril-like structures and globular aggregates of aged $A\beta_{25-35}$ (Maurice, Lockhart and Privat, 1996). The aggregated form of $A\beta_{25-35}$ (9 nmol/mouse) was injected into intracerebroventricular (i.c.v.) by Halminton microsyringe with a 27

gauge stainless-steel with needle length of 3.0 mm. Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg) intraperitoneal. Needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, distance between the eyes and ears are equal, and perpendicular to the plane of the skull. Peptide or vehicle was delivered over 10 sec. Sixty sec. after injection mice exhibited normal behavior (Laursen and Belknap, 1986; Maurice, Lockhart and Privat, 1996).

7. Behavioral Testing

7.1 Determination of spatial learning and memory by Morris water maze (MWM)

Morris water maze (MWM) test was performed to investigate spatial memory of animal (D'Hooge and De Deyn, 2001). It consisted of a circular water tank, painting with black color, which was 70 cm in diameter and 13 cm depth of water was maintained at 25 ± 1 °C. A platform (6 cm diameter) was situated 1 cm below the surface of water. The pool was divided into four equal quadrants with the platform in a fixed location in one of the quadrants. The pool was placed in a test room and surrounded by many cues in a fixed position (Figure 9). Daily swimming consists of four trials in which the mice were placed in the water from four different starting quadrant points and given 60 sec to find the platform. Mice were allowed to rest on it for 10 sec. A maximum of 60 sec was allowed during which the mice had to find the platform and climb onto it for 10 sec. Latency to escape onto the hidden platform was recorded. This was conducted for 5 consecutive days. On the sixth day, each mouse was subjected to a 60 sec probe trial in which the platform was removed and the time spent in quadrant was recorded (Watanabe et al., 2003).

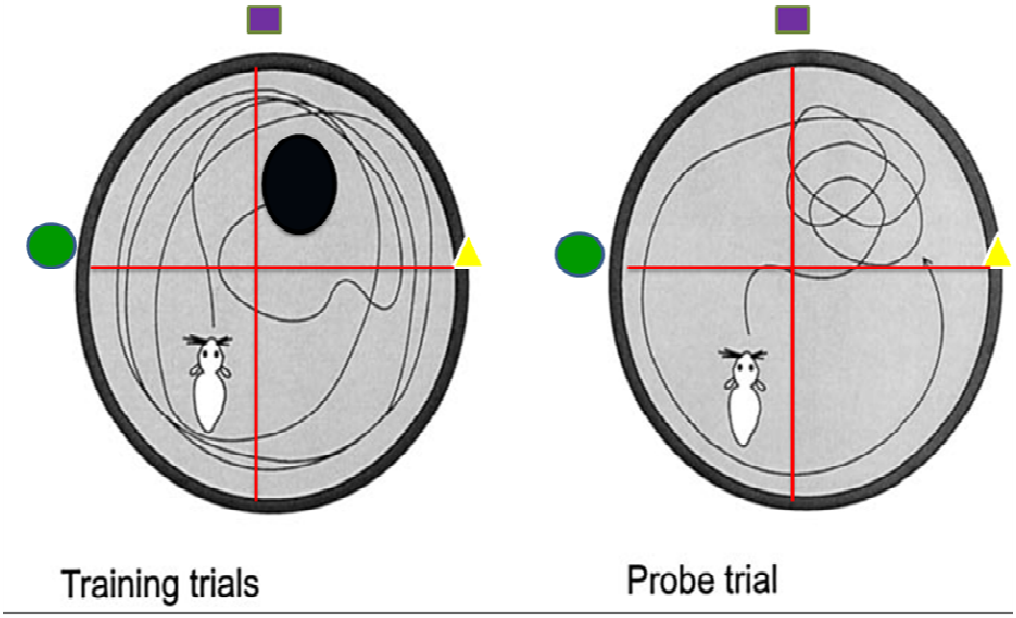


Figure 9 Equipment for Morris water maze test

7.2 Determination of passive avoidance by step-down test

The step-down test was examined to confirm the ability to learn and memory. An apparatus consisted of plexiglass chamber (Figure 10). The inside dimension of the chamber are, length 35 cm; width 23 cm; and height 20 cm. The grid floor was made of stainless steel (diameter 3 mm, gap 11 mm) and a plastic platform (5 cm diameter, 4 cm height) fixed in one corner. Electric shock (0.4 mA) was delivered to the grid floor with an isolated pulse stimulator .The procedure was divided acquisition trial and retention trial. Briefly, an acquisition trial, each mouse was placed on the platform to get adapted to environment for 3 minutes without electric shock. Then electric shocks were delivered to the grid, mice escaped from the grid floor back up onto the platform. The cut-off time was 300 sec. Twenty-four hours after training, mice were placed on the platform for the retention trial. The electric shocks were still delivered for 300 sec. The step-down latency (time taken to descent) and number of errors (number of descent) in 300 sec were recorded with improved retention reflected by increased latency and a reduction in errors (Luo, Yin and Wei, 2003).

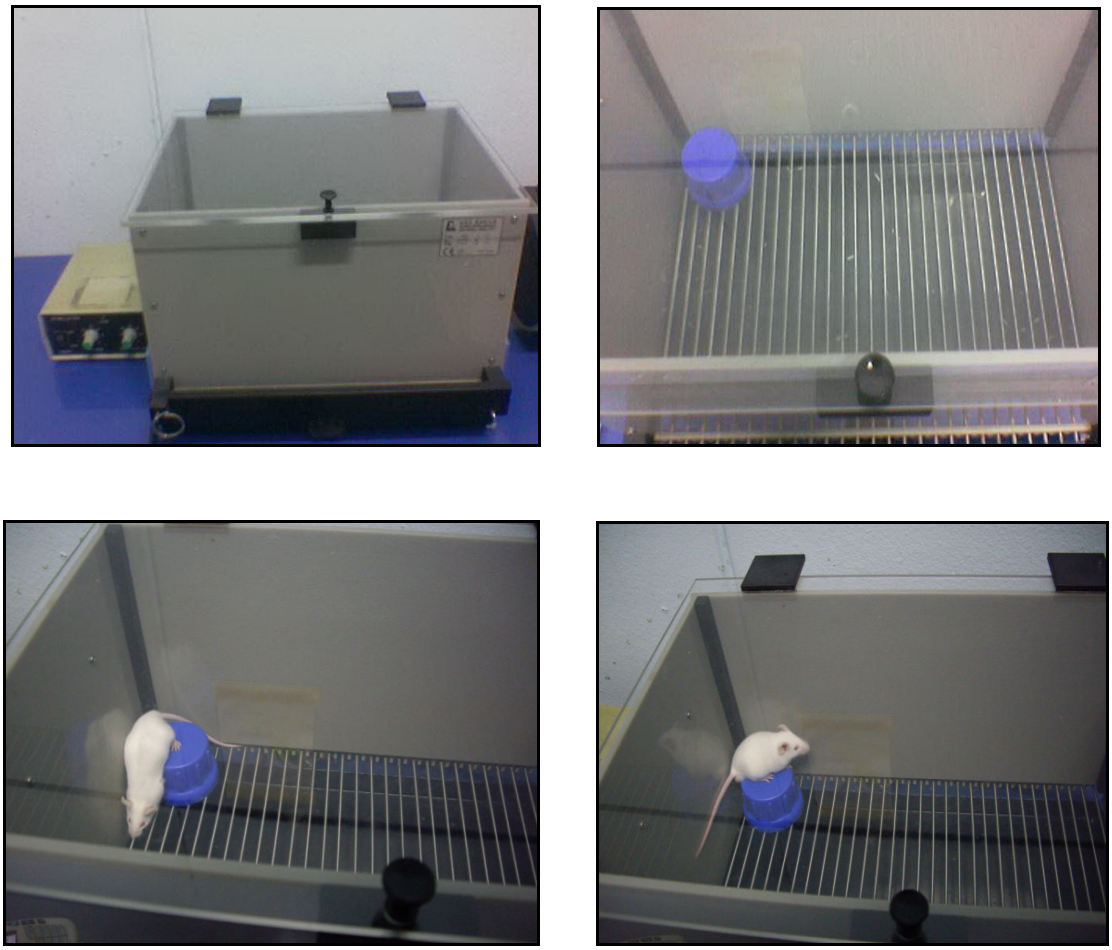


Figure 10 Equipment for step-down test

7.3 Determination of motor function by locomotor activity test

Locomotor activity was assessed to determine motor function. Each animal was placed in an activity cage (UGO Basile, Comerico, Italy) consisting of plexiglass chamber and counter (Figure 11). The inside dimension of the chamber are length 35 cm; width 23 cm; and height 20 cm. The grid floor was made of stainless steel (diameter 3 mm, gap 11 mm), connecting to the circuit of counting unit. The numbers or counts of movements were recorded by the electric counting machine for 5 minutes intervals. The apparatus was placed in light, sound attenuated and ventilated testing room with other behavioral testing apparatus (Gupta, Veerenda Kumar and Srivastava, 2003).

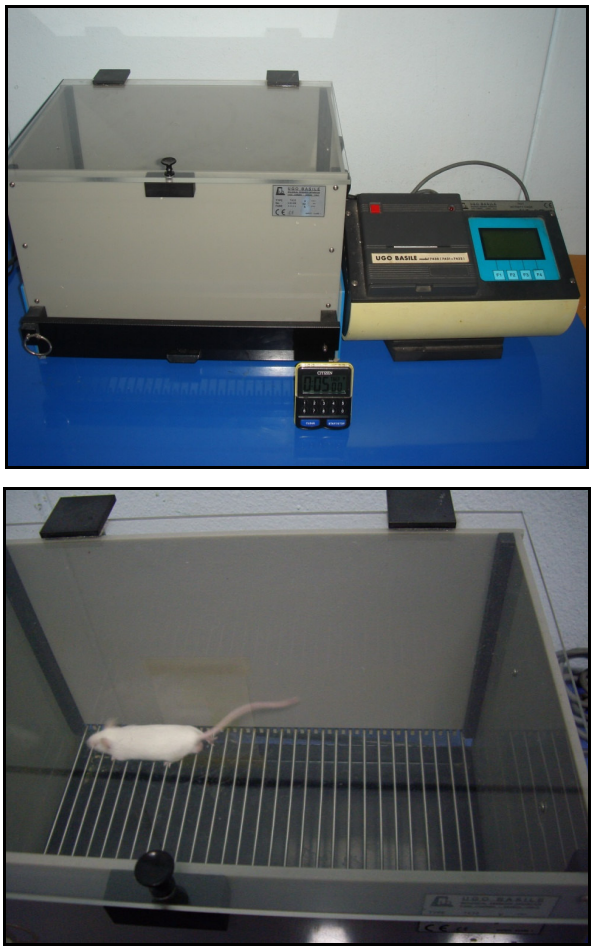


Figure 11 Equipment for locomotor activity test

After behavioral testing in day 15 after $A\beta_{25-35}$ injection, mice were decapitate and wash with ice-cold saline then kept at -80 ^{o}C for examination level of lipid peroxidation by biochemical assay and histological by cresyl violet staining technique.

8. Biochemicals Analysis

8.1 Lipid peroxidation assay

8.1.1Tissue 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 lipid peroxidation.

8.1.2 Measurement of lipid peroxidation

Malondialdehyde (MDA) used as an indicator of lipid peroxidation, was measured as describe by Gupta, Veerenda Kumar and Srivastava (2003). The reagent, 1.5 ml acetic acid (20%), 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodiumdodecyl sulphate (8.1%) were added to 0.1 ml of tissue samples, and then heated at 100 ^{o}C for 60 minutes. After the mixture was cooled with tap water and added 5 ml of *n*-butanol : pyridine (15 : 1) and 1 ml of distilled water and then vortex vigorously. Then the tissue samples were centrifuged at 2500 rpm for 20 min, supernatant were separated and measure by using a spectrophotometer at absorbance 532 nm. The concentration of MDA was expressed as µmol/g tissue (Gupta, Veerenda Kumar and Srivastava, 2003).

9. Histological Examination

After the completion of the behavioral experiment, four animals from each group were used for investigation of neuronal damage in hippocampal formation (CA1 and CA3) with cresyl violet staining technique (Figure 12). The mice were decapitated then removed whole brain and quickly frozen in dry ice. Subsequently, coronal sections (10 µm thick) were cut at the level of hippocampus (approximately 1.5 mm caudal to the bregma) by using cryostat and stained with 1 % cresyl violet for the microscopic observation. Evaluation of cell loss was determined by calculating the density of surviving pyramidal neurons in the hippocampus (CA1 and CA3) by using a computerized image analysis system (Nikon Eclips 200, software: Image Pro Plus 6.1). The subfields of hippocampus (CA1 and CA3) were photographed at 40x magnifications, after background subtraction and gray scale threshold determination. The area of surviving pyramidal neurons was computed as percentage of the total area (8574 µm^2). Measurements were carried out on both ipsi- and contralateral of the hippocampus. Average the percentage area of surviving pyramidal neurons on 3 sections per animal and the average were used for further statistical analysis (Farkas et al., 2006; He et al., 2008).

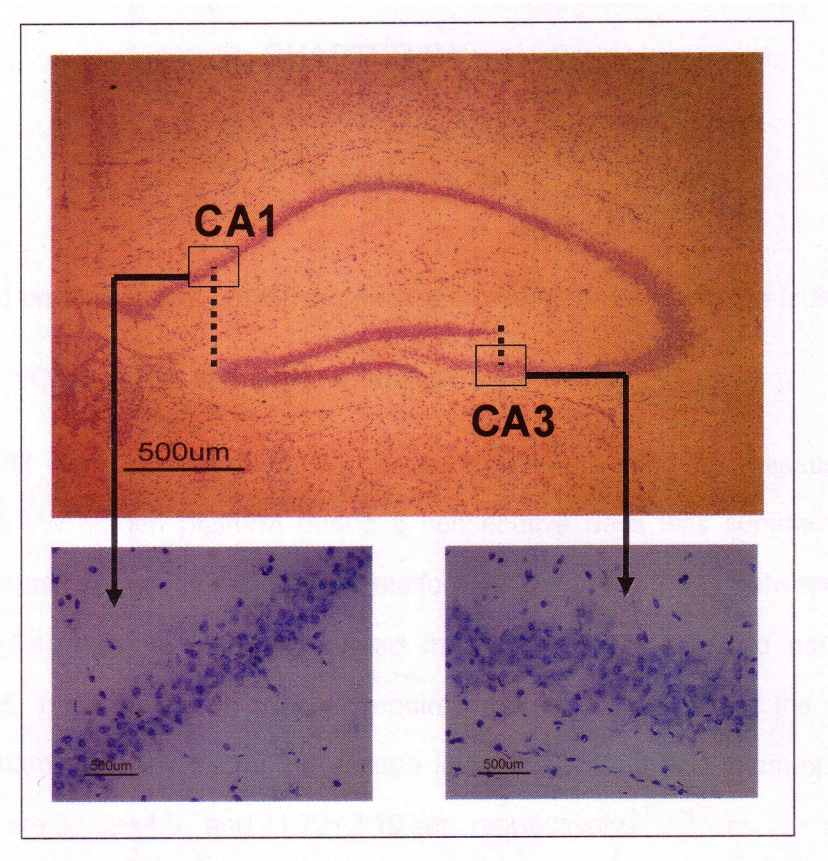


Figure 12 Selection areas of CA1 and CA3 in hippocampus

10. Statistical Analysis

The data of behavioral, biochemical test and morphology study are expressed as mean ± S.E.M. Statistical analysis was carried out by one-way analysis of variance. Tukey's HSD tests were used for post hoc comparison with $p < 0.05$ was considered to indicate statistical significance.

CHAPTER IV

RESULTS

The present study aimed to investigate the effects of the standardized extract of *Centella asiatica* ECa 233 on cognitive deficits induced by an intracerebroventricular (i.c.v.) injection of β-amyloid peptide (25-35) into male ICR mice.

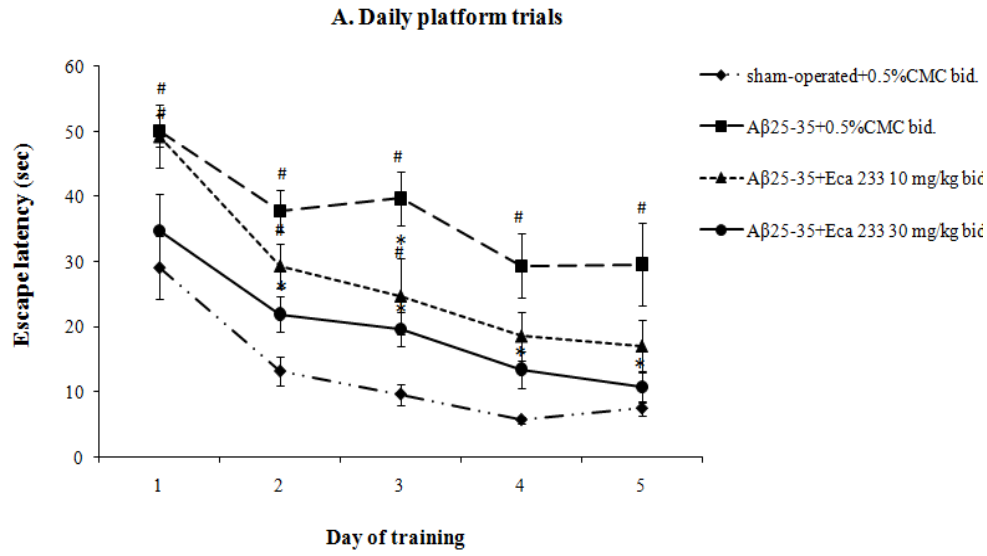
I. Effects of ECa 233 given by pre-treatment protocol

1. Effects of ECa 233 on learning and memory deficit induced by $A\beta_{25-35}$

1.1 Morris water maze test

As shown in Figure13, $A\beta_{25-35}$ treated mice significantly demonstrated impairment in learning and memory in MWM task with escape latency of 29.60 ± 6.32 sec. in day 5 while the respective value in sham-operated group was found to be 7.46 ± 1.18 sec. Accordingly in probe trial on day 6, $A\beta_{25-35}$ treated mice were comparatively found to spend lesser time in the quadrant where the platform was removed than did the sham-operated group. ECa 233 at dose of 10 and 30 mg/kg, B.W., p. o., twice daily given 7 days before the injection of β-amyloid and throughout experiment could significantly improved the learning and memory deficit induced by $A\beta_{25-35}$. Mice receiving ECa 233 at dose of 10 mg/kg, B.W. significantly the escape latency on day 3 with the escape latency of 24.73 ± 5.86 sec. At the dose of 30 mg/kg, B.W. significant difference was detected on day 2, 3, 4 and 5 with the escape latency of 21.98 ± 2.74 sec, 19.60 ± 2.60 sec, 13.49 ± 2.82 sec and 10.77 ± 2.44 sec, respectively (Figure 13A). In probe trial, the percentage of time spent in the target quadrant were 34.59 ± 1.33 %, 22.14 ± 1.79 %, 29.35 ± 2.18 % and 29.68 ± 1.35 % for sham-operated group, $A\beta_{25-35}$ treated group and the group receiving ECa 233 at dose 10 and 30 mg/kg, B.W., p. o., twice daily, respectively (Figure 13B).

A).



B).

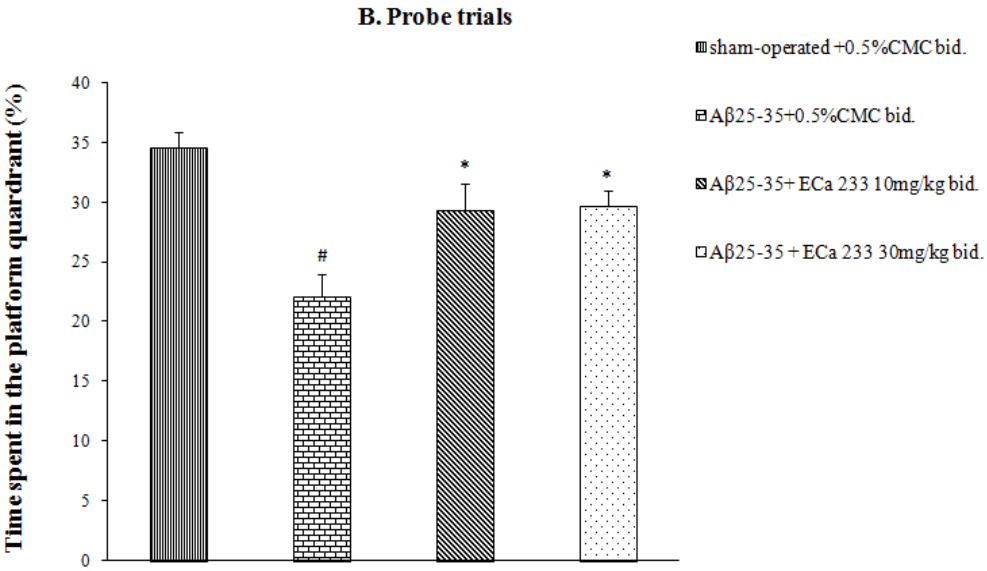
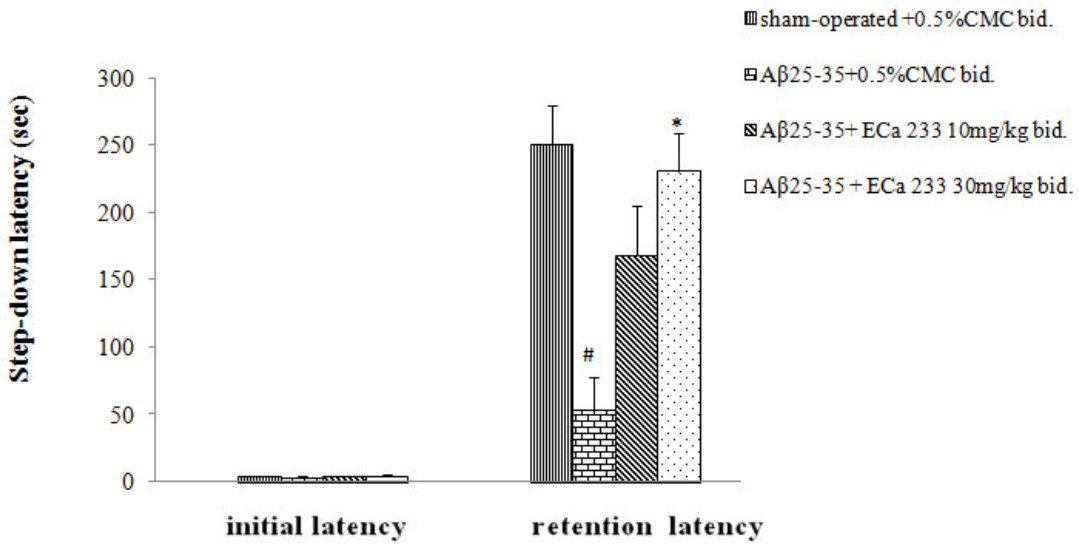


Figure 13 Effects of ECa 233 given by pre-treatment protocol, on learning and memory deficit induced by $A\beta_{25\text{-}35}$ in Morris water maze test. The hidden platform trials (A) were carried out on day 7-12 after injection of β-amyloid peptide (25-35). The probe trial (B) was carried out on day 12 after the β-amyloid peptide (25-35) injection. Each data points represents the mean ± S.E.M. of values from 10 mice (n = 10). $^{\#}P<0.05$ denotes statistically significant difference from sham-operated group. $^{*}P<0.05$ denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

1.2 Step-down test

Step-down latency and number of errors were recorded in step-down test carried out on day 13-14 after the injection of β-amyloid peptide (25-35). As shown in Figure 14. $A\beta_{25-35}$ treated mice significantly demonstrated impairment in learning and memory with step-down latencies of 53.04 ± 24.92 sec while the values in sham-operated group were 249.66 ± 29.80 sec. Accordingly, number of error in $A\beta_{25-35}$ treated mice were comparatively higher than those of the sham-operated (2.90 ± 0.45 vs 0.70 ± 0.33). Administration of ECa 233 significantly improved performance in step-down test in terms of both latency and errors (Figure 14A and 14B). In comparison to $A\beta_{25-35}$ treated group longer step-down latency was observed in both 10 or 30 mg/kg B.W. ECa 233 treated group (167.94 ± 37.10 sec and 230.10 ± 28.98 sec, respectively). However, a statistical difference was demonstrated only in higher dose of ECa 233 (30 mg/kg B.W.) while both doses of ECa 233 significantly improved number of error of $A\beta_{25-35}$ treated group.

A).



B).

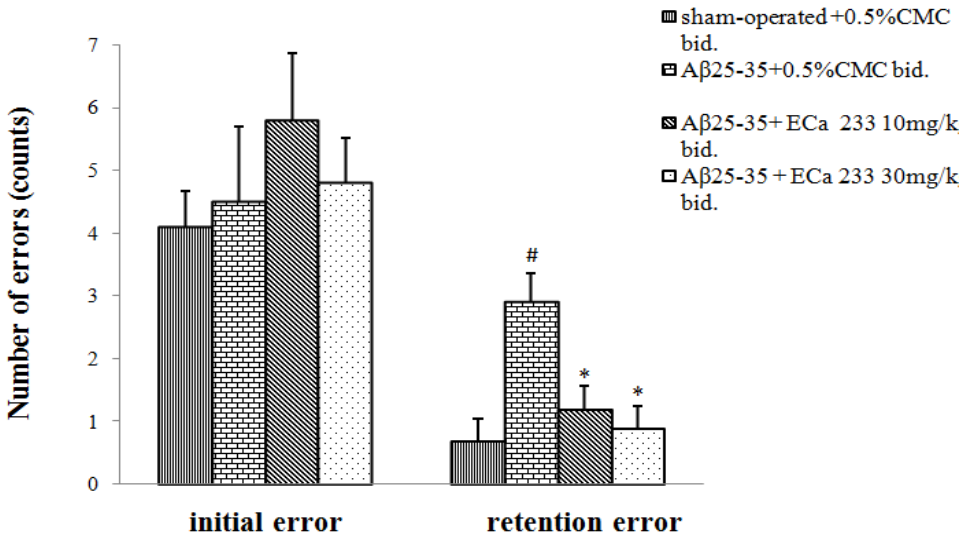


Figure 14 Effects of ECa 233 given by pre-treatment protocol, on step-down passive avoidance task. Step-down latency (A) and number of errors (B). The task was carried out on day 13-14 after the start of β-amyloid peptide injection. Each data points represents the mean ± S.E.M. of values from 10 mice (n = 10). # P<0.05 denotes statistically significant difference from sham-operated group. * P<0.05 denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

2. Effects of ECa 233 on locomotor activity

By using an activity cage, locomotor activity of each group of the animals was measured. Neither the i.c.v. administration of $A\beta_{25-35}$ nor the oral administration of ECa 233 at all test dose exerted any significant effects on locomotor activity of the mice (Figure 15).

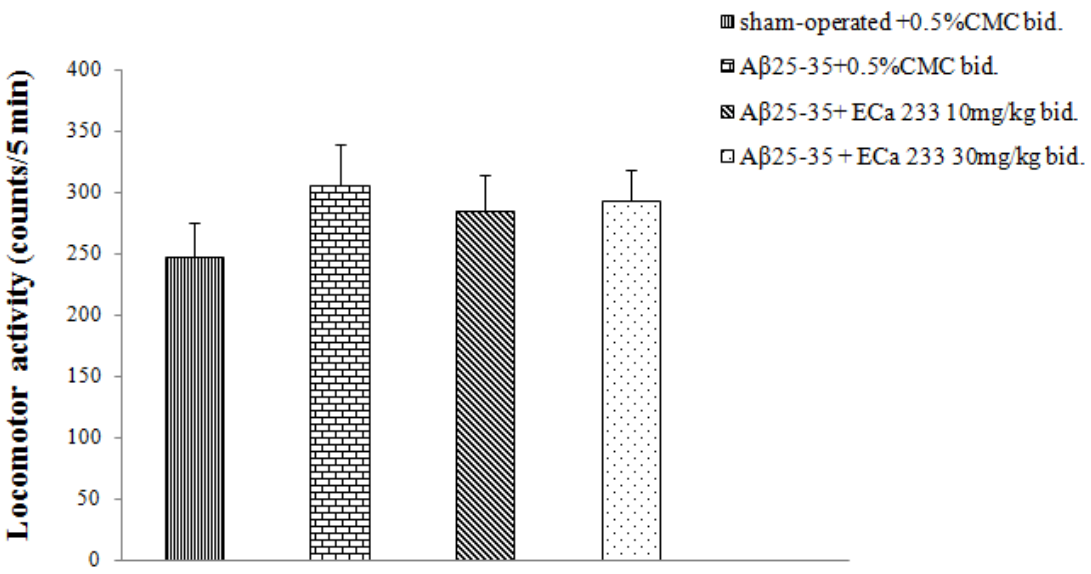


Figure 15 Effects of ECa 233 given by pre-treatment protocol, on locomotor activity. The task was carried out on day 15 after the start of β-amyloid peptide injection. Columns indicate mean ± S.E.M. of values from 10 mice (n=10). # P<0.05 denotes statistically significant difference from sham-operated group. * P<0.05 denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

3. Effects of ECa 233 on levels of brain malondialdehyde

Level of brain lipid peroxidation was expressed in term of MDA. In $A\beta_{25-35}$ treated group MDA level was significantly increased in comparison with those of sham-operated group. MDA level of $A\beta_{25-35}$ and sham-operated group were 41.86 ± 2.93 and 22.58 ± 1.70 µmol/g tissue, respectively. Administration of ECa 233 at dose 10 and 30 mg/kg, twice a day significantly reduced MDA levels to 23.28 ± 2.50 and 17.61 ± 3.19 µmol/g tissues, respectively (Figure 16).

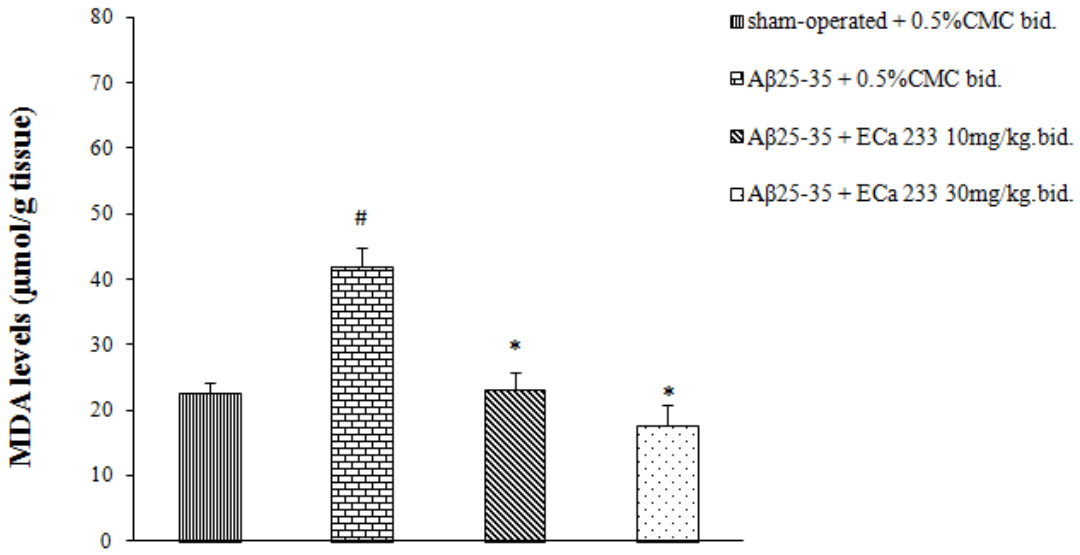


Figure 16 Effects of ECa 233 given by pre-treatment protocol, on levels of brain malondialdehyde in $A\beta_{25-35}$ injected mice. Columns indicate mean ± S.E.M. of values from 6 mice (n=6). $^{\#}$ $P<0.05$ denotes statistically significant difference from sham-operated group. * $P<0.05$ denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

4. Effects of ECa 233 on CA1 and CA3 pyramidal neurons in hippocampal area

Photographs of neurons in the hippocampal CA1 and CA3 regions were illustrated in Figure 17. Cresyl violet staining was employed to examine a histological change. In the hippocampus marked morphological changes were visualized in $A\beta_{25-35}$ group. The brain of mice treated with $A\beta_{25-35}$ exhibited a significant cell loss in the hippocampal CA1 and CA3 areas. The neuronal densities in the CA1 subfield both in ipsilateral and contralateral were reduced to 67.14 ± 9.12 % and 70.91 ± 4.47 % of the values of sham-operated group, respectively. While, the neuronal densities in the CA3 subfield both in ipsilateral and contralateral were 67.85 ± 3.93 % and 67.51± 7.80 % of the values for sham-operated group, respectively. Mice received ECa 233 at dose of 10 and 30 mg/kg, B.W., p. o., twice daily given 7 days before the injection of β-amyloid and throughout experiment could attenuated $A\beta_{25-35}$ induced cell loss in CA1 subfield. Density of CA1 neurons in 10 mg/kg B.W. ECa 233 were found to be 94.12 ± 6.04 % and 81.03 ± 10.18 % in ipsi- and contralateral whereas the corresponding values for CA3 neurons were 90.99 ± 5.08 % and 85.25 ± 11.00 %. Similar effects were noted in 30 mg/kg B.W. ECa 233 treated group. Density of CA1 were found to be 91.45 ± 4.47 % and 91.42 ± 2.20 % in ipsi-and contralateral whereas the corresponding values for CA3 neurons were 98.11± 3.84 % and 91.22 ± 1.55 % (Figure 18A). Since response profile of cell loss observed in both ipsi- and contralateral CA1 and CA3 regions were rather similar in all treatment groups, thus, the density of neuronal cells on both sides were summated and subsequently compared for the difference among groups (Figure 18B). The results indicate that an i.c.v. injection of $A\beta_{25-35}$ induced neuronal degeneration in CA1 and CA3 regions which could be prevented by an orally given ECa 233.

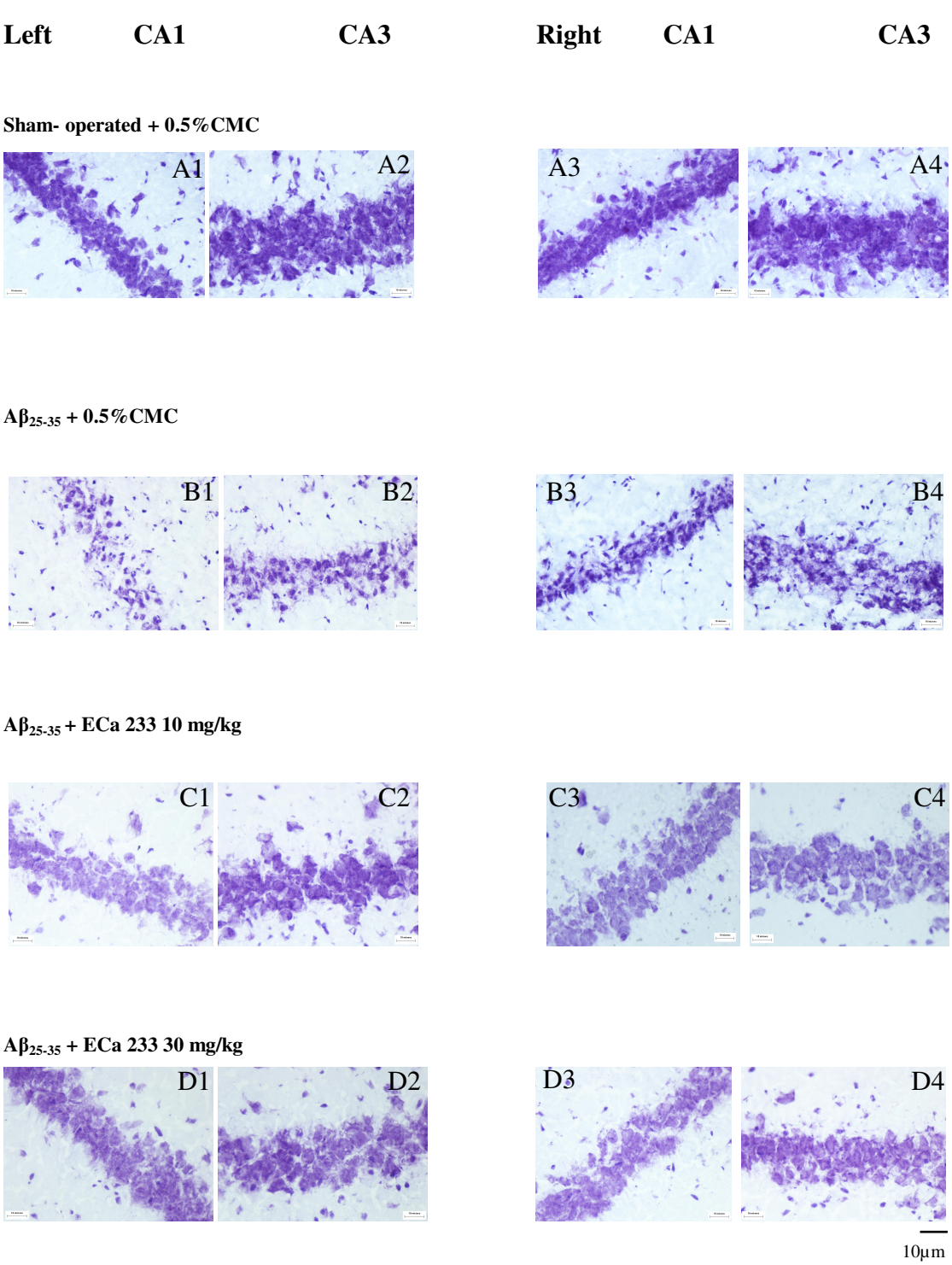
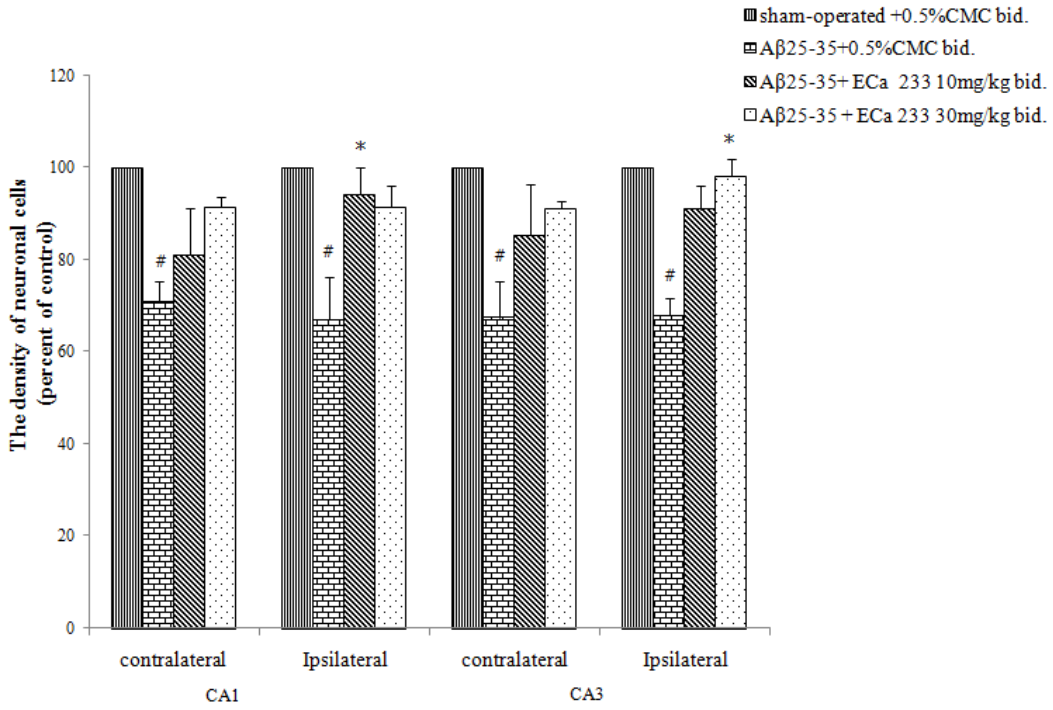


Figure 17 Cresyl violet staining of CA1 and CA3 subfields in transverse left and right hippocampal region given by pre-treatment protocol. A1-A3 in sham-operated + 0.5% CMC, B1-B4 in $A\beta_{25-35}$ + 0.5% CMC, C1-C4 in $A\beta_{25-35}$+ ECa 233 10 mg/kg bid. and D1-D4 in $A\beta_{25-35}$+ ECa 233 30 mg/kg bid. Magnification : 40x. Scale bar are 10µm.

A.)



B.)

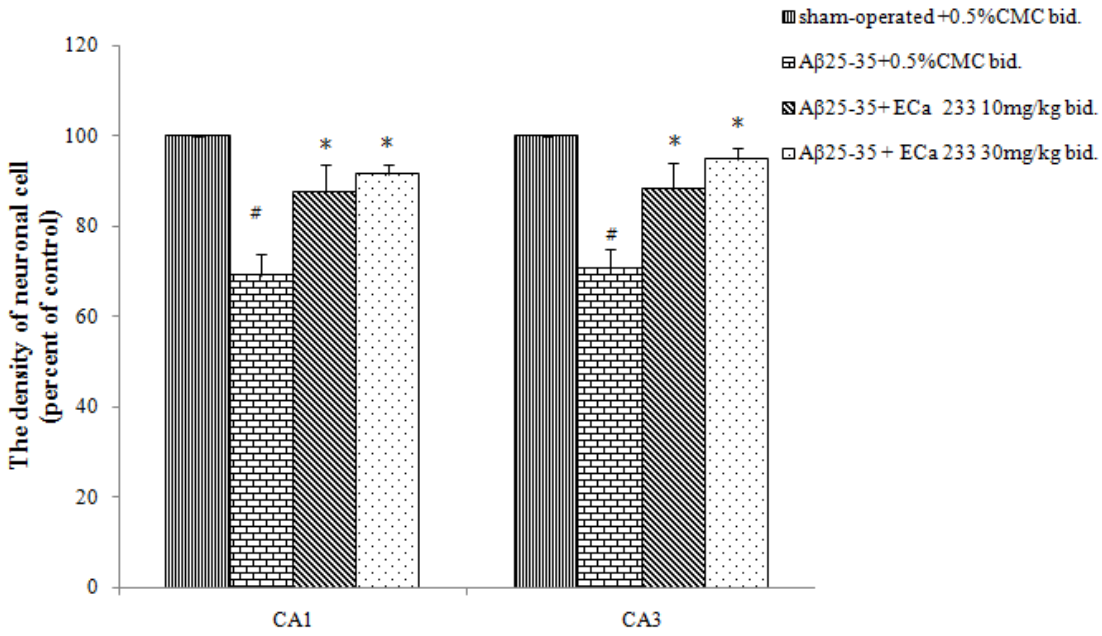


Figure 18 Effects of ECa 233 given by pre-treatment protocol, on density of CA1 and CA3 pyramidal neurons in hippocampal area. Columns indicate mean ± S.E.M. of values from 4 mice (n=4). # P<0.05 denotes statistically significant difference from sham-operated group. * P<0.05 denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

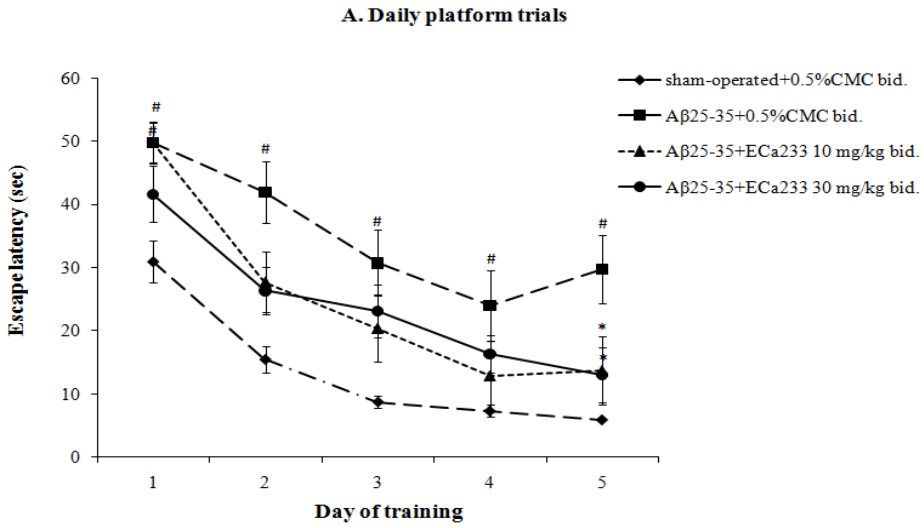
II. Effects of ECa 233 given by post-treatment protocol

1. Effects of ECa 233 on learning and memory deficit induced by $A\beta_{25-35}$

1.1 Morris water maze test

$A\beta_{25-35}$ treated mice significantly demonstrated impairment in learning and memory in MWM task with escape latency of 29.72 ± 5.39 sec in day 5 while the respective value in sham-operated group was found to be 5.92 ± 0.90 sec. In addition in probe trial on day 6, $A\beta_{25-35}$ treated mice were comparatively found to spend lesser time in the quadrant where the platform was removed than did the sham-operated group. Administration of ECa 233 at dose of 10 and 30 mg/kg, B.W., p. o., twice daily given for 15 consecutive days after the injection of β-amyloid could significantly improved the learning and memory deficit induced by $A\beta_{25-35}$. Mice receiving ECa 233 at dose of 10 and 30 mg/kg, B.W. significantly the escape latency on day 5 with the escape latency of 13.70 ± 4.40 sec and 12.95 ± 3.56 sec, respectively (Figure 19A). In probe trial, the percentage of time spent in the target quadrant were 33.04 ± 1.06 %, 24.47 ± 1.37 % , 28.47 ± 1.44 % and 30.30 ± 0.88 % for sham-operated group, $A\beta_{25-35}$ treated group and the group receiving ECa 233 at dose 10 and 30 mg/kg, B.W., p. o., twice daily, respectively (Figure 19B).

A).



B).

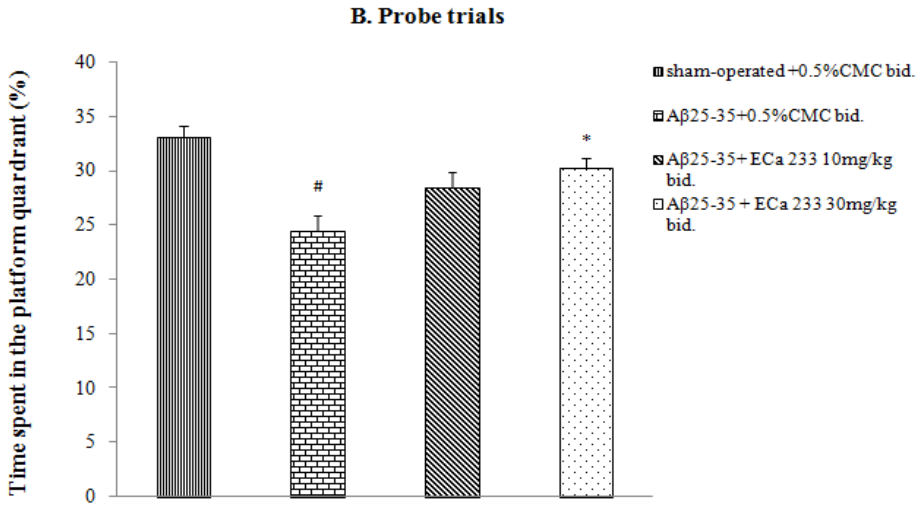


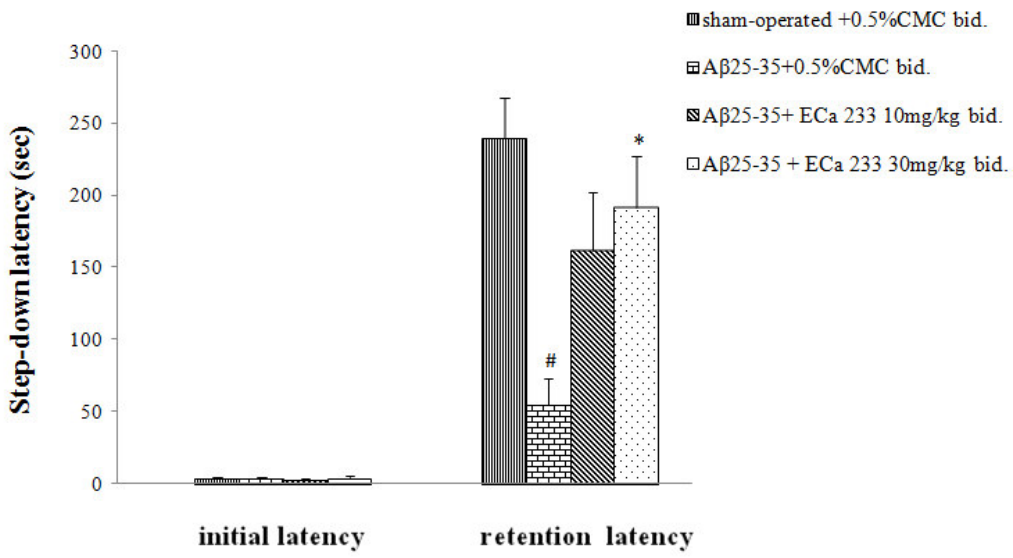
Figure 19 Effects of ECa 233 given by post-treatment protocol, on learning and memory deficit induced by Aβ $_{25\text{-}35}$ in Morris water maze test. The hidden platform trials (A) were carried out on day 7-12 after injection of β-amyloid peptide (25-35). The probe trial (B) was carried out on day 12 after the β-amyloid peptide (25-35) injection. Each data points represents the mean ± S.E.M. of values from 10 mice (n = 10). $^{\#}$P<0.05 denotes statistically significant difference from sham-operated group.

* P<0.05 denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

1.2 Step-down test

As shown in Figure 20, $A\beta_{25-35}$ treated mice significantly demonstrated impairment in learning and memory with step-down latencies of 54.60 ± 18.61 sec while the values in sham-operated group were 240.00 ± 27.59 sec. Accordingly, number of error in $A\beta_{25-35}$ treated mice were comparatively higher than those of the sham-operated group (3.60 ± 0.70 vs 0.80 ± 0.29). Administration of ECa 233 significantly improved performance in step-down test in terms of both latency and errors (Figure 20A and 20B). In comparison to $A\beta_{25-35}$ treated group, longer step-down latency was observed in both 10 or 30 mg/kg B.W. ECa 233 treated group (161.52 ± 40.69 sec and 191.10 ± 35.74 sec, respectively). However, a statistical difference was demonstrated only in higher dose of ECa 233 (30 mg/kg B.W.) while both doses of ECa 233 significantly improved number of error of $A\beta_{25-35}$ treated group.

A).



B).

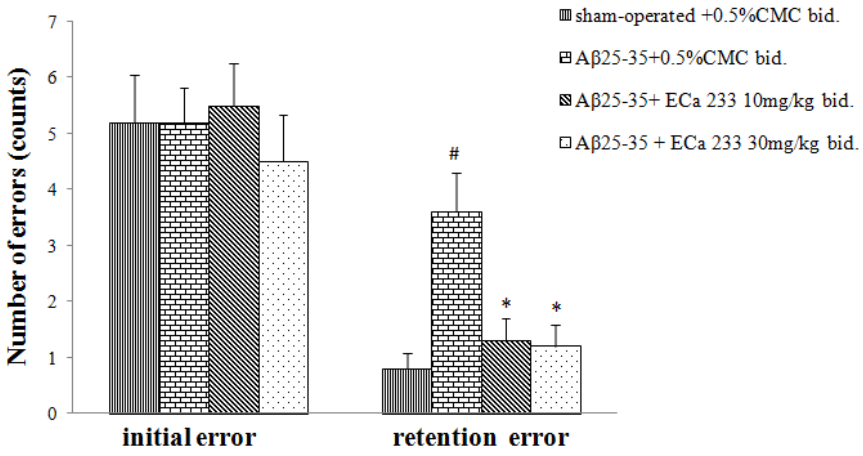


Figure 20 Effects of ECa 233 given by post-treatment protocol, on step-down passive avoidance task. Step-down latency (A) and number of errors (B). The task was carried out on day 13-14 after the start of β-amyloid peptide injection. Each data points represents the mean ± S.E.M. of values from 10 mice (n = 10). $^{\#}$ $P<0.05$ denotes statistically significant difference from sham-operated group. * $P<0.05$ denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

2. Effects of ECa 233 on locomotor activity

Neither the i.c.v. administration of $A\beta_{25-35}$ nor the oral administration of ECa 233 at all test dose exerted any significant effects on locomotor activity of the mice (Figure 21).

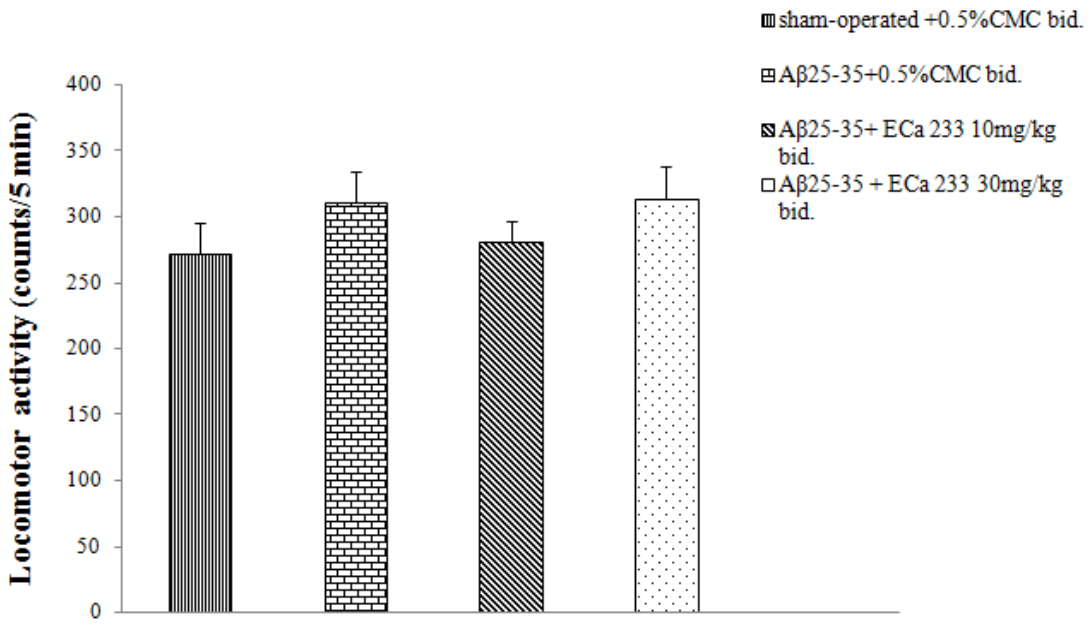


Figure 21 Effects of ECa 233 given by post-treatment protocol, on locomotor activity. The task was carried out on day 15 after the start of β-amyloid peptide injection. Columns indicate mean ± S.E.M. of values from 10 mice (n=10). # P<0.05 denotes statistically significant difference from sham-operated group. * P<0.05 denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

3. Effects of ECa 233 on levels of brain malondialdehyde

In $A\beta_{25-35}$ treated group MDA level was significantly increased in comparison with those of sham-operated group. MDA level of $A\beta_{25-35}$ treated group and sham-operated group were 64.58 ± 8.484 and 20.48 ± 2.65 µmol/g tissues, respectively. Administration of ECa 233 at dose 10 and 30 mg/kg, twice a day significantly reduced MDA levels to 38.93 ± 6.26 and 35.46 ± 5.33 µmol/g tissues, respectively (Figure 22).

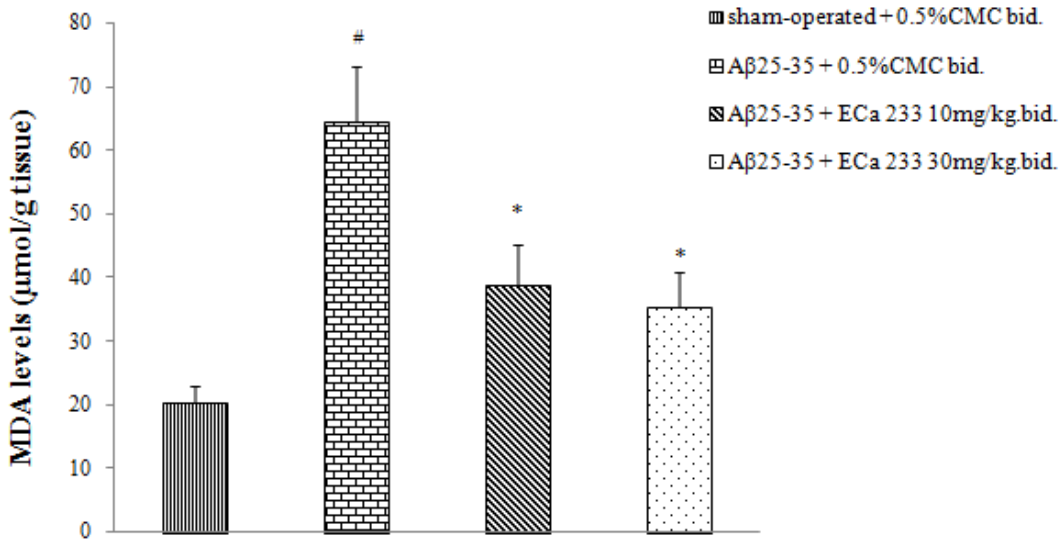


Figure 22 Effects of ECa 233 given by post-treatment protocol, on levels of brain malondialdehyde in $A\beta_{25-35}$ injected mice. Columns indicate mean ± S.E.M. of values from 6 mice (n=6). $^{\#}$ $P<0.05$ denotes statistically significant difference from sham-operated group. * $P<0.05$ denotes statistically significant difference from β amyloid peptide (25-35) injected group.

4. Effects of ECa 233 on CA1 and CA3 pyramidal neurons in hippocampal area

Photographs of neurons in the hippocampal CA1 and CA3 regions were shown in Figure 23. The brain of mice treated with $A\beta_{25-35}$ exhibited a significant cell loss in the hippocampal CA1 and CA3 areas. The neuronal densities in the CA1 subfield both in ipsilateral and contralateral were reduced to 67.64 ± 6.79 % and 69.20 ± 10.00 % of the values of sham-operated group, respectively. While, the neuronal densities in the CA3 subfield both in ipsilateral and contralateral were 69.31 ± 6.60 % and 63.03 ± 5.37 % of the values of sham-operated group, respectively. Administration with ECa 233 at dose of 10 and 30 mg/kg, B.W., p. o., twice daily given for15 consecutive days after the injection of $A\beta_{25-35}$ in CA1 subfield. Density of CA1 neurons in 10 mg/kg B.W. ECa 233 were found to be 84.64 ± 5.68 % and 81.36 ± 8.52 % in ipsi- and contralateral whereas the corresponding values for CA3 neurons were 81.91 ± 3.51 % and 80.10 ± 9.88 %. Similar effects were noted in 30 mg/kg B.W. ECa 233 treated group. Density of CA1 were found to be 93.73 ± 3.82 % and 86.76 ± 2.58 % in ipsi-and contralateral whereas the corresponding values for CA3 neurons were 80.50 ± 4.66 % and 88.48 ± 3.99 % (Figure 24A). Since response profile of cell loss observed in both ipsi- and contralateral CA1 and CA3 regions were rather similar in all treatment groups, thus, the density of neuronal cells on both sides were summated and subsequently compared for the difference among groups (Figure 24B). These data indicate that an i.c.v. injection of $A\beta_{25-35}$ induced neuronal degeneration in CA1 and CA3 regions which could be prevented by an orally given ECa 233.

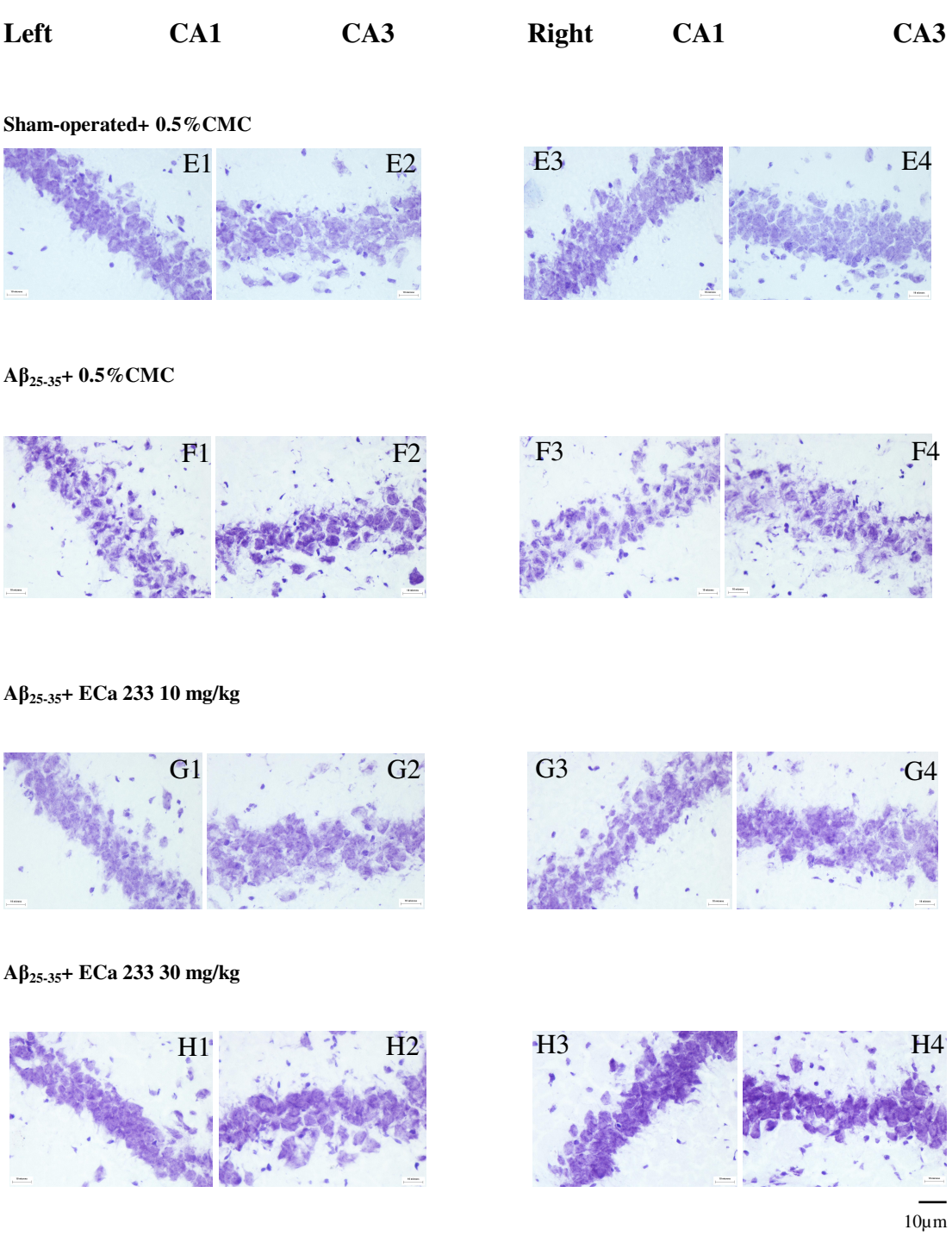
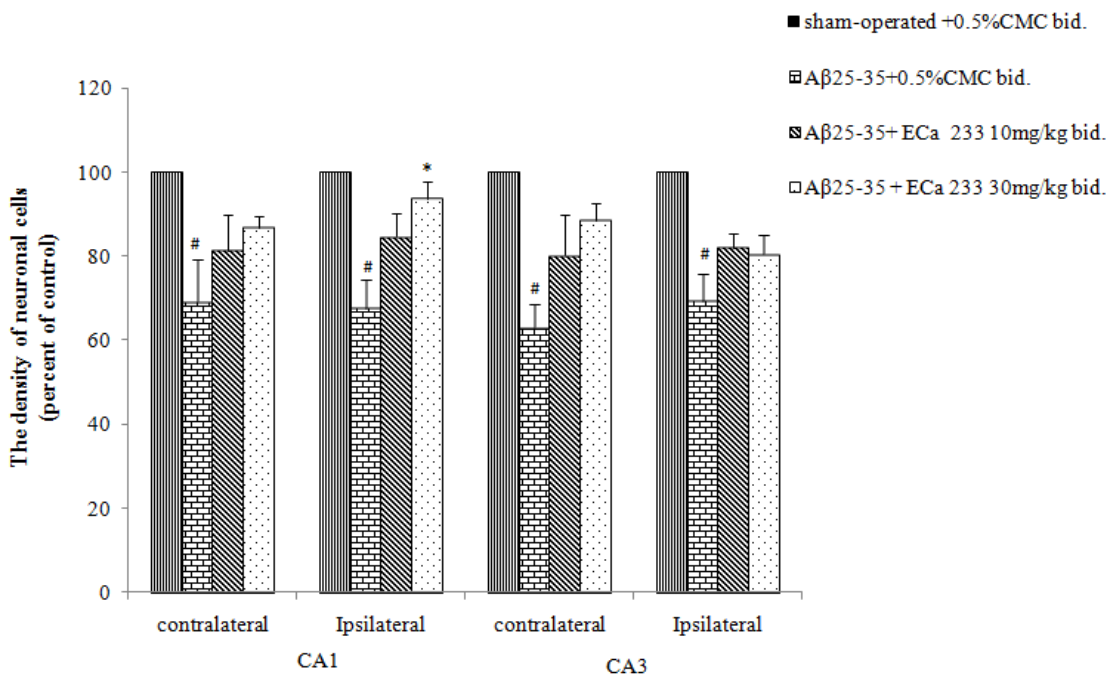


Figure 23 Cresyl violet staining of CA1 and CA3 neurons in transverse left and right hippocampal region given by post-treatment protocol. E1-E3 in sham-operated+0.5% CMC, F1-F4 in $A\beta_{25-35}$ + 0.5% CMC, G1-G4 in $A\beta_{25-35}$+ ECa 233 10 mg/kg bid. and H1-H4 in $A\beta_{25-35}$ + ECa 233 30 mg/kg bid. Magnification : 40x. Scale bar are 10μm.

A.)



B.)

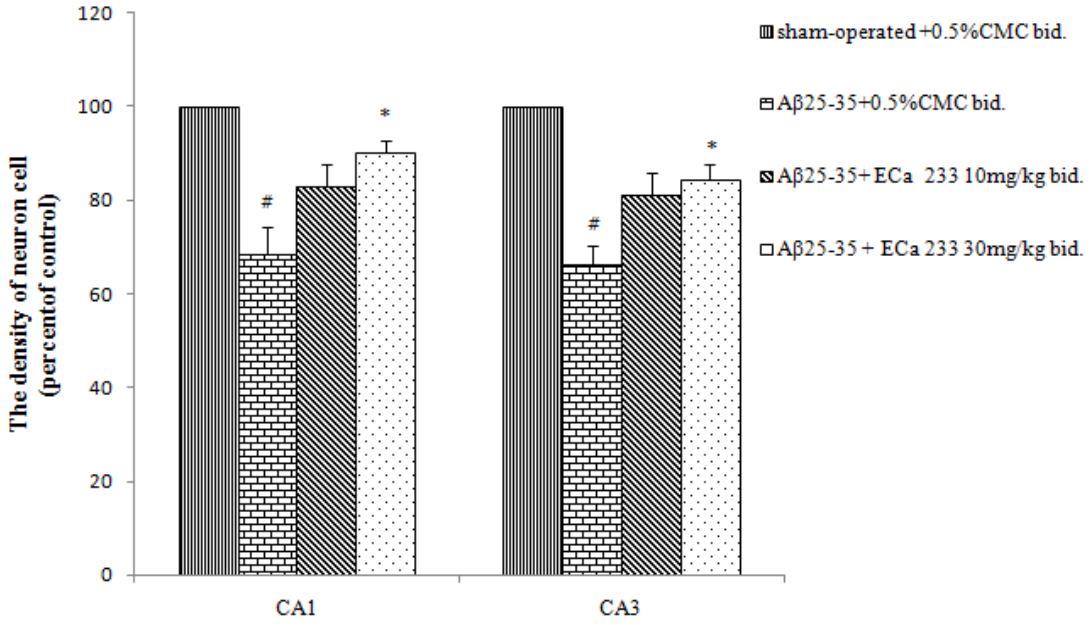


Figure 24 Effects of ECa 233 given by post-treatment protocol, on density of pyramidal cell CA1and CA3 in hippocampal area. Columns indicate mean ± S.E.M. of values from 4 mice (n=4). # P<0.05 denotes statistically significant difference from sham-operated group. * P<0.05 denotes statistically significant difference from β-amyloid peptide (25-35) injected group.

CHAPTER V

DISCUSSION AND CONCLUSION

There are many evidences supporting that amyloid β-peptide (Aβ) may play a role in the progression of Alzheimer's disease (Behl, 1997). Potentially Aβ can cause neurotoxic including learning and memory impairment as well as induce oxidative stress. Previous studies have shown that acute exposure of aged $A\beta_{25-35}$ to hippocampal cultures induces apoptosis-mediated neuronal toxicity during 6 days incubation. Cognitive dysfunction in several learning and memory tests in mice have been also reported (Lockhart et al., 1994; Maurice, Lockhart and Privat, 1996; Tsunekawa et al., 2008). The $A\beta_{25-35}$ is a subset of $A\beta_{1-42}$ located at the C-terminal end in the hydrophobic domain cleavage from APP molecule. This short peptide containing the 11-amino acid sequence (25-35) and is believed to be a key factor influencing cognitive function in AD (Tsunekawa et al., 2008). Several studies on neurotoxic effects of $A\beta_{25-35}$ demonstrated that continuous or acute i.c.v. injection of mice or rats with $A\beta_{25-35}$ induced impairments of memory and delayed neurodegeneration in the hippocampus was observed (Maurice, Lockhart and Privat, 1996; Kim et al., 2008; Tsunekawa et al., 2008). Indeed, the intracerebroventricular (i.c.v.) administration of $A\beta_{25-35}$ into rodent brain induced memory deficits, oxidative stress, biochemical and histological changes (Kim et al., 2008).

In consistent with previous studies demonstrating that acute single i.c.v. of $A\beta_{25-35}$ significantly induced impairment of the spatial memory assessed by MWM test (Maurice, Lockhart and Privat, 1996; Um et al., 2006). In the present study we demonstrated that intracerebroventricular (i.c.v.) injection of $A\beta_{25-35}$ induced learning and memory deficits in Morris water maze task. During days 1-5, mice treated with $A\beta_{25-35}$ spent much longer time to find the platform than did the sham-operated group. Accordingly, in probe trial on day 6, $A\beta_{25-35}$ treated mice were comparatively spent lesser time in the quadrant where the platform had been removed than did the sham-operated group. Oral administration of ECa 233 10 mg/kg for 7 days prior to the injection of $A\beta_{25-35}$ and being continued throughout the experiment was found to significantly reduce the escape latency of $A\beta_{25-35}$ treated mice on day 3, while ECa 233 at dose 30 mg/kg significant reduced the escape latency on day 2, 3, 4 and 5 and

spent time in the quadrant longer than those exhibited by Aβ $_{25-35}$ treated mice in retention test. Longer time was needed to detect the significant improvement of MWM performance in mice receiving ECa 233 in the post-treatment protocol. Administration of ECa 233 at dose of 10 and 30 mg/kg, B.W., p. o., twice daily for 15 consecutive days after the injection of β-amyloid demonstrated could significantly reduce the escape latency on day 5. They spend longer time in the quadrant than those exhibited by Aβ $_{25-35}$ treated mice as well. Thus, ECa 233 at dose of 10 and 30 mg/kg B.W., p. o., twice daily in both the pre-treatment and post-treatment protocols could significantly improve the learning and memory deficit induced by $A\beta_{25-35}$.

In line with previous report, single i.c.v. injection of $A\beta_{25-35}$ significantly impaired the performance in step-down test (Yamaguchi and Kawashima, 2001). Significant improvement of the deficit in learning and memory seen in $A\beta_{25-35}$ treated mice was noted in the ECa 233 treated group. Administration of ECa 233 at the dose of 10 and 30 mg/kg B.W. twice daily, either by pre-treatment or post-treatment, significantly decreased numbers of error. Increment of step-down latency was found in both doses used, however, a statistical significance was observed exclusively in the 30 mg/kg B.W. ECa 233-treated group in both protocols.

The participation of stimulation or inhibition of motor activity that might affect the results observed in MWM test and step-down test were excluded by the finding that ECa 233 showed no effect on locomotor activity in both protocols. Taken all together, the results obtained in MWM and step-down tests demonstrated the ameliorating effects of ECa 233 on $A\beta_{25-35}$ induced deficit in learning and memory.

Oxidative stress causing lipid peroxidation plays an important role in AD and is one of the pathways of neuronal damage in pathogenesis of AD. Lipid peroxidation induced loss of membrane integrity leading to cellular dysfunction, such as loss of Ca^{2+} homeostasis, disruption of signal pathway and activation of nuclear transcription factors and apoptotic pathways and finally neuronal cell death (Varadarajan et al., 2000; Mattson, 2004). It is well known that oxidative damage is regarded as the fundamental pathogenic mechanism of Alzheimer's disease. The ability of Aβ to induce oxidative damage in cell culture is well documented (Stepanichev et al., 2004). Also in the studied of Butterfield and Lauderback (2002) who demonstrated that lipid peroxidation was an end product of the oxidative stress whereby Aβ could induce

neurotoxicity. In the present experiment, at the end of behavioral test, the brain tissues of mice were removed and oxidative stress in brain was measured. It was found that lipid peroxidation in the cortex, measured as MDA level, was significantly increased in $A\beta_{25\text{-}35}$ treated group. These data agree well with previous studies demonstrating similar result in mice and rats (Varadarajan et al., 2000; Fu, Dong and Sun, 2006). Administration of ECa 233 either by pre-treatment or post-treatment inhibited the increase of lipid peroxidation induced by $A\beta_{25\text{-}35}$ in mice. These findings be explained by a strong antioxidant property of *C. asiatica* previously reported (Doknark, 2003; Salaoh, 2003; Veerendra Kumar and Gupta, 2003)

Histological examination was carried out at 15 days after the i.c.v. administration of $A\beta_{25–35}$. Hippocampus is a brain region playing a central role in learning and memory process. Hippocampus damage is one of the most important causes of amnesia in human disorders accompanied by neurodegeneration such as vascular dementia and Alzheimer's disease (Stepanichev et al., 2004). In the experiment, 15 days after the i.c.v. administration of $A\beta_{25–35.}$ Evaluation of cell loss was determined by calculating the density of pyramidal cells CA1 and CA3 subfield of the hippocampus. The result showed that the densities of neuronal cell in hippocampal were significantly decrease in $A\beta_{25\text{-}35}$ group (CA1 and CA3) both ipsi- and contralateral. Indicating a selective neurotoxic action of the injected peptide rather than any specific physical trauma as a result of the injection (Maurice, Lockhart and Privat, 1996). These findings are consistent with the previous studies in mice and rats (Maurice, Lockhart and Privat, 1996; Stepnichev et al., 2004). In accordance with the results in behavioral test, against damage induced by $A\beta_{25\text{-}35}$ and subsequently neuronal cell integrity and function were preserved. Administration of ECa 233 either by pre-treatment or post-treatment protocol demonstrated protective effects against cell loss induced by $A\beta_{25\text{-}35}$. Neuronal cell loss in CA1 and CA3 area of the hippocampus of all specimens examined were more or less well preserved than those observed in $A\beta_{25\text{-}35}$ treated group. However significant increases were not consistently observed in all groups. Inconsistency observed might be in part due to the method use in which the extent of cell loss was determined by density of cells stained by cresyl violet.

Base on the results obtained, ameliorating effects of ECa 233 on deficit in learning memory, previously reported in transient cerebral ischemic and reperfusion

model (2VO), was again demonstrated in β-amyloid model. Oxidative stress causing cell damage leading to cell death and subsequently compromised neuronal function was a common pathway shared by these two models (Stepanichev et al., 2004; Ninyaporn, 2006). Protective or restorative effects of ECa 233, though mainly could be accounted by its antioxidant property. It is likely that some other mechanism also way underlie the results observed.

In conclusion, our findings demonstrate that intracerebroventricular (i.c.v.) administration of $A\beta_{25-35}$ peptide into rodent brain induced oxidative stress seen as the occurrence of lipid peroxidation causing neuronal damage in hippocampus and leading to impairment of learning and memory in behavioral tests. Pre-treatment and post-treatment of ECa 233 at a dose 10 and 30 mg/kg B.W., twice a day is effective in ameliorating cognitive deficits induced by intracerebroventricular injection of $A\beta_{25-35}$ in mice.

In addition, ECa 233 exhibited protection against $A\beta_{25-35}$ induced cell loss in hippocampus and suppression of a rise in brain MDA caused by $A\beta_{25-35.}$ Therefore, ameliorating effects of ECa 233 an impairment of learning and memory induced by $A\beta_{25-35}$ could likely be accounted by antioxidant property of ECa 233 which protected hippocampal neurons against oxidative stress generated by $A\beta_{25-35.}$

The present study confirmed a positive effect on learning and memory deficit of ECa 233, a standardized extract of an indigenous herb, *C. asiatica*, with well defined characteristics with regards to composition of active ingredients physico-chemical property and toxicity profile (Tantisira, 2009). In consideration to the unmet need for medication to alleviate dementia in the elderly which is the fast growing population in our country, further extensive studies are needed to clarify some other pharmacological effects as well as their respective underlying mechanism to bring ECa 233 into an alternative or an adjunctive in the treatment or prevention of neurodegenerative diseases.

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APPENDICES

APPENDIX A

Effects of ECa 233 given by pre-treatment protocol, on learning and memory deficit induced by $A\beta_{25-35}$ in Morris water maze test

Group name	Escape latency in the MWM (sec)					
	n	Day1	Day2	Day3	Day4	Day5
sham-operated + 0.5%CMC bid.	10	29.0620 ± 4.83218	13.1720 ± 2.27169	9.6310 ± 1.60932	5.7060 ± 0.50209	7.4640 ± 1.18238
$A\beta_{25-35}$ + 0.5%CMC bid.	10	50.0520 ± 2.40913[#]	37.8180 ± 3.27121[#]	39.7170 ± 4.18924[#]	29.3650 ± 4.92847[#]	29.6060 ± 6.32656[#]
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	10	49.3140 ± 4.80800[#]	29.3670 ± 3.42926[#]	24.7390 ± 5.86859[#*]	18.5720 ± 3.74534	17.0340 ± 4.11342
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	10	34.8160 ± 5.65087	21.9850 ± 2.74487[*]	19.6090 ± 2.60221[*]	13.4900 ± 2.82559[*]	10.7765 ± 2.44278[*]

Table 2 : Effects of ECa 233 given by pre-treatment protocol, on learning and memory deficit induced by $A\beta_{25-35}$ in Morris water maze test. The task was carried out on day 7-12 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

[#] $P<0.05$ denotes statistically significant difference from sham-operated group

[*] $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Probe test - Time spent in the platform quadrant in Morris water maze task on day 12 after β - amyloid peptide (25-35) injection

Group name	Probe Trials (%)	
	n	Day6
sham-operated + 0.5%CMC bid.	10	34.5920 ± 1.33119
$A\beta_{25-35}$ + 0.5%CMC bid.	10	22.1490 ± 1.79613[#]
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	10	29.3510 ± 2.18071[*]
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	10	29.6810 ± 1.35248[*]

Table 3 : Effects of ECa 233 given by pre-treatment protocol, on spatial memory in the Morris water maze task. The probe trial was carried out on day 12 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

[#] $P<0.05$ denotes statistically significant difference from sham-operated group

[*] $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by pre-treatment protocol, on step-down passive avoidance task

	Group name	sham-operated + 0.5%CMC bid.	$A\beta_{25-35}$ + 0.5%CMC bid.	$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.
	n	10	10	10	10
Step-down latency (sec)	Day1	3.6600 ± 0.71805	2.9400 ± 0.99378	3.3000 ± 1.26254	4.0200 ± 1.35070
	Day2	249.6600 ± 29.80328	53.0400 ± 24.92873[#]	167.9400 ± 37.10929	230.1000 ± 28.98182[*]
Number of error	Day1	4.1000 ± 0.56667	4.5000 ± 1.20416	5.8000 ± 1.07290	4.8000 ± 0.71181
	Day2	0.7000 ± 0.33500	2.9000 ± 0.45826[#]	1.2000 ± 0.35901[*]	0.9000 ± 0.34801[*]

Table 4 : Effects of ECa 233 given by pre-treatment protocol, on step-down passive avoidance task. Step-down latency and number of errors were carried out on day 13-14 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

[#] $P<0.05$ denotes statistically significant difference from sham-operated group

[*] $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by pre-treatment protocol, on spontaneous locomotor activity (counts/5minutes)

Group name	sham-operated + 0.5%CMC bid.	$A\beta_{25-35}$ + 0.5%CMC bid.	$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.
n	10	10	10	10
Locomotor activity (counts/5minutes)	247.1000 ± 28.69435	306.1000 ± 33.62752	285.0000 ± 29.97925	292.9000 ± 25.45691

Table 5 : Effects of ECa 233 given by pre-treatment protocol, on spontaneous locomotor activity (counts/5minutes). The locomotor measurement was carried out on day 15 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

[#] $P<0.05$ denotes statistically significant difference from sham-operated group

[*] $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by pre-treatment protocol, on level of brain lipid peroxidation

Group name	sham-operated + 0.5%CMC bid.	$A\beta_{25-35}$ + 0.5%CMC bid.	$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.
n	6	6	6	6
MDA levels (µmol/g tissue)	22.5853±1.70364	41.8613±2.93238[#]	23.2805±2.50369[*]	17.6195±3.19111[*]

Table 6 : Effects of ECa 233 given by pre-treatment protocol, on level of brain lipid peroxidation. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

[#] $P<0.05$ denotes statistically significant difference from sham-operated group

[*] $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by pre-treatment protocol, on CA1 and CA3 pyramidal neurons in hippocampal area

Group name	n	Contralateral (%)		Ipsilateral (%)	
		CA1	CA3	CA1	CA3
sham-operated + 0.5%CMC bid.	4	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
$A\beta_{25-35}$ + 0.5%CMC bid.	4	70.91 ± 4.47$^{\#}$	67.51± 7.80$^{\#}$	67.14 ± 9.12$^{\#}$	67.85 ± 3.93$^{\#}$
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	4	81.03 ±10.18	85.25 ± 11.00	94.12 ± 6.04*	90.99 ± 5.08
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	4	91.42 ± 2.20	91.22 ± 1.55	91.45 ± 4.47	98.11± 3.84*

Table 7 : Effects of ECa 233 given by pre-treatment protocol, on CA1 and CA3 pyramidal neurons in hippocampal area. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

$^{\#}$ $P<0.05$ denotes statistically significant difference from sham-operated group

* $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by pre-treatment protocol, on summation of ipsi- and contralateral in CA1 and CA3 pyramidal neurons in hippocampal area

Group name	n	CA1 (%)	CA3 (%)
sham-operated + 0.5%CMC bid.	8	100 ± 0.00	100 ± 0.00
$A\beta_{25-35}$ + 0.5%CMC bid.	8	69.03 ± 4.76 #	70.78± 4.04#
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	8	87.57 ±6.00*	88.15 ± 5.76*
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	8	91.43 ± 2.30*	94.71 ± 2.58*

Table 8 : Effects of ECa 233 given by pre-treatment protocol, on summation of ipsi- and contralateral in CA1 and CA3 pyramidal neurons in hippocampal area. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

# $P<0.05$ denotes statistically significant difference from sham-operated group

* $P<0.05$ denotes statistically significant difference from β-amyloid peptide (25-35) injected group

APPENDIX B

Effects of ECa 233 given by post-treatment protocol, on learning and memory deficit induced by $A\beta_{25-35}$ in Morris water maze test

Group name	Escape latency in the MWM (sec)					
	n	Day1	Day2	Day3	Day4	Day5
sham-operated + 0.5%CMC bid.	10	30.9740 ± 3.90102	15.4970 ± 3.32581	8.7130 ± 2.10384	7.3290 ± 0.98136	5.9200 ± 0.90509
$A\beta_{25-35}$ + 0.5%CMC bid.	10	49.6950 ± 3.24069$^{\#}$	41.8725 ± 4.84613$^{\#}$	30.6922 ± 5.23368$^{\#}$	23.9980 ± 5.57869$^{\#}$	29.7210 ± 5.39873$^{\#}$
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	10	49.8380 ± 4.45639$^{\#}$	27.7115 ± 3.69161	20.3980 ± 4.19559	12.8830 ± 2.89787	13.7060 ± 4.40825*
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	10	41.5880 ± 3.87749	26.3260 ± 4.35863	23.1320 ± 4.39694	16.2940 ± 3.57031	12.9590 ± 3.56318*

Table 9 : Effects of ECa 233 given by post-treatment protocol, on learning and memory deficit induced by $A\beta_{25-35}$ in Morris water maze test. The task was carried out on day 7-12 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. of escape latency. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

$^{\#}$ $P<0.05$ denotes statistically significant difference from sham-operated group

* $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Probe test -Time spent in the platform quadrant in Morris water maze task on day 12 after β - amyloid peptide (25-35) injection

Group name	Probe Trials (%)	
	n	Day6
sham-operated + 0.5%CMC bid.	10	33.0440 ± 1.06803
$A\beta_{25-35}$ + 0.5%CMC bid.	10	24.4720 ± 1.37485[#]
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	10	28.4730 ± 1.44789
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	10	30.3040 ± 0.88886[*]

Table 10 : Effects of ECa 233 given by post-treatment protocol, on learning and memory deficit induced by $A\beta_{25-35}$ in Morris water maze test. The probe trial was carried out on day 12 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 (P<0.05) was considered as statistically significant.

[#] P<0.05 denotes statisticallysignificant difference from sham-operated group

[*] P<0.05 denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by post-treatment protocol, on step-down passive avoidance task

	Group name	sham-operated + 0.5%CMC bid.	$A\beta_{25-35}$ + 0.5%CMC bid.	$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.
	n	10	10	10	10
Step-down latency (sec)	Day1	3.2400 ± 1.00876	3.3600 ± 0.69397	3.0000 ± 0.73756	4.0800 ± 0.95833
	Day2	240.0000 ± 27.59609	54.6060 ± 18.61567[#]	161.5200 ± 40.69209	191.1000 ± 35.74748[*]
Number of error	Day1	5.2000 ± 0.85375	5.2000 ± 0.62893	5.5000 ± 0.74907	4.5000 ± 0.83333
	Day2	0.8000 ± 0.29059	3.6000 ± 0.70238[#]	1.3000 ± 0.39581[*]	1.2000 ± 0.38873[*]

Table 11 : Effects of ECa 233 given by post-treatment protocol, on step-down passive avoidance task. Step-down latency and number of errors were carried out on day 13-14 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

[#] $P<0.05$ denotes statistically significant difference from sham-operated group

[*] $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by post-treatment protocol, on spontaneous locomotor activity (counts/5minutes)

Group name	sham-operated + 0.5%CMC bid.	$A\beta_{25-35}$ + 0.5%CMC bid.	$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.
n	10	10	10	10
Locomotor activity (counts/5minutes)	272.3000 ± 23.40183	310.5000 ± 23.54110	280.6000 ± 15.22367	313.4000 ± 24.47094

Table 12 : Effects of ECa 233 given by post-treatment protocol, on spontaneous locomotor activity (counts/5minutes). The locomotor measurement was carried out on day 15 after the start β - amyloid peptide (25-35) injection. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

$^{\#}$ $P<0.05$ denotes statistically significant difference from sham-operated group

* $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by post-treatment protocol, on level of brain lipid peroxidation

Group name	sham-operated + 0.5%CMC bid.	$A\beta_{25-35}$ + 0.5%CMC bid.	$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.
n	6	6	6	6
MDA levels (µmol/g tissue)	20.483±2.653	64.5882±8.48426[#]	38.9337±6.26679[*]	35.4675±5.3353[*]

Table 13 : Effects of ECa 233 given by post-treatment protocol, on level of brain lipid peroxidation. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

[#] $P<0.05$ denotes statistically significant difference from sham-operated group

[*] $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by post-treatment protocol, on CA1 and CA3 pyramidal neurons in hippocampal area

Group name	n	Contralateral (%)		Ipsilateral (%)	
		CA1	CA3	CA1	CA3
sham-operated + 0.5%CMC bid.	4	100 ±0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
$A\beta_{25-35}$ + 0.5%CMC bid.	4	69.20 ± 10.09#	63.03 ± 5.37#	67.64 ± 6.79#	69.31 ± 6.60#
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	4	81.36 ± 8.52	80.10 ± 9.88	84.64 ± 5.68	81.91 ± 3.51
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	4	86.76 ± 2.58	88.48 ± 3.99	93.73 ± 3.82*	80.50 ± 4.66

Table 14 : Effects of ECa 233 given by post-treatment protocol, on CA1 and CA3 pyramidal neurons in hippocampal area. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

# $P<0.05$ denotes statistically significant difference from sham-operated group

* $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

Effects of ECa 233 given by post-treatment protocol, on summation of ipsi- and contralateral in CA1 and CA3 pyramidal neurons in hippocampal area

Group name	n	CA1 (%)	CA3 (%)
sham-operated + 0.5%CMC bid.	8	100 ± 0.00	100 ± 0.00
$A\beta_{25-35}$ + 0.5%CMC bid.	8	68.42 ± 5.66 #	66.18± 4.15#
$A\beta_{25-35}$ + ECa 233 10 mg/kg bid.	8	82.99 ± 4.77	81.01 ± 4.86
$A\beta_{25-35}$ + ECa 233 30 mg/kg bid.	8	90.23 ± 2.41*	84.47 ± 3.15*

Table 15 : Effects of ECa 233 given by post-treatment protocol, on summation of ipsi- and contralateral in CA1 and CA3 pyramidal neurons in hippocampal area. The values expressed as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and Tukey for comparison. A significant value of P less than 0.05 ($P<0.05$) was considered as statistically significant.

# $P<0.05$ denotes statistically significant difference from sham-operated group

* $P<0.05$ denotes statistically significant difference from β - amyloid peptide (25-35) injected group

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PRESENTATION	**A. Kam-eg**, B. Tantisira and M. H. Tantisira (2009) Preliminary Study of Effects of a Standardized Extract of *Centella asiatica* ECa 233 on Deficit of Learning and Memory Induced by an Intracerebroventricular Injection of β-Amyloid Peptide in Mice. Proceedings of the 31th Annual Meeting of the Pharmacology and Therapeutics Society of Thailand, Theme: From Basic to New Therapeutic Approaches. 18-21 March, Khonkaen, Thailand.