Neuropharmacological Activity of *Apium graveolens* L. and its Neuroprotective Effect on MPTP-Induced Parkinson-Like Symptoms

Phetcharat Boonruamkaew

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Physiology

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Candidate

ชื่อวิทยานิพนธ์ ฤทธิ์ทางประสาทเภสัชวิทยาของสารสกัดขึ้นฉ่ายและฤทธิ์ปกป้องอาการโรคพาร์กินสันที่ถูกเหนี่ยวนำด้วยสาร เอ็ม พี ที พี

ผู้เขียน นางสาวเพชรรัตน์ บุญร่วมแก้ว

สาขาวิชา สรีรวิทยา

ปีการศึกษา 2559

บทคัดย่อ

โรคพาร์กินสันมีอุบัติการณ์เป็นอันดับที่สองรองจากโรคอัลไซเมอร์ของกลุ่มโรคความเสื่อมของระบบประสาท ซึ่งมีผลกระทบต่อทั้งด้านสังคมและเศรษฐกิจ การเสื่อมของเซลล์ประสาทที่สร้างสารสื่อประสาทโดพามีนในวิถีประสาท nigrostriatal ทำให้เกิดความผิดปกติได้ทั้งระบบประสาทสั่งการและไม่ใช่ระบบประสาทสั่งการ เช่น ความบกพร่องในการเรียนรู้และจดจำ หรือ เกิดอาการซึมเศร้าตามมาได้ ในปัจจุบันนี้กลไกการเกิดโรคพาร์กินสันยังไม่ทราบแน่ชัด อย่างไรก็ตามสารอนุมูลอิสระเป็นปัจจัยที่สำคัญของการเกิดโรคพาร์กินสัน การรักษาด้วยยาในปัจจุบันมีเป้าหมายมุ่งเน้นการเพิ่มปริมาณสารสื่อประสาทโดพามีน หรือการเพิ่มการรอดชีวิตของเซลล์ประสาทที่สร้างสารสื่อประสาทโดพามีน อย่างไรก็ตามการรักษาด้วยยาแผนปัจจุบันก่อให้เกิดผลข้างเคียงมากมายจากการใช้ยา และการตอบสนองต่อการรักษาทางยาไม่คงที่หลังจากมีการใช้ยาอย่างต่อเนื่องเป็นระยะเวลานาน จึงทำให้ยาไม่สามารถไปถึงเป้าหมายที่ต้องการได้ ดังนั้นการรักษาด้วยทางเลือกอื่นจึงเป็นหัวข้อที่น่าสนใจ ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินฤทธิ์ทางเภสัชวิทยาและฤทธิ์ปกป้องระบบประสาทของสารสกัดขึ้นฉ่ายในหนูที่ถูกเหนี่ยวนำให้เกิดอาการเลียนแบบโรคพาร์กินสัน

หนูถีบจักรเพศผู้สายพันธุ์ C57BL/6 วัยเจริญพันธุ์ได้รับการป้อนสารสกัดขึ้นฉ่ายผ่านทางปากในขนาดต่างกันคือ 65, 125, 250, 375, และ 500 มก. ต่อ กก. น้ำหนักตัว เป็นระยะเวลา 4 สัปดาห์ ผลการศึกษาพบว่าสารสกัดขึ้นฉ่ายขนาด 125 มก. ต่อ กก. น้ำหนักตัวมีฤทธิ์ต้านอาการวิตกกังวล อาการซึมเศร้า ในขณะที่ขนาด 250 มก. ต่อ กก. น้ำหนักตัวมีฤทธิ์เพิ่มความจำทั้งแบบ spatial และ non-spatial ในหนูทดลอง ทั้งนี้สารสกัดขึ้นฉ่ายขนาดดังกล่าวยังมีผลในการปกป้องการเรียนรู้และจดจำในสภาวะที่มีความบกพร่องจากการเหนี่ยวนำด้วยสาร scopolamine ได้ดีขึ้น รวมทั้งยังมีผลปกป้องเซลล์ประสาทในสมองบริเวณต่างๆของ cerebral cortex, hippocampus และ striatum

โดยกลไกการออกฤทธิ์เป็นผลมาจากการยับยั้งการทำงานของเอนไซม์ acetylcholinesterase การลดการสร้างสารอนุมูลอิสระจากการวัดระดับ malondialdehyde เพิ่มการทำงานของเอนไซม์ต้านการสร้างสารอนุมูลอิสระชนิด glutathione peroxidase และเปอร์เซ็นต์ยับยั้งของ superoxide anion และยับยั้งการทำงานของเอนไซม์ monoamine oxidase ชนิด A ซึ่งเกี่ยวข้องโดยตรงกับกลไกของอาการวิตกกังวลและซึมเศร้า การทดลองต่อมาได้ทำการวิจัยเช่นเดียวกับการศึกษาฤทธิ์ของสารสกัดขึ้นฉ่ายต่อระบบประสาทส่วนกลาง ยกเว้นขนาดของสาร โดยให้สารสกัดขนาด 125, 250, และ 375 มก. ต่อ กก. น้ำหนักตัวเป็นระยะเวลา 3 สัปดาห์ และเหนี่ยวนำให้หนูมีอาการเลียนแบบโรคพาร์กินสันด้วยสาร เอ็ม พี ที พี ผลการศึกษาพบว่าสารสกัดขึ้นฉ่ายขนาด 375 มก. ต่อ กก. น้ำหนักตัวมีฤทธิ์ปกป้องและบรรเทาอาการเลียนแบบโรคพาร์กินสันในหนูทดลองได้อย่างมีประสิทธิภาพโดยกลไกการออกฤทธิ์เป็นผลมาจากการลดลงของสารอนุมูลอิสระ การเปลี่ยนแปลงการทำงานของเอนไซม์ monoamine oxidase ชนิด A และ B และการยับยั้งกระบวนการอักเสบในระบบประสาท ส่งผลให้มีการเพิ่มขึ้นของการรอดชีวิตของเซลล์ประสาทที่สร้างสารสื่อประสาทโดพามีนในสมองส่วน substantia nigra จึงสรุปได้ว่า สารสกัดขึ้นฉ่ายเป็นสมุนไพรทางธรรมชาติที่มีศักยภาพในการพัฒนาเป็นสารต้านความวิตกกังวล ซึมเศร้า เพิ่มการเรียนรู้และจดจำ และปกป้องอาการเลียนแบบโรคพาร์กินสัน ทั้งนี้กลไกการออกฤทธิ์ และการพัฒนาไปเป็นรูปแบบยาหรืออาหารเสริมสุขภาพของสารสกัดยังต้องมีการศึกษาวิจัย ต่อไป

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Author	Miss Phetcharat Boonruamkaew
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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. This condition has a great impact on both social and economic aspects. The degeneration of dopaminergic neurons in nigrostriatal pathway produces both motor and non-motor disorders such as learning and memory impairment or progressive depression. At present, the precise underlying mechanism of neurodegeneration in PD is still unclear. However, piles of scientific evidence point out that oxidative stress is one of the important factors. The therapeutic efficacy of drugs used nowadays, which targets at an increasing of dopamine level or dopaminergic neuron survival is still limited. Current pharmacotherapy usually produces various side effects and the drug response is not stable after prolonged treatment and cannot reach to the target. Thus, an alternative medicine has drawn several attentions. The current study aimed to determine the neuropharmacological activity and neuroprotective effect of *A. graveolens* on MPTP-induced Parkinson-like symptoms in mice. Young adult male C57BL/6 mice were orally received *A. graveolens* crude extract at various doses ranging from 65, 125, 250, 375, and 500 mg/kg BW for 4 weeks, respectively. The results displayed that *A. graveolens* at a dose of 125 mg/kg BW exhibited anxiolytic and antidepressant effects while *A. graveolens* at a dose of 250 mg/kg BW showed the cognitive enhancing effect in both spatial and non spatial memories. *A. graveolens* was also found to alleviate cognitive deficit-induced by scopolamine. Moreover, *A. graveolens* can prevent neurons survival in various regions of cerebral cortex, hippocampus. The proposed mechanisms related to the inhibition of acetylcholinesterase enzyme, regulating the malondialdehyde level and percentage of inhibition of superoxide anion via the increasing of glutathione peroxidase activity, and decreasing the activity of monoamine oxidase enzyme which directly associated with anxiety and depression. This study further determined the neuroprotective effect of *A. graveolens*

on MPTP-induced mice model. The experiment has been performed as mention earlier via oral gavage except that the mice were orally given of *A. graveolens* at various doses ranging from 125, 250, and 375 mg/kg BW for 3 weeks, respectively. Then, the animals were injected with MPTP to induce Parkinson-like symptoms. Our results demonstrated that *A. graveolens* at dose of 375 mg/kg BW showed the peak neuroprotective effect against MPTP induction. The possible underlying mechanism may occur via the reduction of oxidative stress, alteration of monoamine oxidase enzyme activity and suppression of the inflammation process attributed to the increase of dopaminergic neuron in substantia nigra. In conclusion, *A. graveolens* has a high potential to be developed as an anxiolytic, anti-depressant or cognitive enhancer and as the agent to reduce motor disorder in animal model of PD. However, further investigation of possible underlying pathway of *A. graveolens* and the development of drug still required.

Excellent Portion of My Thesis is dedicated for My Parents and All of Entire Teaching staffs

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Sincere appreciation and gratitude is also expressed to my co-advisor, Dr. Morakot Sroyraya, teaching and guiding the immunohistochemistry study as well.

I would like to thank to Assoc. Prof. Dr. Somsak Tiamkao, who offering Diazepam, Aricept, Tidomet plus, and Fluoxetine drugs for this experiment, Division of Neurology, Department of Medicine, Faculty of Medicine, Khon Kaen University, Thailand. I would like to express my gratitude to Dr. Wanida Sukketsiri for her kindness and providing the *A. graveolens* crude extract.

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LIST OF ABBREVIATIONS

AADC	aromatic amino acid decarboxylase
AAV	adeno-associated virus
AChE	acetylcholinesterase
AD	Alzheimer's disease
A. graveolens	*Apium graveolens* L.
ANOVA	analysis of variance
Apaf-1	apoptotic protease activating factor-1
APP	amyloid precursor protein
ATChI	acetylthiocholine iodide
BBB	blood brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	bone derived neurotrophic factor
BKF	Bangkok Forestry Herbarium
BSA	bovine serum albumin
BuPh	*3-n*-butylphthalide
°C	degree Celsius
CCl_4	carbon tetrachloride
Cl^-	chloride ion
cm	centimeter
CNS	central nervous system
COMT	catechol-O-methyltransferase
COX	cyclooxygenase
COX2	cyclooxygenase 2
CSF	cerebrospinal fluid
Cu/Zn SOD	copper/zink superoxide dismutase

LIST OF ABBREVIATIONS (cont.)

DA	dopamine
DAB	diaminobenzidine
DAT	dopamine transporter
DEHP	di-(2-ethylhexyl) phthalate
DI	deionized water
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPX	distyrene, a plasticizer, and xylene
DNA	deoxyribonunucleic acid
DNR	Daunorubicin
DTNB	dithiobisnitrobenzoate
DTT	dithiothreitol
DW	distill water
EDTA	ethylenediaminetetraacetic acid
g	gram
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GDNF	glial cell line derived neurotrophic factor
GI	gastrointestinal
GPe	external part of globus pollidus
GPi	internal part of globus pollidus
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSH	oxidized glutathione
GTP-CH1	guanosine triphosphate- cyclohydrolase1

LIST OF ABBREVIATIONS (cont.)

h	hour
H_2O	water
HRP	horadish peroxidase
Hsp70	heat shock protein 70
HSV-1	Herpes simplex virus 1
hTH	human tyrosine hydroxylase
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
Iba-1	ionized binding adaptor-molecule 1
IHC	immunohistochemistry
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal injection
KCl	potassium chloride
KH_2PO_4	potassium phosphate monobasic
L	liter
µl	microliter
LB	lewy bodies
LC	locus coeruleus
LD_{50}	median lethal dose
L-NBP	*L-3-n*-Butylphthalide
LPS	lipopolysaccharide
M	molar
µm	micrometer
mM	millimolar
MAO-A	monoamine oxidase-typeA

LIST OF ABBREVIATIONS (cont.)

MAO-B	monoamine oxidase-type B
MDA	malondialdehyde
mg	milligram
mg/ml	milligram per milliliter
mg/kg BW	milligram per kilogram body weight
min	minute
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6- tetrahydro-pyridine
ml	milliliter
NaCl	sodium chloride
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	sodium phosphate dibasic
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium
NF-κB	nuclear factor kappa B
β-NADPH	beta-nicotinamide adenine dinucleotide phosphate
nm	nanometer
nmol	nanomole
NO	nitric oxide
N-S-E-W	North-South-East-West
NSS	normal saline solution
O_2	dioxygen

LIST OF ABBREVIATIONS (cont.)

$O_2^{\cdot -}$	superoxide anion
O.D.	optical density
OH•	hydroxyl
6-OHDA	6-hydoxydopamine
$ONOO^-$	peroxynitrite
PBS	phosphate buffer saline
PET	positron emission tomography
PD	Parkinson's disease
PI3K	phosphatidylinositol 3-kinase
REM	rapid eye movement
RNAi	ribonucleic acid interfere
ROOH	lipid peroxide
rpm	round per minute
RT	room temperature
s	second
S.D.	standard of deviation
SDS	sodium dodecyl sulphate
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticularis
SOD1	superoxide dismutase1
Std	standard
STN	subthalamic nucleus
TBA	thiobarbitutic acid
TCS	transcranial sonography
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl

LIST OF ABBREVIATIONS (cont.)

TGF-β	transforming growth factor-beta
TH	tyrosine hydroxylase
TMP	1,1,3,3-tetramethoxy propane
U	unit
v/v	volume by volume
w/w	weight by weight
XIAP	X-linked inhibitor of apoptosis protein
XO	xanthine oxidase

CHAPTER 1
INTRODUCTION

1.1 Background and Rationale

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease, affecting 1 to 2 % of the population over the age of 65 years (Moghal et al., 1994; Olanow et al., 2007). PD is characterized by bradykinesia, tremor, rigidity, and impaired postural reflexes and leads to disability and affects quality of life (Schrag et al., 2000). In addition, it puts considerable emotional stress and economic burden on caregivers (Schrag et al., 2000). With the increasing age of the general population, the prevalence of PD will rise steadily. In 2010, Chulalongkorn Comprehensive Movement Disorder Centre reported that the number of PD patients in Thailand was approximate 1% of aging population over the age of 50-75 years (Chulalongkorn Comprehensive Movement Disorder Centre, 2010). It was also reported that Thai aging population in 2014 was 6,647,000, 11.36 % of all Thai population (Institute for population and social research of Mahidol University, 2014). Therefore, the estimated prevalence of PD in Thailand will be approximate 755,099 cases. Based on the increasing prevalence of this disease with the advancing age, the Parkinson's related problem is increasing its important nowadays.

PD is a common, chronic, and debilitating degenerative disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Many reports have suggested that

the pathogenesis of PD is significantly sustained by oxidative stress (Hunot et al., 1996; Jenner and Olanow, 1996), inflammatory processes (McGeer et al., 1988; Hunot et al., 1999), and apoptosis (Hartmann et al., 2000), although the underlying mechanisms are incompletely understood. A wide range of therapeutic agents have failed to limit disease progression. Nowadays, the standard drugs for PD treatment are levodopa, dopamine agonists, and monoamine oxidase-type B (MAO-B) inhibitor but unfortunately, these drugs are expensive and produce abundant severe adverse effects, such as motor fluctuations and dyskinesia (Waters, 1992; Factor, 2004). Consequently, the development of effective neuroprotective therapies remains a major challenge in PD. Due to the advance of biotechnology; stem cell implantation has gained much interest for PD treatment (Correia et al., 2005). However, the cost expenditure for this strategy is very high and the procedure is invasive. Moreover, the stem cell will be implanted to the patients are not specific enough to differentiate to dopaminergic neuron and exert the function compensate to the old neurons. In addition, the deep brain stimulation is also reported to be effective in treating PD (Benabid et al., 2006). Nowadays, this strategy is also high cost and usually recommended for the late state of patients who are not response for drug treatments. In addition, the neuropsychiatric disorders of depression and anxiety are commonly found in PD patients and affect intensively quality of life. On the other hand, it stills unclear about the pathophysiological pathway of these symptoms (Nègre-Pagès et al., 2010; Kano et al., 2011; Yamanishi et al., 2013).

Hence, the developments of novel protective compound and therapeutic strategies against these diseases which have low cost, non-

invasive, produce less side effects are still required. Recent data showed that free radicals play an important role on the neurodegeneration in PD and antioxidants could protect against this condition (Prasad et al., 1999).

It has been known that nature provides human beings with a myriad of plants possessing various medicinal properties. Plants have been used by human beings since immemorial times to cure diseases and to promote relief from ailments. There were times when they were the most important sources of medicine for people. However, this old form of therapeutics began to lose its importance, being more and more replaced by synthetic remedies. In the recent decades, the ancient use of plants was a lead for scientists in their search for new substances endowed with therapeutic properties. It was estimated that nearly 25% of the modern drugs directly or indirectly originated from plants (De Smet, 1997). Natural products in the diet are known to exhibit a variety of biological effects, including antioxidant, anti-inflammatory, anti-carcinogenic, anti-mutagenic and anti-aging effects (Hasani-Ranjbar et al., 2012). They have relatively low side-effects and a long history of human use. Thus, edible phytochemicals may be good candidates for therapeutic and/or preventive application in PD.

Thailand abounds with medicinal plants many of which have been successfully employed to cure the Thais of a wide range of sickness including the sickness which related to the dysfunction of the central nervous system. Many Thai medicinal plants have been claimed to possess the central nervous system including the *Apium graveolens* L. Several previous studies demonstrated that this medicinal plant and active compound from this plant showed many beneficial effects including anti-hypertension (Ko et al, 1991; Tsi and Tan, 1997; Branković et al., 2010;

Zhang et al., 2012), anti-cancer (Gusman et al, 2001; Patel et al., 2007; Lin et al., 2008), antimicrobial effects (Momin and Nair, 2001; Misic et al., 2008), antioxidant activity (Popovic et al, 2006; Wei and Shibamoto, 2007; Jain et al., 2009; Yao and Ren, 2011; Iswantini et al., 2012), anti-inflammation properties (Al Hindawi et al., 1989; Momin and Nair, 2002; Ovodora et al., 2009; Feng et al., 2012),inhibited acetylcholinesterase (AChE) enzyme (Gholamhoseinian et al, 2009; Szwajgier and Borowiec, 2012). Moreover, the chemical analysis data showed that the luteolin was reported to be the one active ingredient in *A. graveolens* that can inhibit lipopolysaccharide (LPS) which played an important role in decreased dopamine recycling, tyrosine hydroxylase (TH) activity, expressed tumor necrosis factor-α, nitric oxide and superoxide that effect on dopamine synthesis in neurons and glial cells (Chen et al., 2008). Since *A. graveolens* and its active compound showed some effect on the central nervous system, therefore it was possible that *A. graveolens* should have the potential to possess many neuropharmacological activity and neuroprotective effect against PD. Unfortunately, less supported documents about these effects were available. Thus, the present study was set up to determine the neuropharmacological activity and neuroprotective effect against PD of *A. graveolens*.

1.2 Review of Literatures

1.2.1 Anatomy and physiology of basal ganglia

The basal ganglia (basal nuclei) has been regarded as a group of the dorsal striatum (including putamen and caudate nucleus), the internal part of globus pollidus (GPi), the external part of globus pollidus (GPe), the substantia nigra (consist of GABAergic neurons in the par reticulate (SNpr) and dopaminergic neurons in the par compacta (SNpc)) and subthalamic nucleus (STN) of thalamus which are motor control system.

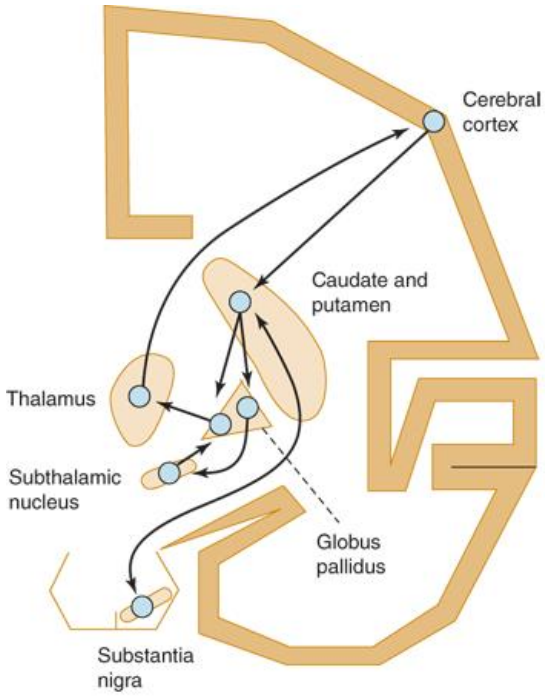


Figure 1 The basic neuronal circuitry of the basal ganglia (Aminoff et al., 2009)

The motor activity is controlled by the intrinsic basal ganglia-thalamocortical circuitry between the cerebral cortex, basal ganglia and thalamus was shown in Figure 2. The main neurotransmitters and their

stimulating (+) or inhibitory (-) signals are exhibited. In PD patient, there was degeneration of the SNpc, leading to overactivity in the indirect pathway (red dot) and increased the glutamatergic signal from the STN.

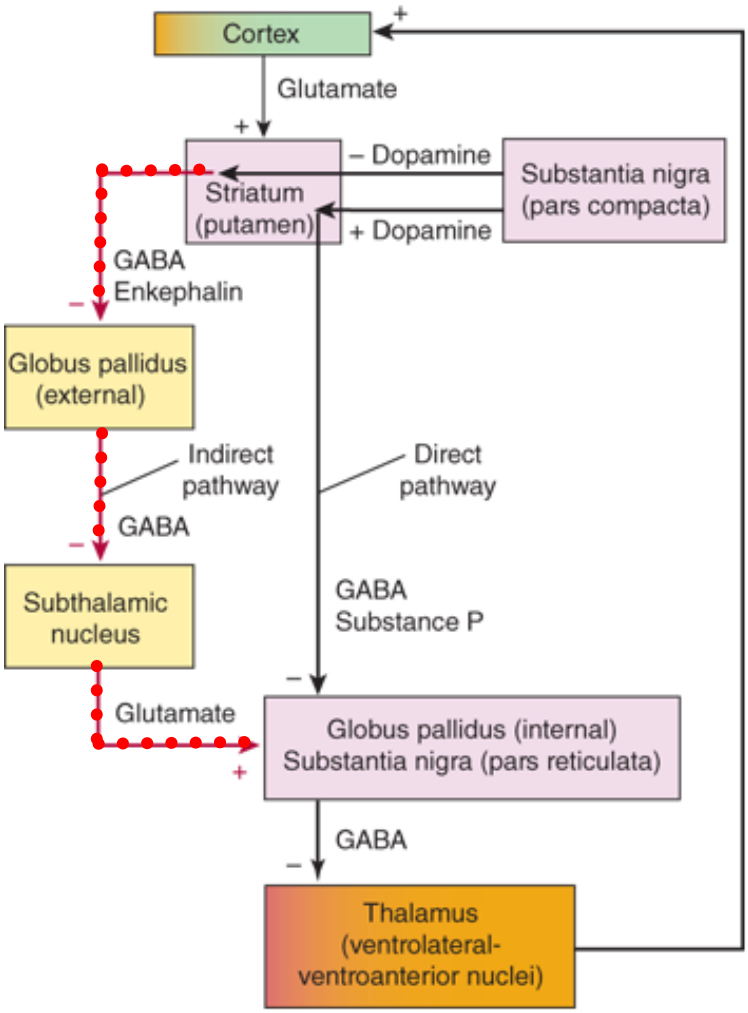


Figure 2 The functional circuitry between the cerebral cortex, basal ganglia, and thalamus. GABA; gamma-aminobutyric acid (Aminoff, 2009)

1.2.2 Parkinson's disease (PD)

PD is classically characterized by a loss of dopaminergic neurons in SNpc which projects to the striatum resulting in dopamine (DA) level decrease as shown in Figure 3A. The cardinal characterizations of PD are a motor syndrome featuring bradykinesia,

tremor, cogwheel rigidity, and postural instability (Dauer and Przedborski, 2003).

- **Tremor** was primarily differentiated clinical sign about 50-70% of PD patients (Pal et al., 2002; Jankovic, 2008). Tremor is relatively 3-5 Hz rhythmic movement of the thumb and index finger while the hand is at rest like pill-rolling and then disappearing with movement (Hallett, 2003), ordinary in one hand relate to contralateral upper limb or ipsilateral lower limb. In progressive PD, the tremor can be diminished or finally disappears (Toth et al., 2008). The mechanism of resting tremor is not entirely clear but it may be due to unmasking of a pathologic central oscillator at 3-5 Hz.

- **Rigidity** is one type of increased muscle tone equal in both antagonist and agonist muscles. They were divided into 2 types including sustained like plastic or lead pipe and intermittent as cogwheel that is usually. The pathogenesis of rigidity in PD is multifactorial, associated with abnormal long-latency reflexes which are mediated by a loop through the sensorimotor cortices afterward abnormal excitability of this central loop occurred (Rothwell et al., 1983), background muscle contraction, and even changes in muscle and joint function (Hallett et al., 2003).

- **Bradykinesia** is slowness of voluntary movements and poverty of normal related movements as akinesia that is an absent voluntary movement (Marsden, 1994). The mechanism underlying bradykinesia is elucidated, related to decrease cortical activation from impaired function in basal ganglia-thalamocortical circuitry (Galvan and Wichmann, 2008) which is a failure of basal ganglia’s output to reinforce the cortical mechanism (Berradelli et al., 2001).

- Postural instability is significant impairment of posture is rare in early PD and usually occurs about 5 years after onset of the disease (Marttila and Rinne, 1977). The posture may display slightly flexion of the neck or trunk with slightly lean to one side. The abnormalities of gait consist of reduced arm swing, slow gait, early fatigue, stride length shortening, discontinuous shuffle, or tripping over objects, sometimes in related to incapacity to turn immediately and ankle dystonia. Afterwards, the disease progresses, the steps are shorter, unstable. There are high tendency to fall and become be freezing gait which is difficult to initiate or stop gait when turning or crossing the obstacles (Schwab et al., 1969). The pathological mechanism of postural instability and gait disturbance are likely multifactorial (Nallegowda et al., 2004) such as visual disorder, imbalance, sensory distortion and pain at joints.

The early nonspecific symptoms of PD such as fatigue, shoulder joint stiffness, somatosensory disturbance, muscle cramps, sleep and rapid eye movement (REM) sleep disorder, etc.

Furthermore, non-motor manifestations are autonomic, psychiatric and cognitive dysfunction are being increasingly diagnosis as part of the clinical syndrome (Taylor et al., 1990; Owen et al., 1992; Frank et al., 2004; Carbon and Eidelberg, 2006) due to neuronal loss in PD was not only limited to dopaminergic neuron in SNpc but also affected pedunculopontine nucleus (Zweig et al., 1989), cholinergic neurons in the basal nucleus of Maynert (Whitehouse et al., 1983; Nakano et al., 1984), noradrenergic neurons in the locus coeruleus (LC) (Man et al., 1983; Jellinger, 1991) and serotonergic neurons of the raphe nuclei (Halliday et al., 1990). The neurons were degenerated in PD to accumulate cytoplasmic inclusion bodies consist of α-synuclein, referred to lewy bodies (LB).

Alpha-synuclein is 140 amino acid protein with an average molecular weight of 19 kDa. The normal function of α-synuclein is not clearly, but it may have chaperone activities, modulate tyrosine hydroxylase (TH) that is the rate limiting step enzyme in dopamine synthesis and play a key role in synaptic vesicle trafficking in dopaminergic neurons (Ostrerova et al., 1999; Murphy et al., 2000; Perez et al., 2002). The filaments of LBs are derived from aggregated forms of α-synuclein which is toxic to neurons.

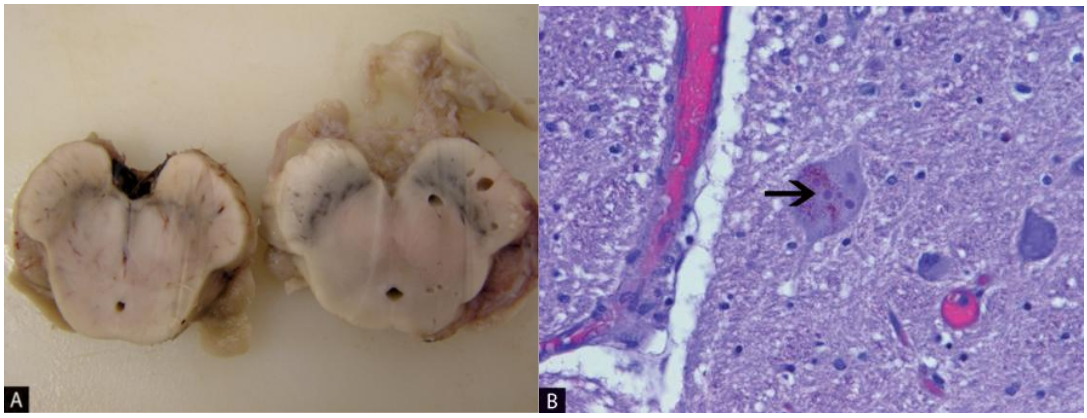


Figure 3 The brainstem on the left side of the image has a pale SNpc compared to the age-matched control on the right side of the image (A). The neuron in the center of the image has several Lewy bodies which are the hallmark of PD, and rich in a protein known as α-synuclein, visible in this section as basophilic homogeneous cytoplasmic inclusions surrounded by a clear halo (arrow), hematoxylin and eosin, 400× (B) (Kamp et al., 2010).

The association between features and pathological stages of PD can divide into 6 stages (Braak et al., 2004).

Stage 1 olfactory bulb and medulla oblongata lesions in dorsal nucleus of cranial nerves IX and X, intermediate reticular formation olfactory bulb and anterior olfactory nucleus

Stage 2 pontine tegmentum pathology of stage1 plus lesions in caudal raphe nuclei, gigantocellular reticular nucleus, and ceruleus-subceruleus complex

Stage 3 midbrain pathology of stage 2 plus lesions in SNpc

Stage 4 basal prosencephalon and mesocortex pathology of stage 3 plus prosencephalic lesions, anteromedial temporal mesocortex, and allocortex (CA-2 plexus)

Stage 5 neocortex pathology of stage 4 plus lesions in prefrontal cortex and sensory association neocortical areas

Stage 6 neocortex pathology of stage 5 plus lesions in first-order sensory association areas, premotor cortex, and even primary sensory and motor cortices

1.2.3 Pathophysiological pathways of PD

The underlying pathophysiological mechanisms of PD are associated with the role of oxidative stress (Blesa et al., 2015), neuroinflammation (Sawada et al., 2006) and apoptosis (Singha and Dikshit, 2007) so many factors to cause of PD pathology. The informed understanding of the mechanisms is elucidated. Therefore, the surrogate diagnosis in PD is difficult to identify.

The biomarkers for PD include clinical tests, imaging analysis, cerebrospinal fluid collection, blood tests and genetics studies. The clinical tests consist of motor test to evaluate performance on motor tasks to determine disability, pharmacological challenges to examine response to dopamine agonists such as levodopa to differentiate PD from other Parkinsonian syndromes and olfaction test to assess sense of smell

that is lost early in the course of PD (Merello et al., 2002; Michell et al., 2004). The imaging analysis include cerebral radiotracer PET scan imaging using fluorodopa as radiotracer to analyze uptake of fluorodopa to flurodopamine that correlates with declining motor function and progression of the disease which is the most extensively modality for PD diagnosis, and transcranial sonography (TCS) utilizes color-coded duplex ultrasound to image the SNpc (Becker and Berg, 2001; Ravina et al., 2005). The blood and cerebrospinal fluid (CSF) collections to examine possible markers such as mitochondrial complex I level, monoamine oxidase-B (MAO-B) activity, dopamine transporter (DAT), proteosome and α-synuclein as shown in Figure 3B (Hass et al., 1995; Zhou et al., 2001; Caronti et al., 2001; Blandini et al., 2006; El-Agnaf et al., 2006). The genetics studies of *SNCA*, *PARK2*, *UCH-L1*, *PINK1*, *MAPT*, etc. are indicators of pathogenic pathway of PD (Pankratz and Foroud, 2004; Forman et al., 2005).

1.2.4 The related factors in PD pathology

There are many possible factors which are related to pathological occurrence in PD (Marras and Tanner, 2004; Wang et al., 2011; Lee et al., 2012; Dardiotis et al., 2013; Moretto and Colosio, 2013).

1. Agriculture-associated factors; rural living, farming, gardening, well water drinking, and fungicide/ herbicide/ pesticide exposure

2. Work-related factors; organic solvent/ carbon monoxide/ wood preservative/ metal/ glue/ paint/ lacquer exposure, paper/ wood pulp factory, carpentry, cleaning/ chemical manufacturing, iron ore mining, and construction job

3. Disease of infection; *Nocardia* is gram-positive, mostly found in soil matter and infection was acquired by inhalation of the bacteria or through traumatic injury. Finally they lead to be the slowly progressive pneumonia.

4. Other factors; high fat nutrition, mood swing, trauma of head injury, general anesthesia, and very hard physical job

1.2.5 Therapeutic alternatives for PD

The treatment of PD is so important to promote patients can do daily life activities. Although the PD is completely curable but slow or maintain progression are the gold targets and the pharmacotherapy for PD is remarkably alternatives.

- The drug therapy for PD

Therapeutics drug for PD patient can divide into 5 groups include dopamine precursor, dopamine agonists, MAO-B inhibitors, catechol-O-methyltransferase (COMT) inhibitors and muscarinic antagonists (Figure 4), however the effective and gold target of drug treatment in PD is levodopa. Levodopa is dopamine precursor, aromatic amino acid ring, was found in nut as *Macuna pruriens* to treat PD according to Ayurveda of India (Manyam et al., 2004; Tharakan et al., 2005; Dhanasekaran et al., 2007) and levodopa has been used to ameliorate PD symptoms especially bradykinesia, rigidity and resting tremor since 1960 that levodopa was absorbed at the small intestine via amino acid transportation, half-life is about 1-1.5 h, was converted to dopamine by enzyme aromatic amino acid decarboxylase (AADC) and COMT to 3-O-methyldopa. The only 5-10% of levedopa can cross blood brain barrier (BBB) due to peripheral degradation.

However the effect of drug treatment for PD are satisfied but the drugs are expensive and cause of many serious side effects such as dyskinesia, hallucination, brain edema or swelling, hypotension, sleep disturbance, and depression (Finley, 2001). Therefore, the novel therapeutic is still required to develop alternative medicine.

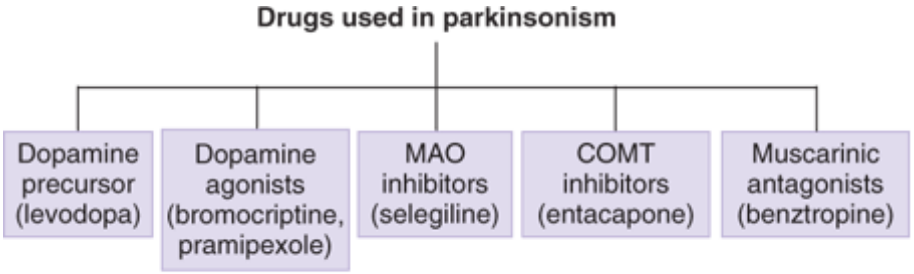


Figure 4 The drug treatment for PD (Trevor et al., 2009)

- **Potential gene therapy for PD**

Recently, the gene therapy is the good alternatives but it stills controversial. The suitable genes with the most influential vector systems will enable the novel therapeutic approaches for patients with PD (Table 1-3).

Table 1 Categories of candidate genes related to familial PD

Symptomatic	**Transmitter enzymes**		**Gene for inhibition of overactive neurons**	
	- TH - GTP-CH1 - AADC		- GAD	
Restorative	Antiapoptotic molecules	Neurotrophic factors	Free radical scavengers	Anti-α–synuclein toxicity
	- bcl-2 - Apaf-1 - Dominant negative inhibitor - X-chromosome-linked inhibitor	- GDNF - BDNF	- Cu/Zn SOD	- Hsp70 - β-synuclein - inhibitor of transglutaminase - ribozyme - RNAi of α–synuclein - α–synuclein antibody

Modified from Fleming et al., 2005; Factor and Weiner, 2008; Houlden and Singleton, 2012; Klein and Westenberger, 2012; Kumar et al., 2012; Marras et al., 2012; Toft and Wszolek, 2013.

Table 2 The gene replacement for PD

Replacement	Locus	Gene	Proposed function	References
PARK 1	4q21-22	*α-synuclein*	Presynapstic	Polymeropoulos et al., 1996
PARK 2	4q25-27	*Parkin*	E3 ubiquitin ligase	Kitada et al., 1998
PARK 3	2p13	*?*		
PARK 4	Erroneous locus-identical to *PARK1*			
PARK 5	4p14	*UCH-L1*	Ubiquitin recycling enzyme	Wintermeyer et al., 2000
PARK 6	1p25-36	*Pink1*	Mitochondrial	Bonifati et al., 2003
PARK 7	1p36	*DJ1*	Oxidative stress response	Valente et al., 2004
PARK 8	12p11.2q13.1	*LRRK2*	Kinase	Di Fonzo et al., 2006
PARK 9	1p36	*ATP13A2*		
PARK 10	1p32	*?*		
PARK 11	2q36-27	*?*		
PARK 12	Xq21-q25	*?*		
PARK 13	2p12	*HTRA2*		
PARK 14	22q13.1	*PLA2G6*		
PARK 15	22q12-q13	*FBX07*		
PARK 16	1q32	*?*		
PARK 17	16q11.2	*VPS35*		
PARK 18	3q27.1	*EIF4G1*		
PARK 19	1p32	*DNAJC6*		
PARK 20	21q22	*SYNJ1*		

Adapted from Polymeropoulos et al., 1996; Kitada et al., 1998; Wintermeyer et al., 2000; Bonifati et al., 2003; Valente et al., 2004; Di Fonzo et al., 2006.

Table 3 The viral vectors for gene therapy

Virus	Integration in host genome	Target cells	Advantages	Disadventages
HSV-1	No	Neurons	Large insert size, latency	Cytotoxicity
Adenovirus	No	Glia>Neurons	High titer, efficient	Immunogenicity
AAV	Ch 19, some episomal	Neurons>Glia	Nonpathogenic	Low titer
Retrovirus	Random integration	Dividing cells only	Most experience	For *ex vivo* gene transfer only
Lentivirus	Random integration	Dividing and nondividing, neurons	Low immune reaction	No packaging cell line, concerns about HIV virus

Adapted from Factor and Weiner, 2008.

The type of viral vectors for gene therapy in PD patient as following,

1. *Ad* Vectors

Adenovirus based therapies for PD has been used extensively in rodent model. First, *Ad* vector encoding the *TH* gene, when injected into the striatum of 6-hydoxydopamine (6-OHDA)-induced rats, resulting in decrease of amphetamine-induced rotational behavior (Horellou et al., 1994) and was observed for 1-2 weeks following gene transfer but was a vigorous inflammatory response, gliosis and local tissue damage.

Ad vector expression of transforming growth factor-beta (TGF-β) family member and glial cell line derived neurotrophic

factor (GDNF) have also been examined in 6-OHDA-lesioned rats which protected the dopaminergic neuron from chemical damage was observed for up to 6 weeks post-induced (Choi-Lundberg et al., 1997).

The oxidative stress is an one important factor leading to be PD. Cu/Zn superoxide dismutase (SOD1) play a key role in cellular response to oxidative stress so *Ad* vector overexpressing SOD1 shown the neuroprotective effect on DA neurons within nigrostriatal pathway in the striatum of an MPP^+-induced rat model (Barkats et al., 2006).

2. *AAV* vectors

Recombinant *AAV* vectors displayed great benefit of PD gene therapy. *AAV* vector encoding *β-galactosidase* or *human tyrosine hydroxylase (hTH)* were injected to 6-OHDA-induced rat brain and exhibited long-term transgene expression for 3 months and then functional recovery (Kaplitt et al., 1997). *AAV* vector delivering *hTH* and *aromatic amino acid decarboxylase (AADC)* genes to MPTP-treated monkey brain shown that *AAV* vector could be long-term transgene expression and avoided significant toxicity (During et al., 1998). The use of *AAV* vector to demonstrate glutamic acid decarboxylase (GAD) such as GAD_{65} and GAD_{67} to change excitatory neuron to inhibitory neuron which the result displayed that they increased GABA release in the SNpc and injected to protect 6-OHDA lesion rats (Luo et al., 2003). Recently, *AAV* vector overexpressing *Parkin* and *DJ-1* gene to SNpc of an MPTP mouse model of PD. The result displayed inhibition of DA neuron death for 1 week following MPTP treatment (Paterna et al., 2007). Therefore, the overexpressing of *Parkin* and *DJ-1* were considered as candidates for gene therapy in PD.

3. Lentivirus vectors

Lentiviral vector-based therapies popularly have been used in rodent and nonhuman primate experiments. The self-inactivation of lentivirus expressing *GDNF* displayed neuroprotective effect in 6-OHDA rat and MPTP nonhuman primate models of PD and normal aged rhesus monkeys (Bensadoun et al., 2000; Déglon et al., 2000; Kordower et al., 2000). The long-term striatal overexpression of *GDNF* caused of dose-dependent *TH* downregulation for 6 weeks after injection. The mechanism underlying of *GDNF* mediated repression *TH* expression is elucidated but proposed that high level of *GDNF* possessed hyperactivating effect which finally sustains DA neuron in long-term (Georgievska et al., 2004). Recently, the E3 ubiquitin ligase, *Parkin*, the mutant gene related to autosomal recessive juvenile PD exhibited the neuroprotective activity to DA neuron after induced PD experiments. The wild-type *Parkin* was overexpressed through lentiviral vector to SNpc of 6-OHDA rat model 2 weeks before lesioning, the result shown that the significant inhibition of DA neuron death at 1 and 3 weeks after induced go along with behavioral improvement of both amphetamine-induced rotational and cylinder test lasting 20 weeks (Vercammen et al., 2006).

4. *HSV* vectors

HSV amplicon is gene transfer vector to direct transgene expression was used with either 9kb TH or preproenkephalin promoter in *vivo*. The results exhibited that using preproenkephalin with *HSV* amplicon sustained specificity to DA neuron and expression duration (Kaplitt et al., 1994). 6-OHDA-induced rats were treated with *HSV* amplicon inserting of *hTH* (During et al., 1994). Lately, researchers

compared the neuroprotective properties of BDNF and GDNF on DA neuron death using *HSV-1* vector. Their results concluded that GDNF has more potent in recovery of behavioral and biochemical parameters of 6-OHDA-induced mice when compared to either GDNF-BDNF coexpression or BDNF alone (Sun et al., 2014).

5. Nonviral vectors

The purpose of using nonviral vectors is pleasing because of lack of immunogenic or cytotoxic viral gene products. Nonviral delivery of genes includes naked DNA, polycationic polymers, DNA attached to positively charged metal particles or DNA encapsulated with in cationic lipids. The *TH* expressing plasmid, encapsulated in PEGylated immunoliposomes which were targeted the brain with transferring receptor-specific at BBB via IV injection to 6-OHDA-lesioned rats. The results displayed that the plasmid was effectively crossing BBB and occurred transient *TH* level normalization within striatum of 6-OHDA-lesioned rats (Zhang et al., 2003).

1.2.6 *A. graveolens*

A recent decade, *A. graveolens* (Chinese celery) is one kind of medicinal plant that has the effect on CNS. Many previous studies shown that fresh *A. graveolens* 200 g include calcium (Teng et al., 1985), vitamin C and trace elements such as potassium, magnesium, vitamin A, B1, B2, B6, phosphorus and iron (Mitra et al., 2001; Belal, 2011). The polyphenols existing in *A. graveolens* is flavones 20-140 mg/kg (Herrmann K, 1976; Hertog et al., 1992; Crozier et al., 1997; Justesen et al., 1998; U.S. Dept. of Agriculture National Nutrient Database) which

consist of apigenin, luteolin, and quercetin (Tang et al., 1990; Anthony and Dweck, 2009), α-Tocopherol, glucosides (Ching and Mohamed, 2001; Kitajima et al., 2003; Chun et al., 2006), and phylloquinone (Damon et al., 2005) as shown in Figure 5. In addition, the pure compounds for example L-tryptophan, *L-3-n*-Butylphthalide (*L*-NBP), *3-n*-butyl-5-dihydrophthalide (Momin and Nair, 2001; Momin and Nair, 2002), 5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-l-benzopyran-one and 2, 3-dihydro-6-hidroxy-5-benzofuran carboxylic acid (Iswantini et al., 2012) were also found in this plant.

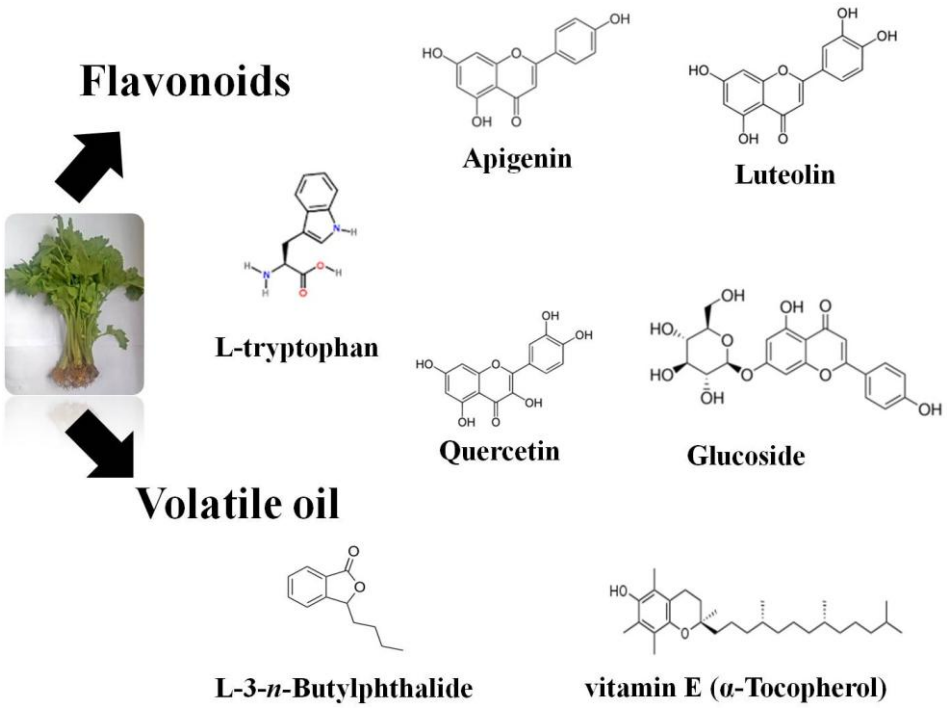


Figure 5 The chemical analysis showed the main flavanoids and volatile oil compounds existing in *A. graveolens* (Herrmann K, 1976; Hertog et al., 1992; Crozier et al., 1997; Justesen et al., 1998; U.S. Dept. of Agriculture National Nutrient Database; Ching and Mohamed, 2001; Momin and Nair, 2001; Momin and Nair, 2002; Damon et al., 2005; Kitajima et al., 2003; Chun et al., 2006).

Many previous reviews showed that celery and some active ingredient extract have many neuropharmacological activities.

1. Antioxidant activity and anti-inflammatory process

The essential oil of celery seed exhibited *in vitro* scavenging activity for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and displayed the greatest inhibitory activity as 23% toward malondialdehyde (MDA) formation. The main compound of the oil in celery seed was limonene (composition, 74.6%) (Wei and Shibamoto, 2007). The extracts of celery leaves and roots in n-butanol showed that *in vitro*, they are good radical scavengers of OH•, DPPH• radicals and reduce lipid peroxide intensity in liposomes. *In vivo* studies were associated with activities of glutathione peroxidase, catalase, glutathione content in liver and blood of mice by induced with carbon tetrachloride (CCl_4) (Popovic et al, 2006). The methanolic extract of celery seed prevented hepatotoxicity which induced by di-(2-ethylhexyl) phthalate (DEHP) (Jain et al., 2009). There are antioxidant effects based on phenolic content in celery (Yao and Ren, 2011). The pure compounds of celery stems and leaves were 5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-l-benzopyran-4-one and 2,3-dihydro-6-hidroxy-5-benzofuran carboxylic acid can inhibit xanthine oxidase (XO) as 85.44% (Iswantini et al., 2012). The pure compound of celery seeds, 3-hydroxymethyl-6-methoxy-2,3-dihydro-1H-indol-2-ol showed the inhibition of cyclooxygenase (COX) and topoisomerase in *in vitro* (Al Hindawi et al., 1989; Momin and Nair, 2002) as same as the pure compound of celery stem can inhibit inflammation in *in vitro* (Ovodova et al., 2009). Apigenin and *DL-3-n*-butylphthalide were the pure compound of celery displayed antioxidant effect of cell that induced by H_2O_2 and

decreased mitochondrial dysfunction (แสงแข และ มั่นเขตต์กรณ์, 2543; Huang et al., 2010) and *DL-3-n*-butylphthalide also prevented neuronal cell death from neuro-inflammation via glial cells activation (Feng et al., 2012).

2. Enhance memory and neuroprotective effects

The water extract of celery roots can inhibit AChE enzyme in *in vitro* as 100% (Szwajgier and Borowiec, 2012) which conform to the methanolic extract of celery stems can prevent neuronal PC12 cells from inducing with amyloid beta (Park et al., 2009). On the other hand, the methanolic extract of celery leaves inhibited the enzyme only 4.7% (Gholamhoseinian et al, 2009). The pure compound, *L-3-n*-butylphthalide from celery seeds, stems and leaves amilorated cognitive impairment, promoted long-term spatial memory, decreased of amyloid-β and controlled amyloid precursor protein (APP) production in transgenic AD mice (Peng et al., 2010). The high concentration of pure compound, luteolin from celery inhibited LPS which played an important role in decreased dopamine recycling, TH activity, expressed tumor necrosis factor-α, nitric oxide and superoxide that affected on dopamine synthesis in neurons and glial cells (Chen et al., 2008).

3. Anti-hypertensive effect

The ethanolic and water extract of celery stems and leaves 0.5-15 mg/kg displayed anti-hypertensive effect 46 % and 14% respectively in experimental rabbits. However, the ethanolic extract can decrease heart contractility and heart rate than atropine in rat (Branković et al., 2010). In addition, the pure compound of celery leaf stalks was apigenin showed vasodilated effect in rat (Ko et al, 1991; Zhang et al.,

2012) and *3-n*-butylphthalide (BuPh) can inhibit calcium uptake resulting in vasodilation and low systolic blood pressure (Tsi and Tan, 1997).

4. Enhancing neural stem cells growth and mature neurons

The water extract of celery stems, leaves, and roots for 3 days to test in stem cell and then induced differentiation of neural stem cells to neuron cells, astrocytes and oligodendrocytes accurately (Tie-qiao et al., 2006). The pure compound, apigenin can promote differentiation of mature neuronal cells in both *in vitro & in vivo* (Taupin, 2009).

5. Anti-cancer effect

There was previous study found that the pure compound, apigenin exhibited higher potential effect than terpene in enhancing effect with the cancer treatment drug as daunorubicin (DNR) in *in vitro* of K562/S, K562/ADR and GLC4/S, and GLC4/AD cancer cell (แสงแข และ มั่นเขตต์กรณ์, 2543). Moreover, apigenin also played a key role in many cancer chemopreventions such as liver, gastrointestinal (GI) systems, thyroid, lung, and prostate cancer via inhibition of abnormal cell growth, cell cycle, and leading to be apoptosis program and prevented oxidative stress (Patel et al., 2007). The pure compound, luteolin from celery played a role in inducing cell to apoptosis pathway via activation Tumor suppressor p53 and inhibiting of abnormal cell growth via phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor kappa B (NF-κB) and X-linked inhibitor of apoptosis protein (XIAP) (Lin et al., 2008).

6. Gastroprotective effect

The ethanolic extract of celery stems and leaves for 72 h at concentration of 250 and 500 mg/kg displayed gastric mucosa prevention and inhibiting gastric secretion in rat resulting in gastric ulcer prevention (Al-Howiriny et al., 2010). Furthermore the volatile oil of celery seeds at concentration of 300 mg/kg can prevent peptic ulcer in rat 98% by decreased gastric secretion and acidic condition in stomach (Baananou et al., 2012).

7. Antimicrobial effects

The methanolic extract of celery seeds at concentration of 100 µg/ml showed antifungal effects of *Candida albicans* and *Candida parapsilasis* which the pure compounds of celery seeds were sedanolide, senkyunolide-N and senkyunolide-J (Momin and Nair, 2001). Further the volatile oil of celery roots and leaves that extracted with liquid carbon dioxide after analyzed active compounds in celery roots were limonene, carvone and *3-n*-butylphthalide and celery leaves were limonene higher than celery roots and low level of carvone. For celery leaves exhibited antibacterial activities of *Escherichia coli, Hafnia alvei, Bacillus cereus, Enterococcus faecalis, Enterobacter aerogenes* and *Staphylococcus aureus* while celery roots displayed antibacterial effect on *Bacillus cereus* and *Enterococcus faecalis* (Sipailiene et al., 2005). Furthermore, the volatile oil from celery seed that extracted with carbon dioxide and water showed high antibacterial activity of *Staphylococcus aureus*, *Listeria monocytogenes*, and *Listeria ivanovii* in *in vivo* and clinic which the content of the oil were sedanenolide, sedanolide and *3-n*-butylphthalide (Misic et al., 2008).

8. Toxicity test.

There was acute toxicity test in rat using single dose of 0.25-12 g/kg fresh celery followed for every 6 h, 14 days. demonstrated that LD_{50} means 7.55 g/kg which indicated wide therapeutic dose (Al-Howiriny et al., 2010) and intraperitoneal injection of the pure compound, apigenin in adult male mice at concentration of 25 mg/kg daily until 7 days caused of sperm density decreased and infertility occurred (Li et al., 2010). In addition, the acute oral toxicity of 70% methanolic extract of *A. graveolens* at dose of 2.5 and 5 g/kg BW were also determined. It was found to be safe up to 5 g/kg BW (Choosri et al., 2016).

1.2.7 1-Methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP)

MPTP is a neurotoxin precursor to MPP^+, which causes reversible symptoms of PD by destroying dopaminergic neurons in the SNpc of the brain. This substance itself is not toxic, and as a lipophilic compound can cross the BBB. Once inside the brain, MPTP is metabolized into the toxic cation 1-methyl-4-phenylpyridinium (MPP^+) by the enzyme MAO-B of glial cells. MPP^+ kills primarily dopamine-producing neurons in a part of the brain called the SNpc and then interferes with complex I of the electron transport chain, a component of mitochondrial metabolism, which leads to cell death and causes the buildup of free radicals, toxic molecules that contribute further to cell destruction (Figure 6).

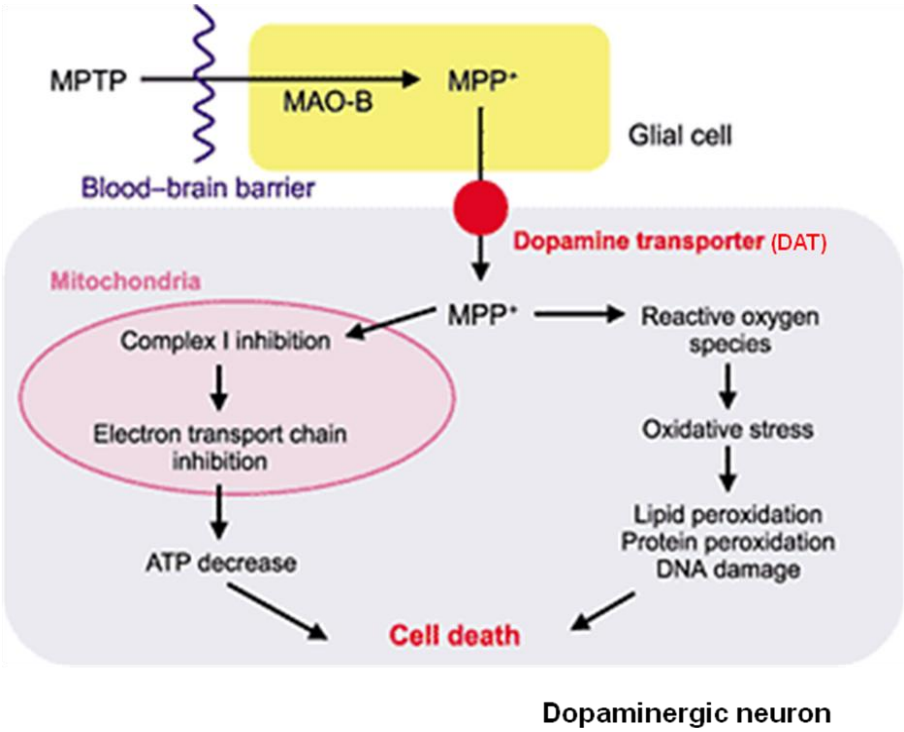


Figure 6 The MPTP-induced dopaminergic neurons death via oxidative stress and mitochondrial complex I inhibition pathways (Langston et al., 1983).

1.2.8 Ionized binding adaptor-molecule 1 (Iba-1)

Iba-1 is the marker of activated microglia which high expressed in neuroinflammation. There was the proposed mechanism between neuroinflammatory processes and oxidative stress affect to dopaminergic neurons in Parkinson's disease (Figure 6). The microglial cells were activated and then released inducible nitric oxide synthase (iNOS), leading to the nitric oxide (NO) production. In addition, microglial cells also have expressed NADPH oxidase, leading to the high level of superoxide anion ($O_2^{\cdot -}$). Other way, $O_2^{\cdot -}$ and NO might cause of the $ONOO^-$, oxidative damage within dopaminergic neurons. The high concentration of free ferrous iron can cause of the highly reactive OH and oxidative stress to the neuronal cell. Reactive astrocytes highly represent myeloperoxidase, which produces HOCl from H_2O_2 and Cl^- and directly

inflict oxidative damage to dopaminergic neurons. According to these mechanisms of neuronal cell death, neuroinflammatory-related to oxidative damage might also initiate from the damaged dopaminergic neurons via COX 2 expression.

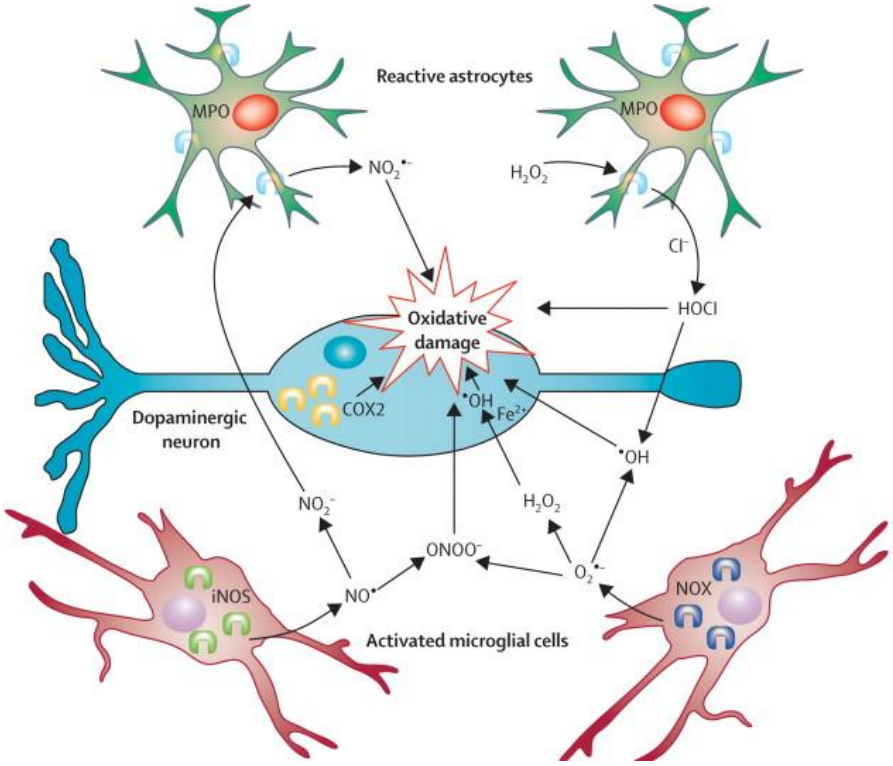


Figure 7 Possible linkage between neuroinflammation and oxidative stress to dopaminergic neurons in PD (Hirsch EC, 2009)

1.2.9 Positive control drugs of this study

- Diazepam

A positive allosteric modulators of the GABA-type A receptors ($GABA_A$), $C_{16}H_{13}ClN_2O$, MW 284.7 g/mol. The bioavailability is high and also long of half-life. The mechanism of action of this drug specifically bound to $GABA_A$ receptor subunits at neuronal synapses of CNS and then activating GABA-mediated chloride ion channel opening leading to promote membrane hyperpolarization for treatment of

acute anxiety, panic disorder, insomnia and other sleep disorders. Diazepam may react with the opioids, ethanol, and other anti-depressant drugs (Kearney, 2012).

- Fluoxetine

A drug of selective serotonin reuptake inhibitor (SSRI) group to delay the reuptake of serotonin and does not affect norepinephrine (NE) and dopamine (DA) recycling in therapeutics dose for the treatment of major depressive, obsessive-compulsive (OCD) and panic disorders. The bioavailability of this drug is quite high, high affinity to binding with protein with MW 309.33 g/mol, the half-life about 1-3 days. However, the high doses of this drug have been displayed to significantly elevate in synaptic norepinephrine and dopamine levels in rats. Hence, DA and NE may act as anti-depressant effect at supratherapeutic doses (60-80 mg) via 5-HT_2C receptors. In addition, fluoxetine might interact with chronic use of MAO inhibitor drug because it is potent inhibitors of the drug-metabolizing cytochrome P-450 enzyme of liver, which leads to many drug interactions such as the serotonin syndrome. This syndrome is characterized by clinical appearances of neuromuscular overactivity, autonomic dysfunction and mental condition disturbances. Due to the long period of synergistic effect of MAO inhibitor and the serotonin uptake inhibitor (Benowitz, 2012).

- Donepezil

Acetylcholinesterase inhibitor or aricept ($C_{24}H_{29}NO_3$, MW 379.492 g/mol) can reversibly inactivate cholinesterases to inhibit degradation of acetylcholine resulting in increasing of acetylcholine concentrations at cholinergic synapses. Donepezil has high bioavailability and affinity to binding with protein and half-life

approximate 70 h. Recently, it was found that this drug exerts the potential NMDA-induced current in cortical neuronal test so it was reported to reduce the risk of AD (Moriguchi et al., 2005; Petersen et al., 2005).

- Vitamin C

The ionic form of L-ascorbic acid ($C_6H_8O_6$), is a water soluble antioxidant vitamin with molecular mass 176.12 g/mol. The bioavailability of this substance is rapid, negligible with protein binding, its half-life depends on plasma concentration and excretion via kidney. The previous reports exhibited that vitamin C acts as a free radical scavenger in various conditions of model including Huntington's disease (Rebec et al., 2003), cerebral ischemia in *in vitro* (MacGregor et al., 2003) and seizure-induced by iron (Yamamoto et al., 2002). In addition, it can ameliorate kainite-induced lipid peroxidation and reduce neuronal loss in hippocampus brain area of rat (MacGregor et al., 1996).

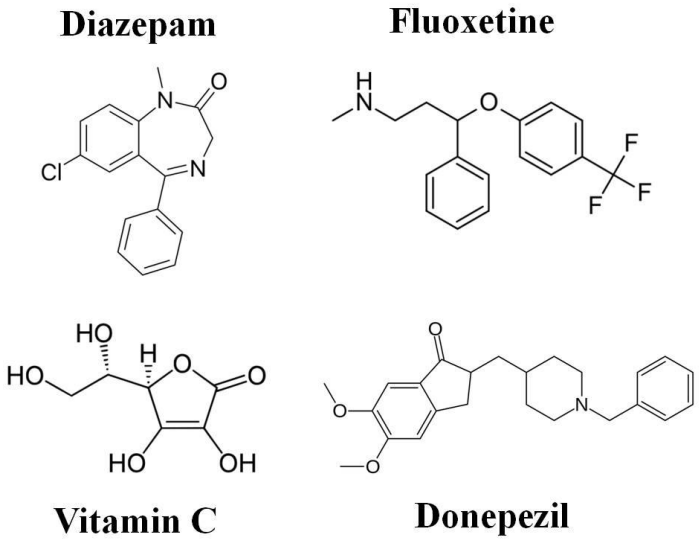


Figure 8 The chemical structure of positive control drugs of this research

1.2.10 MAO-A, B enzymes

- MAO-A enzyme

An enzyme degrades the oxidative deamination of amine neurotransmitters such as 5-HT, DA, and NE. The enzyme was highly represented in neural cells, cardiac cell and locates in the outer mitochondrial membrane. MAO-A level in the brains of patient with major depressive disorder who on drug at least 5 months were approximately elevated 34% as measured from positron emission tomography (Meyer et al., 2006).

- MAO-B enzyme

This enzyme highly expressed in glial cells and mainly degrades DA neurotransmitter. AD and PD are both related to increasing levels of MAO-B in the brain (Nagatsu and Sawada, 2006; Mallajosyula et al., 2009). In addition, the MAO-B activity causes of reactive oxygen species, which directly destroyed cells (Nagatsu and Sawada, 2006). Furthermore, MAO-B levels have been reported to increase with age, suggesting a role in natural age related cognitive decline and the increased of neurological diseases (Kumar and Andersen, 2004).

1.3 Objectives

1.3.1 General experimental aims

This experiment is designed as described following;

1. To determine the potential effect of *A. graveolens* on the central nervous system

2. To evaluate the potential effect of *A. graveolens* as a natural neuroprotectant against PD

1.3.2 Specific objectives

1. To determine the neuropharmacological activities of *A. graveolen*s in adult male mice

2. To determine the cognitive enhancing effect of *A. graveolens* against cognitive deficit condition induced by scopolamine

3. To study the possible mechanisms underlying the neuroprotective effect of *A. graveolen*s

3.1 To determine the alteration of acetylcholinesterase (AChE) activity

3.2 To determine the alteration of free radicals levels; superoxide anion, lipid peroxidation product and the alteration of scavenging enzymes activities including glutathione peroxidase (GPx)

3.3 To determine the alteration of monoamine oxidase-A (MAO-A) enzyme activity

3.4 To determine the number of neuron densities in brain areas of cerebral cortex, striatum and hippocampus

4. To determine the neuroprotective of *A. graveolens* against Parkinson's disease induced by MPTP

5. To determine the possible neuroprotective mechanisms of *A. graveolens* protect against neurodegeneration in animal model of PD

5.1 To determine the alteration of free radicals levels; superoxide anion, lipid peroxide product and the alteration of scavenging enzymes activities including glutathione peroxidase (GPx)

5.2 To determine the alteration of inflammation process; levels of ionized binding adaptor molecule-1 (Iba-1)

5.3 To determine the alteration of survival dopaminergic neuron; levels of tyrosine hydroxylase (TH)

5.4 To determine the alteration of total monoamine oxidase-A, B (MAO-A, B) activities

1.3.3 Hypotheses

1. If *A. graveolens* possesses neuropharmacological activity, the animals received *A. graveolen*s should exert neurobehaviors including cognitive enhancement, anxiolytic and anti-depression differ from vehicle-treated group.

2. If *A. graveolens* has promoted neuronal differentiation, the animals which received *A. graveolen*s should increase the neurons density in brain areas of cerebral cortex, striatum, and hippocampus differ from vehicle treated group.

3. If *A. graveolens* has neuroprotective effect against scopolamine induced cognitive impairment, the animals received *A. graveolen*s should have better cognitive score than that of vehicle-treated group.

4. If *A. graveolens* has neuroprotective effect against PD induced by MPTP, the animals received *A. graveolens* should have more density of dopaminergic neurons than that of non-treated group.

5. If the neuroprotective effect of *A. graveolens* occurred via the decrease in free radicals, the animals received *A. graveolens* with MPTP should show lower lipid peroxidation product but higher antioxidant enzyme activities compared to that of vehicle with MPTP.

6. If the neuroprotective effect of *A. graveolens* occurred via the decrease in inflammation process, the mice received *A. graveolens* with MPTP should show lower Iba-1 protein when compared to that of vehicle with MPTP.

7. If the neuroprotective effect of *A. graveolens* occurred via the decrease in total MAO-A, B activities, the mice received *A. graveolens* with MPTP should show lower total MAO-A, B activities when compared to that of vehicle with MPTP.

1.3.4 Significance of the study

1. To provide the information about neuropharmacological activities of *A. graveolens*

2. To provide the information about cognitive enhancing effect of *A. graveolens* in cognitive deficit induced by scopolamine

3. To provide the information about neuroprotective effect of *A. graveolens* against neurotoxicity and dopaminergic toxicity in PD

4. To provide the information about the possible mechanisms underlying the neuroprotective effects of *A. graveolens*

1.3.5 Scope and limitations of the study

1. Male mice were used in this experiment and the ages were approximately at 8 weeks old.

2. All injections should be performed at the same period to avoid the influence of circadian rhythm.

3. Both physical and psychological stress should be avoided to prevent the influence of stress.

4. In all experiments, control and experiment groups should be performed in parallel at the same period to avoid effect of seasonal changes.

CHAPTER 2
RESEARCH METHODOLOGY

2.1 Methods

2.1.1 Preparation of *Apium graveolens* L. crude extract

A. graveolens were collected from Lampang Herb Conservation, Lampang, Thailand was authenticated at the Forest Herbarium, Bangkok, Thailand (BKF number 188856) and prepared as methanolic extract by Dr. Wanida Sukketsiri, Department of Pharmacology, Faculty of Science, Prince of Songkla University, Thailand. The dried power was extracted with 70% methanol in ratio 1:10 for 72 h. The methanolic fraction was filtered by filtrated paper no.1, dried under vacuum in rotary evaporator and lyophilized with Freeze dryer. The *A. graveolens* methanolic extract was kept in tightly closed and light protected container, and stored at 4^{o}C in a refrigerator until use. The *A. graveolens* extract was freshly prepared everyday by dissolved in distill water in order to obtain the desired concentration before administered orally via intragastric tube once daily. According to our research group's result, the total phenolic (gallic acid) and flavonoids (quercetin) contents in the extract were determined. It was found that *A. graveolens* contains of high phenolic content as 43.406 ± 4.70 mg of gallic acid, 18.206 ± 0.12 mg of quercetin per 100 g dry extract (Choosri et al., 2016).

2.1.2 Animal treatments

Adult male C57BL/6 mice, 8 weeks old were used as experimental model. They were obtained from National Laboratory Animal Center, Mahidol University, Salaya campus and supplied with standard protocol of Southern animal unit, Prince of Songkla University. The weights of the animals on the first day of experiment were 25-30 g. They were randomly housed 5 per cage and maintained in a clean room at a temperature between 23 and 27 °C, with a 12 h light-dark cycle and a relative humidity of 50%. Mice were housed in metabolic cages under the supply of filtered pathogen-free air with access to food and water ad libitum. This study was divided into 2 separated parts as described below. All injections in this study were performed once daily between 8.00-9.00 a.m.

The experimental procedures were performed in accordance with the principles of animal care outlined by Faculty of Science, Prince of Songkla University (MOE0521.11/582).

2.1.3 Experimental protocols

2.1.3.1 Animal preparation

This study was designed to investigate the neuropharmacological activities and the neuroprotective effect of *A. graveolens* against PD. The study was divided into 3 separated parts. The first part was set up to screen the neuropharmacological activities of *A. graveolens* in healthy mice. The second part was carried out to determine the neuroprotective effects in cognitive impairment induced by

scopolamine. The last part was designed to determine the neuroprotective effect of *A. graveolens* to protect against PD model.

2.1.3.2 Neuropharmacological activities of *A. graveolens* in healthy mice

The animals were randomly divided into 5 groups as described following;

Group I: Control group which received no treatment.

Group II: Vehicle feeding group, which received distill water (DW) once daily via oral route for 4 weeks.

Group III - IV: Mice were treated with either donepezil (a cholinesterase inhibitor) or vitamin C (an antioxidant) respectively in order to serve as positive control of cognitive function.

Group V - VI: Mice were treated with either diazepam (a standard drug for treatment of anxiety disorders) or fluoxetine (a most common drug for treatment of depression disorders) respectively in order to serve as positive control of anxiolytic and anti-depressant.

Group VII - XI: Mice received *A. graveolens* at various doses ranging from 65, 125, 250, 375 and 500 mg/kg body weight (BW) once daily for 4 weeks.

Each mouse in group II-IX received the same volume of substance suspension. The cognitive enhancing effect was determined in Morris water maze and object recognition tests whereas the anxiolytic activity and anti-depression effect were assessed using elevated plus maze, hole-board, light-dark box, pentobarbital-induced sleeping

time, stereotypy, forced swimming, and tail suspension tests respectively according to the following time schedule, after single dose, 1, 2, 3 and 4 weeks of substance administration. The memory test was performed within 30 min after the treatment. Then, they were sacrificed and keep the brain areas of cerebral cortex, striatum, and hippocampus in order to measure possible underlying mechanism of *A. graveolens* (Figure 9).

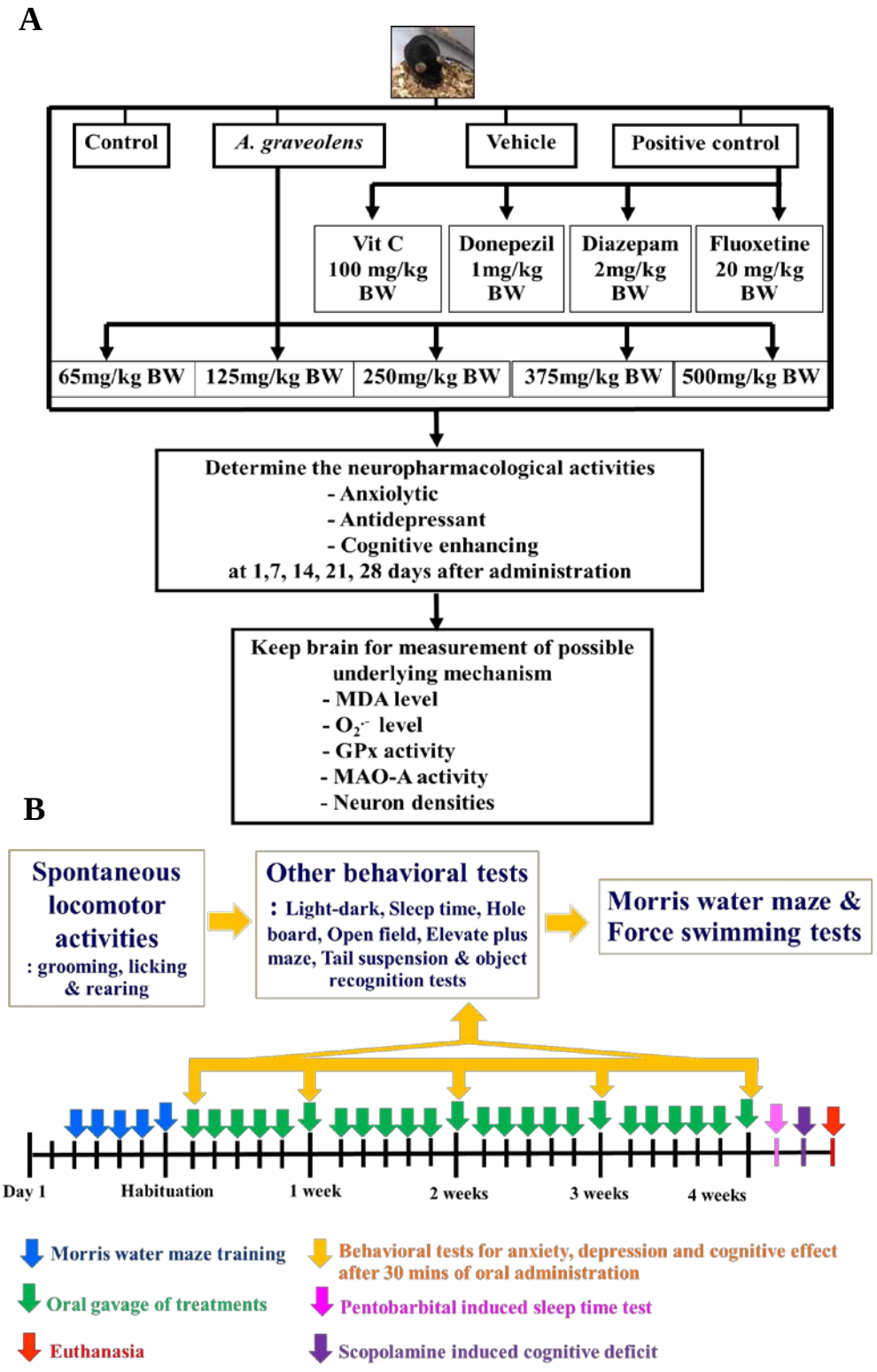


Figure 9 Overview of schematic diagram displays the determination of the effect of oral administration of *A. graveolens* crude extract on neurophamacological activities (A) and experimental diagram in details (B).

2.1.3.3 Neuroprotective effect of *A. graveolens* in cognitive deficit condition induced by scopolamine

All mice were divided into various groups and received the same treatment as mentioned in 2.1.3.2 except that the animals in group II-IV and VII-XI were intraperitoneally (i.p.) injected scopolamine at dose of 3 mg/kg BW within 30 minutes after the last dose of administration (Peterson, 1997; Sun et al., 2007; Wang et al., 2010; Ahmed and Gilani, 2009). Then, they were determined the cognitive function using Morris water maze and object recognition tests and the mice were euthanized to separate the brain areas of cerebral cortex and hippocampus to determine the possible underlying mechanisms of *A. graveolens* (Figure 9B, 10).

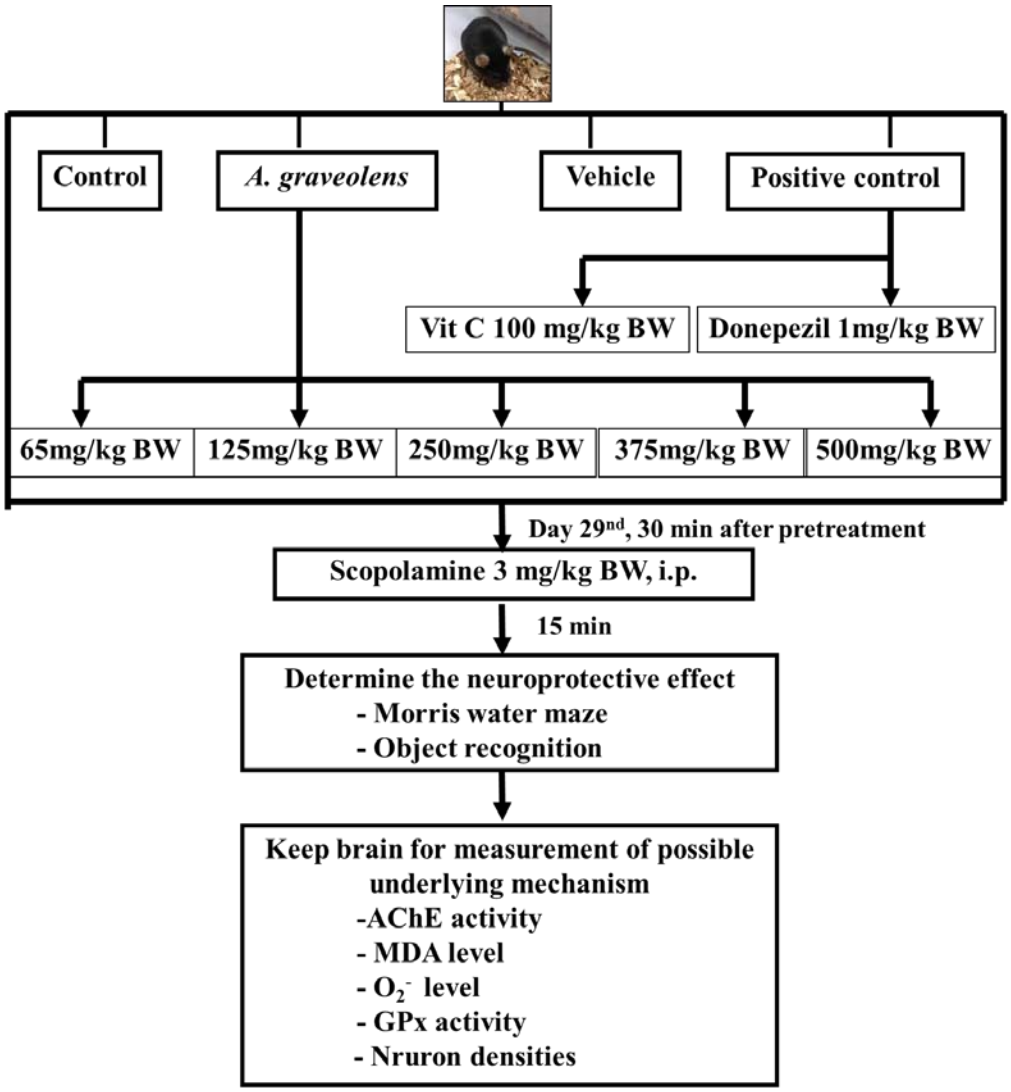


Figure 10 Schematic diagram exhibits the determination of cognitive enhancing effect of oral administration of *A. graveolens* crude extract in cognitive deficit condition induced by scopolamine.

2.1.3.4 Neuroprotective effect of *A. graveolens* in motor deficit condition induced by MPTP

The animals were also divided into various groups as mentioned in 2.1.3.2 except that the animals in positive control group change to tidomet plus 25 mg/kg BW (standard drug for PD treatment) and the administration of each substance was administered 21 days before and 7 days after the i.p. of MPTP. All mice were determined

the motor function using rotarod apparatus, footprint analysis, narrow beam, grid walk, swim, drag and resting tremor tests. Then, they were sacrificed and kept the brain areas of cerebral cortex and striatum in order to measure possible underlying mechanism of *A. graveolens* (Figure 11).

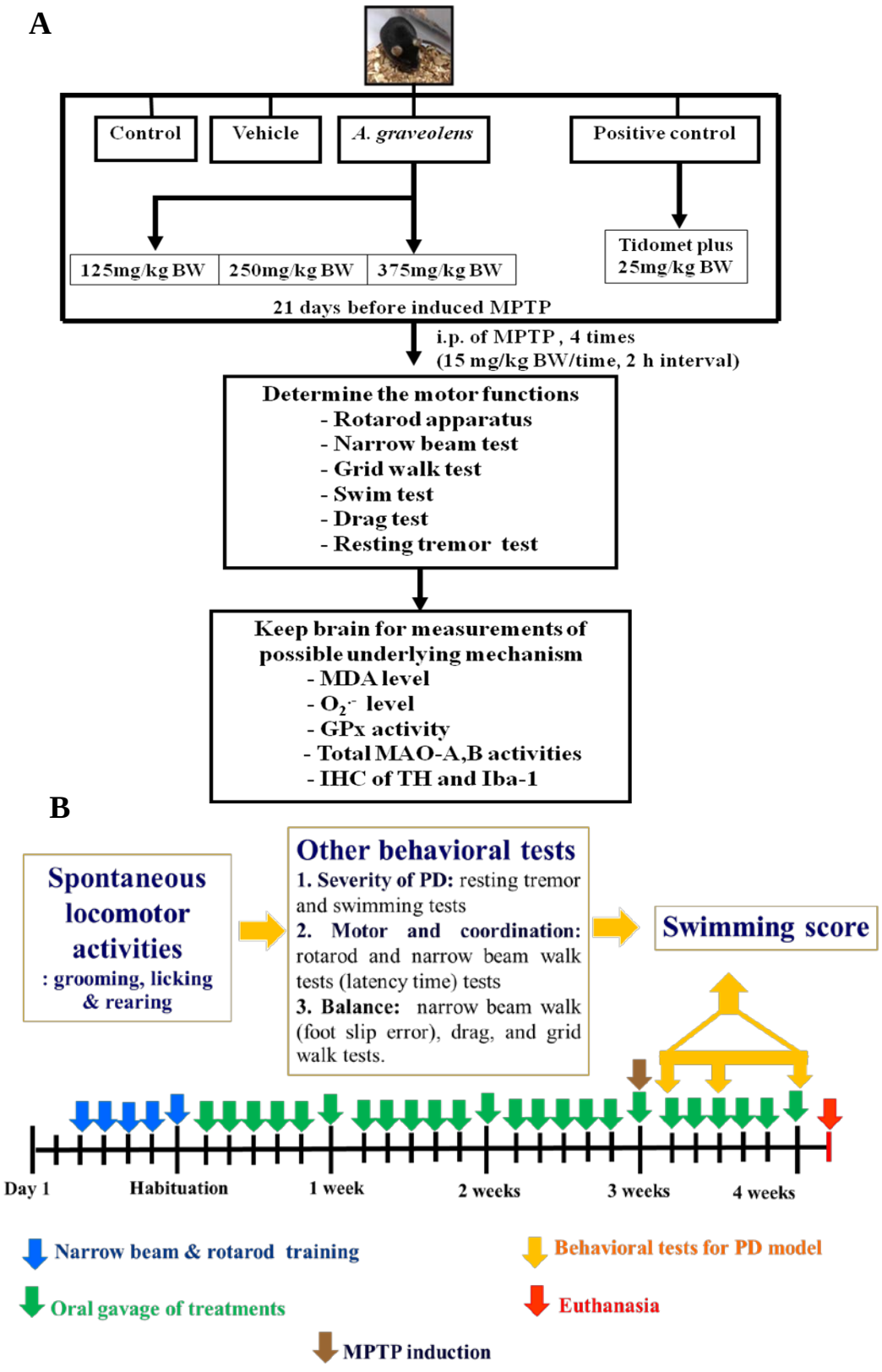


Figure 11 Schematic diagram displays the determination of the neuroprotective effect

of oral administration of *A. graveolens* crude extract in Parkinson-like symptoms condition induced by MPTP (A) and experimental diagram in details (B).

2.1.4 The administration of MPTP protocol

Each animal received four injections of either saline or MPTP solution, the interval between injections was 2 h. MPTP was dissolved in saline and injected i.p. (15 mg/kg BW per injection) in a volume of 1 ml/ 100 g of BW (Sedelis et al., 2001).

2.1.5 Behavioral studies

Behavior testing of all animals was conducted every 7 days by blinded observers. The animals were assessed the changes in all behaviors including

2.1.5.1 Determination of anxiolytic effect including

- Elevated plus maze test

Elevated plus maze apparatus was used to measure the anxiety behavior of the experimental animals. To perform the experiment, experimental animals were placed at the center of the plus maze facing one of the open arms. During 5 min test period, the following data were recorded: number of entries and the time spent in the open and enclosed arms, and the total number of arm entries. If experimental animals were anxious, the percentage of time spent or entries into the open arms decreases (Lister et al., 1987).

- Hole board test

The hold board apparatus was used to determine anxiety in mice. Mice were placed and freely moved on floor of experimental box which was made from plastic (width (W) 40 cm, length (L) 40 cm and height (H) 50 cm) and there are round hole on floor of box 16 holes (diameter 2 cm). The diameters of holes were wide enough for mice can dip into look below for 5 min. The numbers of head dipped of mice were counted (Takeda et al., 1998).

- Light-dark box test

The light/dark test depends on the innate antipathy of rodents to brightly light areas so the light/dark test is useful to measure anxiolytic-like or anxiogenic-like activity in mice. The test apparatus consists of a dark safe compartment and a large illuminated compartment. The scope to which an anxiolytic compound can activate exploratory activity bases on the baseline level of the control group. The time to spend in light box was determined and compared between groups within 6 min (Bourin and Hascoët, 2003).

- Pentobarbital-induced sleeping time test

The anxiety disorders caused an arousal dysfunction which often leads to persistent sleep difficulties. Mice were first orally treated with substance for 30 min before followed by administration of pentobarbital-HCl (50 mg/kg, i.p.). Subsequently, latency of sleep and sleep duration were recorded and compared between groups (Ma et al., 2008).

- Stereotypy

Stereotypy has been categorized as a group of preservative acts in continuum, going from increased rearing, grooming and licking. All animals in each group were determined repetitive characteristic which no obvious goal or function of the behavior which mentioned above.

2.1.5.2 Determination of antidepressant effect including

- Tail suspension test (TST)

Mice were suspended by their tails using adhesive tape to the horizontal bar for 6 min and the immobility time was examined. Typically, the suspended mice were immediately escape-like behaviors, followed by an immobile posture. If antidepressants are given prior to the test, mice exhibited a decrease in duration of immobility (Steru et al., 1985).

- Forced swimming test (FST)

The forced swimming test was based on the observation that mice were forced to swim and display a floating posture in an inescapable cylinder filled with water which was considered as a depression-like behavior (behavioral despair). In the test, antidepressants induced a decrease in immobility time (Borsini et al., 1995). Mice were measured the following behaviors within 3 min (Detke et al., 1995): Immobility-floating in the water without effort and only kept the head above the water, swimming-displaying moderate active movements

around the cylinder and climbing-showing active vigorously motions with forepaws in and out of water, usually touched against the walls.

2.1.5.3 Determination of cognitive enhancing effect including

- Morris water maze test (MWT)

The Morris water maze test is a commonly used cognitive and behavioral assessment tool for testing working spatial memory which was believed to be related to the hippocampal function. Animals were examined in a round polyvinyl water pool (120 diameter, H 50 cm) that were partially added with water (25 °C). Powder was used to provide the water opaque. The pool was divided equally into four quadrants, labeled N-S-E-W. A platform (10 cm diameter) was placed in one of the four quadrants (the target quadrant) and submerged 1.5 cm below the water surface. The training started by the mice was freely swum in the pool for 60s with platform that remained in the same quadrant during the entire experiment. The mice were located the platform using only distal spatial cues available in the experiment. The cues were stilled constant throughout the test. The mice must swim until it climbed onto the platform. After climbing onto the platform, the mice will remain there for 30 s before the commencement of the next trial. If the mice failed to reach the escapes platform within the maximally allowed time of 60 s, it were gently placed on the platform and allowed to remain there for the same amount of time. The time to reach the platform or escape latency in seconds was determined. After 24 h, the retention time was determined wherein the extent of memory consolidation. The

time spent in the target quadrant expressed the degree of memory consolidation that has taken place after learning. Mice were placed into the pool as the training trial, except that the blind platform was removed from the pool. The time of crossing the target platform quadrant was counted for 1 min (Morris, 1984).

- Object recognition test

The object recognition test was performed to determine cognition of shape, color and texture. The apparatus consisted of a circular arena, 100 cm in diameter and H 40 cm wall. Each object was available in triplicate and always thoroughly cleaned with 70% alcohol between sessions to prevent the use of odor cues. In addition, mice could not displace the objects. The order of objects used per subject per session was determined randomly; all locations and combinations of objects were used in a balanced score to reduce biases of measurement. Mice were placed in apparatus, then were recorded of number and time of approach to both objects and calculated according to the equation (Antunes and Biala, 2012).

$$\text{Discrimination index} = \frac{\text{(Time of approach to object2} - \text{Time of approach to object1)}}{\text{(Time of approach to object2} + \text{Time of approach to object1)}}$$

2.1.5.4 Determination of motor function including

- Rotarod apparatus

The rotarod test, mice walk on a rotating rod, was widely used to examine motor and coordination condition. Performance was measured by the duration that an animal stays upon the

drum as a function of drum speed. Mice were allowed to adjust their posture in order to maintain their balance on a rotating rod at speeds of 5, 10, 15 and 20 rounds per min (rpm). The average retention time on the rod was calculated as described previously (Rozas et al., 1997).

- Narrow beam test

In the beam-walking test, mice were trained to cross a series of elevated at a height of 100 cm from the floor, narrow beams (L 100 cm× W 1 cm) to reach an enclosed escape platform. The protocol was measured of latency to traverse the beam and foot slips. The time taken to traverse the beam from one end to other was counted (Pisa, 1998).

- Grid walk test

The grid walking task based on the foot fault task, was a simple way to determine motor impairments of limb functioning and placing deficits during locomotion in experimental animals. Mice were placed on an elevated H 100 cm, W 40 cm, L 60 cm, leveled grid with openings. Mice without Parkinson-like symptoms were typically placed their paws precisely on the frame to maintain themselves while moving along the grid. Each time a paw slipped through an open grid, a “foot fault” was counted (Tillerson et al., 2003).

- Swimming test

Swimming test was performed to determine motor disability using round swimming pool (L 40 cm, W 25 cm, H 16 cm) and filled with the water 12 cm from the floor (maintained

temperature 27 ± 2 ^{o}C). Mice were tested and scored as follow: 0 = no swimming and the head was above the water, 1 = sometimes swim-floating using hind paws, 2 = swim-floating alternation and 3 = swim continuously (Haobam et al., 2005).

- Drag test

The drag test was acted to determine the balance ability using forelimbs in responsible for an externally dynamic stimulus. Mice were gently lifted using the tail and dragged backwards at a persistent speed about 20 cm/s for 120 cm fixed distance. The number of touches occurred by each forepaw was counted (mean between the two forepaws) (Viaro et al., 2008).

- Resting tremor

The resting tremor was performed to evaluate Parkinson-like symptom as resting tremor in mice. Mice were placed in transparent box that was above the floor 500 cm, recorded using VDO for 45 min and determined severity every 3 min as described following; 0 = no observation of resting tremor, 1 = showing less resting tremor of postural muscle only, 2 = exhibiting moderate resting tremor sometimes of head, 3 = obviously resting tremor but not involved in head sometimes, 4 = continuously showing resting tremor and no movement of limbs and head and 5 = displaying resting tremor all time and body (Lundblad et al., 2004).

2.1.6 Histological, immunohistochemical studies and enzyme assay

- Tissue Preparation

The brain of the animals were perfused transcardially with fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3 and they were post fixed in the same fixative overnight at 4°C. Then, they were immersed sequentially for 48-72 h each in a cryoprotectant containing 30% sucrose. Serial sections of tissues containing cerebral cortex and hippocampus were cut frozen on a sliding microtome at 30 µm thickness. Sections were stored in phosphate buffer and they were picked up with the slides coated with a 0.01% aqueous solution of a high molecular weight poly-L-lysine. The serial cut sections were either stored at 4°C or processed immediately.

2.1.6.1 Cresyl violet staining for Nissl substance

Adjacent series of cerebral cortex and hippocampus sections from various groups were stained with 0.5% cresyl violet to determine the neuronal density (Paxions and Chorles, 1981).

2.1.6.2 Immunohistochemistry (IHC) study of tyrosine hydroxylase (TH)

The immunohistochemistry analysis of tyrosine hydroxylase was performed according to the method of Ahmad and co-workers (Ahmad et al., 2005). The coronal brain sections of 5 µm thickness were cut on a rotary microtome. The sections were then transferred to gelatin-coated slides and immersed in wash buffer (sodium

phosphate 100 mM, sodium chloride 0.5 M, Triton X-100, sodium azide) at pH 7.4 for 20 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide and 10% methanol in PBS and incubated for 30 min at room temperature (RT). Thereafter, the slides were washed with PBS, and the sections were overlaid with 20 µl of anti-tyrosine hydroxylase antibodies (2% in PBS) and incubated for 2 h in a humid chamber at RT. The slides were incubated again with 20 µl solution of biotinylated anti-mose IgG (2% in PBS) for 3 h at RT in the humid chamber. Then slides were exposed to streptavidin-peroxidase and the labeled locations were visualized with a solution of diaminobenzidine (DAB) kit. Finally the sections were dehydrated, cover-slipped, and observed under the light microscope.

2.1.6.3 IHC analysis of ionized binding adaptor molecule-1 (Iba-1)

Immunohistochemistry technique of substantia nigra pars compacta (SNpc) was done as described previously (Thangavel et al., 2009; Ghatak and Combs, 2014). The high level expression of Iba-1 occurred from activated microglia cell (Hirsch, 2009). The serial coronal brain sections were cut 5-6 µm in rotatry microtome, placed on slide, dipped into 0.3% H_2O_2 for inhibiting endogenous peroxidase and blocking with 1% BSA for inhibiting non specific binding followed by incubation with the primary rabbit anti-goat Iba-1 for overnight at RT. The sections were then washed and incubated with biotinylated secondary goat anti-rabbit antibody for 2 hr at RT followed by DAB as a substrate to develop brown color. Eventually, the sections were dehydrated, cover slipped, observed under the light microscope.

- Preparation of tissue homogenates

After the last injection of substances, all animals were anesthetized with i.p. injection of pentobarbital sodium at dose of 50 mg/kg BW. Brains were isolated and kept cool in ice buckets. Then the tissues were homogenized in 4 volume of 1.15% KCl with glass Potter-Elvehjim homogenizer (Marzel, 1979).

2.1.6.4 Estimation of malondialdehyde (MDA)

Malondialdehyde (MDA) a measure of lipid peroxidation was measured as described by Ohkawa et al. (1979). Reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecylnsulphate (8.1%) were added to 0.1 ml of processed tissue samples, and then heated at 100 °C for 60 min. The mixture was cooled under tap water and 5 ml of n-butanol-pyridine (15:1), 1 ml of distilled water was added and vortexed vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was separated and the absorbance was measured at 532 nm using a spectrophotometer. The concentration of MDA was expressed as nmol/g tissue.

2.1.6.5 Acetylcholinesterase (AChE) assay

The activity of AChE was measured according to a method developed by Ellman et al. 1961. This method employs acetylthiocholine iodide (ATChI) as a synthetic substrate for AChE. ATChI is broken down to thiocholine and acetate by AChE and thiocholine is reacted with dithiobisnitrobenzoate (DTNB) to produce a

yellow color. The quantity of yellow color which develops over time is a measure of the activity of AChE and can be measured using a spectrophotometer at wavelength of 412 nm (Ellman et al., 1961).

2.1.6.6 Scavenging enzyme assay

Glutathione peroxidase (GPx) is radical scavenging enzyme that catalyzes reduced reaction of hydrogen peroxide compound as lipid peroxide (ROOH) and H_2O_2 by glutathione (GSH) reacted together and caused of oxidized glutathione (GSSH) and H_2O. Afterwards, glutathione reductase (GR) catalyze GSSH by reacted with nicotinamide adenine dinucleotide phosphate (NADPH) resulting to GSH and $NADP^+$. The measurement of absorbance of $NADP^+$ was done at 340 nm (Hussain et al., 1995).

2.1.6.7 Total monoamine oxidase-A, B (MAO-A, B) activities

The homogenates were incubated with 500 µM tyramine plus 500 nM pargyline or 2.5 mM tyramine plus 500 nM clorgyline in order to inhibit the MAO-B or A activities, respectively. The chromogenic solution prepared in the assay mixture contained vanillic acid (1 mM), 4-aminoantipyrine (500 mM), and peroxidase (4 U ml^{-1}) in potassium phosphate buffer (0.2 M, pH 7.6). The MAO-A and B activities were determined by spectrophotometric measurement according to Holt et al., 1997 and measured optical density (O.D.) at 425 nm. The MAO-A and B activities are analyzed as unit/mg protein wherein, one unit is determined as nmol product formed/min.

2.1.6.8 Superoxide ($O_2^{\cdot-}$) anion assay

Superoxide anion is particularly important as the product of the one-electron reduction of dioxygen O_2, which was widely produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens but superoxide is biologically toxic. The $O_2^{\cdot-}$ level was determined by spectrophotometric measurement. The method based on xanthine/ xanthine oxidase (XO) system which converted nitro blue tetrazolium (NBT)-yellow color to formazan-blue color. The reagent mix was prepared of EDTA, NBT, xanthine and xanthine oxidase (XO), mixed with sample and measured at 560 nm compared with standard curve of TEMPOL. The data was expressed as % inhibition which was calculated following the equation (Ukeda et al., 1997).

$$\% \text{ inhibition} = \frac{A-B}{B} \times 100$$

A= OD of reagent only

B= OD of sample

2.1.7 Data analysis

All sections of cerebral cortex, hippocampus, striatum and SNpc were evaluated with the Olympus light microscope model BH-2 (Shinjuku-ku, Tokyo, Japan). For the determination of neuronal density, five representative non-adjacent sections containing cerebral cortex (from bregma 0.7 mm, dorsolateral 9.7 mm, mediolateral 2 mm, anteroposterior 2 mm), hippocampus (from bregma -5.2 mm, dorsolateral 3.8 mm, mediolateral 4.3 mm, anteroposterio 5.1 mm), striatum (from bregma -2.3 mm, dorsolateral 6.7 mm, mediolateral 4.3 mm, anteroposterio 6.3 mm)

and SNpc (from bregma -4.8 mm, dorsolateral 4.2 mm, mediolateral 2.2 mm, anteroposterio 8.2 mm) were selected for analysis. The observer was blind to the treatment at time of analysis. The density neurons in cell number/1.5 mm^2 and TH positive immunoreactived neurons were determined at 40X magnification.

All motor behaviors including stereotypy, elevated plus maze test, hole board test, open field test, light-dark test, sleep time test, forced swimming test, tail suspension test, object recognition test, morris water maze test, rotarod test, footprint analysis, narrow beam test, grid walk test, swim test, drag test and resting tremor score were analyzed using SPSS program version 13.0 (IBM Corp., Armonk, NY, USA).

All tissue homogenates of cerebral cortex, striatum, and hippocampus were evaluated with the aid of spectrophotometer.

2.1.8 Statistical analysis

All data were expressed as mean ± S.D. value. The significant differences between control and treatment and the differences among various groups were compared by ANOVA (Tukey's test). The statistical difference was regarded at p-value < 0.05.

2.2 Materials and Equipment

MPTP and scopolamine were purchased from Sigma-Aldridge company, diazepam, donepezil, tidomet plus and fluoxetine (Sigma-Aldridge company, Ltd., MO, NY, USA) were kindly given by Assoc. Prof. Dr. Somsak Tiamkao, Division of Neurology, Department of Medicine, Faculty of Medicine, Khon Kaen University, Thailand.

CHAPTER 3
RESULTS

3.1 Neuropharmacological activity of *Apium graveolens* L. in healthy mice

3.1.1 Effect on spontaneous motor activities

The effects of *A. graveolens* on spontaneous motor behaviors including grooming, rearing and licking behaviors were demonstrated in Figures 12. The results showed that vehicle and *A. graveolens* at all doses failed to show significant changes in all spontaneous motor behaviors mentioned above.

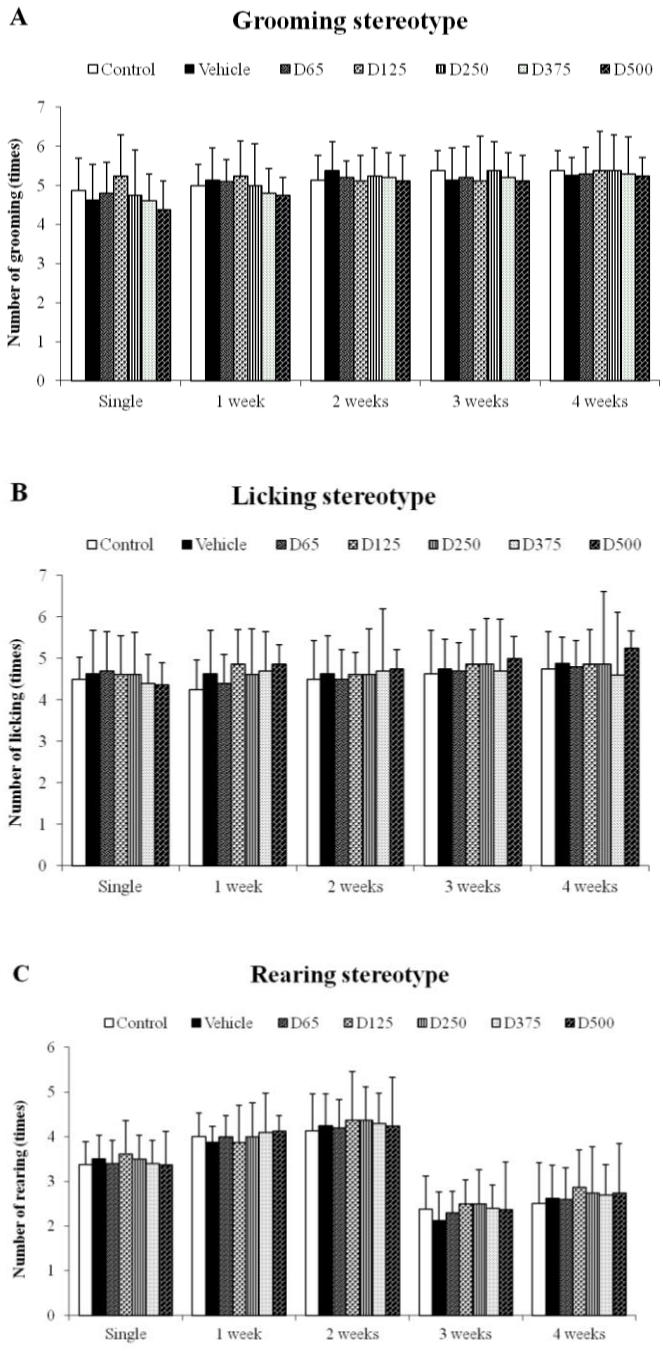


Figure 12 The effect of *A. graveolens* on spontaneous activities of mice subjected to grooming (A), licking (B), and rearing (C). Control: no treatment group, Vehicle: DW-treated group, D65: 65 mg/kg *A. graveolens*-treated group, D125: 125 mg/kg *A. graveolens*-treated group, D250: 250 mg/kg *A. graveolens*-treated group, D375: 375 mg/kg *A. graveolens*-treated group, D500: 500 mg/kg *A. graveolens*-treated group. Data were represented as mean ± S.D., n=8 each.

3.1.2 Anxiolytic activity

The anxiolytic effect of *A. graveolens* crude extract, was examined by dark-light box test (Figure 13), sleep test-induced by pentobarbital (Figure 14), hole board test (Figure 15), open field test (Figure 16), and the last, elevated plus maze test (Figure 17) as indices.

The results showed that vehicle did not exhibit significant difference in all tests. Diazepam, a standard drug for anxiety therapy which was utilized as a positive control in this study, significantly increased time spent of light chamber in dark-light box test, time spent of open arm entry and number of open arm approach in elevate plus maze test, time of central area approach in open field test, sleep duration of sleep test and number of head dip of mice in hole board test while decreased onset to sleep-induced by pentobarbital and number of peripheral area approach in open field test (p<0.05 all). Interestingly, D125 mg/kg BW of *A. graveolens*-treated group showed significantly on elevated time of central area approach, time spent of light chamber and sleep duration whereas reduced the number of peripheral area approach in open field test and onset to sleep-induced by pentobarbital (p<0.05 all) when compare to positive control group. Furthermore, D125 mg/kg BW also increased the number and time of open arms in elevate plus maze test, number of head dip in hole board test (p<0.05 all) when compare to diazepam treated group.

A. graveolens of 125-500 mg/kg BW that were used in this study significantly improved all of anxiolytic- screening tests after 1-4 weeks of treatment (p<0.05 all). The anxiolytic effect of *A. graveolens*

on all behavioral tests also failed to exhibit a dose-dependent effect. The peak effect was found in *A. graveolens* at dose of 125 mg/kg BW.

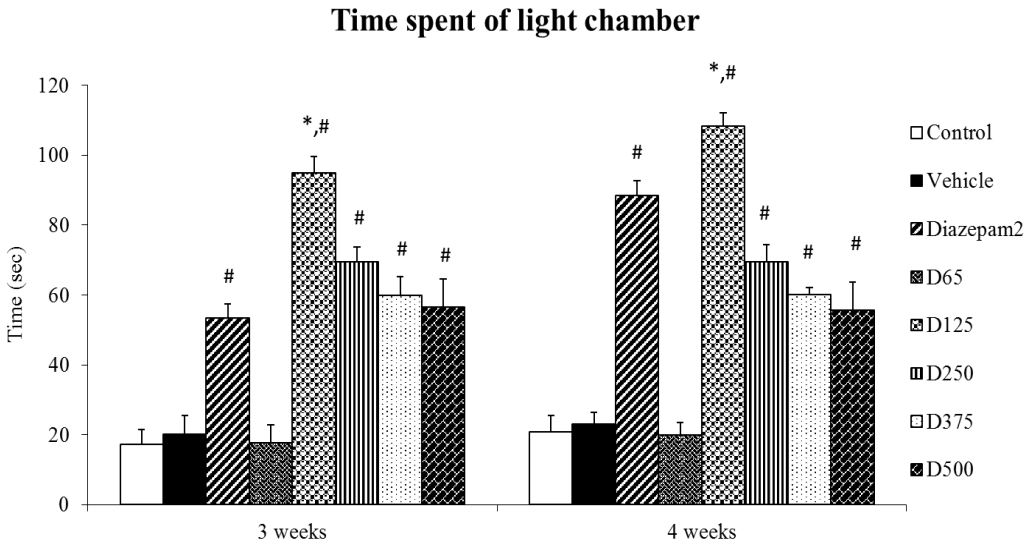


Figure 13 The anxiolytic effect of *A. graveolens* on time spent of light chamber of mice subjected to dark-light box. Control: no treatment group, Vehicle: DW-treated group, Diazepam: 2 mg/kg BW diazepam-treated group, D65: 65 mg/kg *A. graveolens*-treated group, D125: 125 mg/kg *A. graveolens*-treated group, D250: 250 mg/kg *A. graveolens*-treated group, D375: 375 mg/kg *A. graveolens*-treated group, D500: 500 mg/kg *A. graveolens*-treated group. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Diazepam-treated group, $p<0.05$.

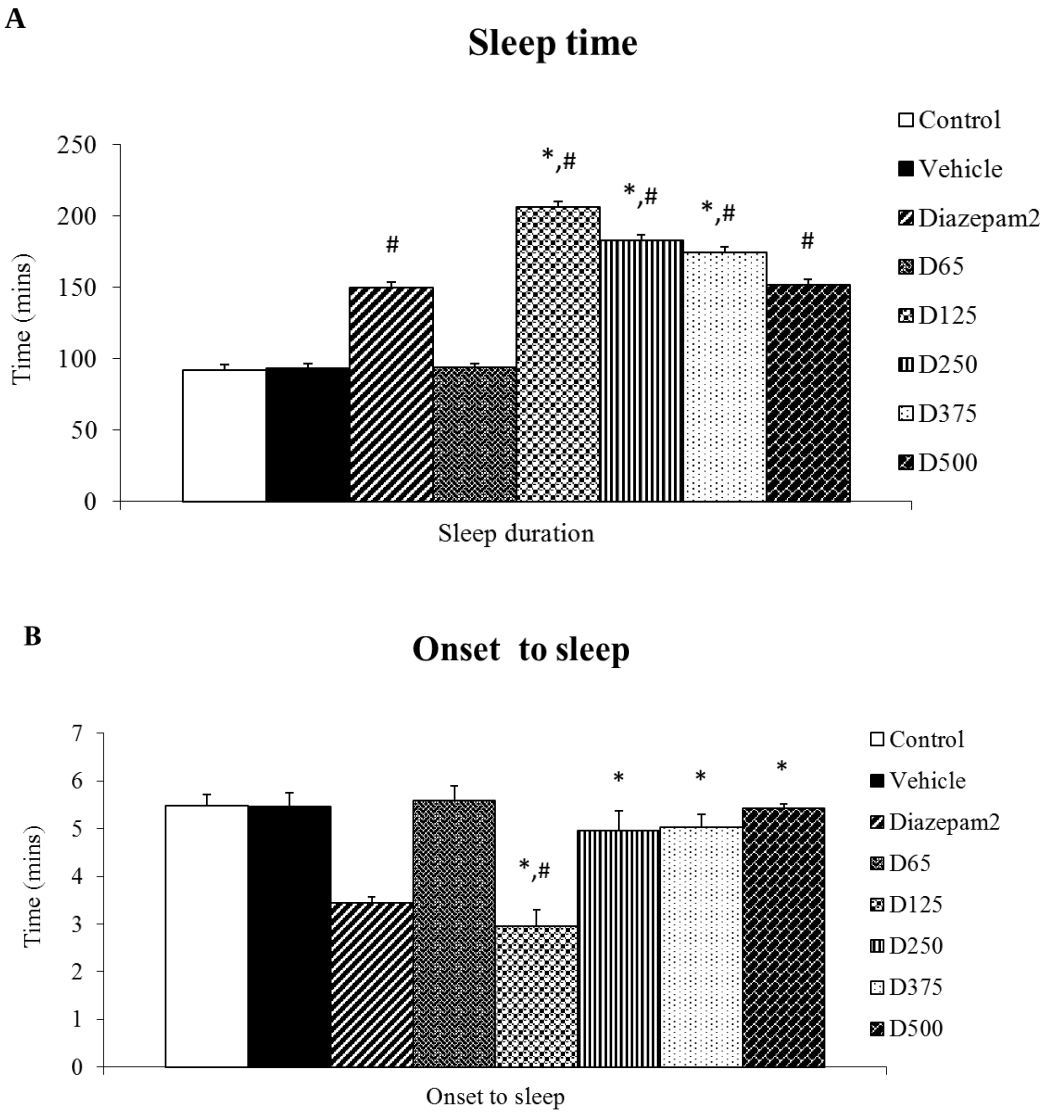


Figure 14 The anxiolytic effect of *A. graveolens* on sleep duration (A) and onset to sleep (B) of mice subjected to sleep test-induced by pentobarbital drug. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Diazepam-treated group, $p<0.05$.

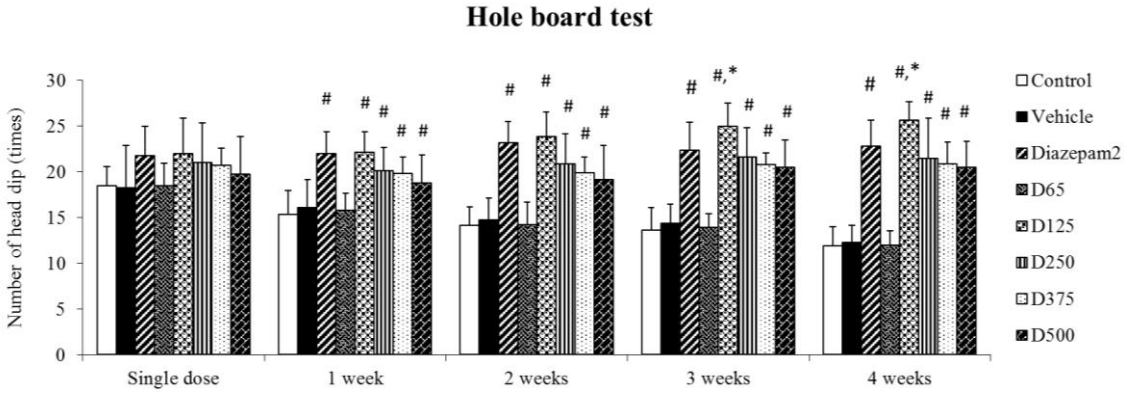


Figure 15 The anxiolytic effect of *A. graveolens* on number of head dip of mice subjected to hole board test. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Diazepam-treated group, $p<0.05$.

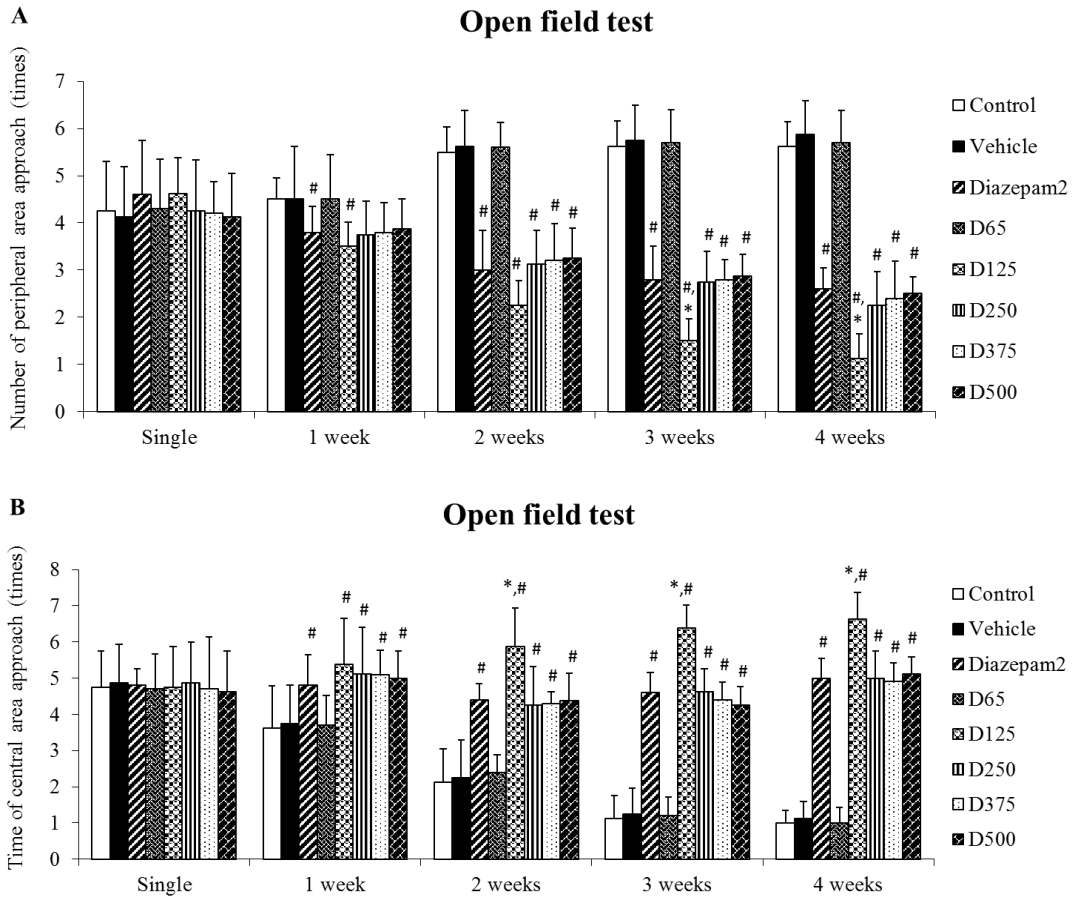


Figure 16 The anxiolytic effect of *A. graveolens* on number of peripheral area approach (A) and time of central area approach (B) of mice subjected to open field test. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Diazepam-treated group, $p<0.05$.

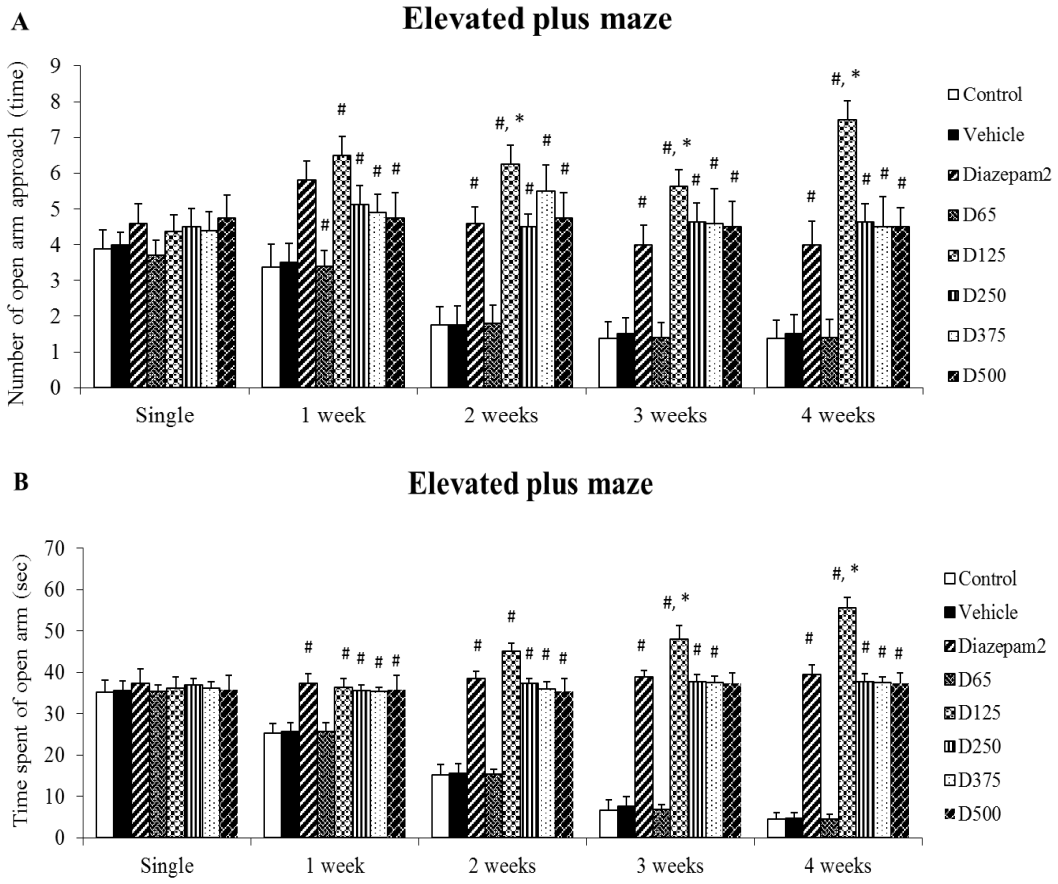


Figure 17 The anxiolytic effect of *A. graveolens* on number of open arm approach (A), time spent of open arm approach (B) of mice subjected to elevated plus maze test. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Diazepam-treated group, $p<0.05$.

3.1.3 Anti-depression activity

The parameters determined in the forced swimming and tail suspension tests were expressed in Figures 18-19. The results of this study displayed that only vehicle treatment did not show significant difference in immobility, climbing times in forced swimming test and immobility times in tail suspension test at all treatment durations used in this study. Fluoxetine, a serotonin uptake inhibitor, significantly reduced immobility time ($p<0.05$ all) but elevated climbing time at all duration of

treatment (p<0.05 all). The significant changes in climbing time induced by fluoxetine were observed after single dose of administration until 4 weeks of treatment (p<0.05 all). *A. graveolens* from 125-500 mg/kg BW produced significant changes in both immobility and climbing times at all treatment duration in the same pattern as those occurred after fluoxetine treatment (p<0.05 all). In tail suspension test, fluoxetine and *A. graveolens* also exhibited significant changes in immobility time that coherent with forced swimming test. Our data failed to show a dose-dependent effect of *A. graveolens*.

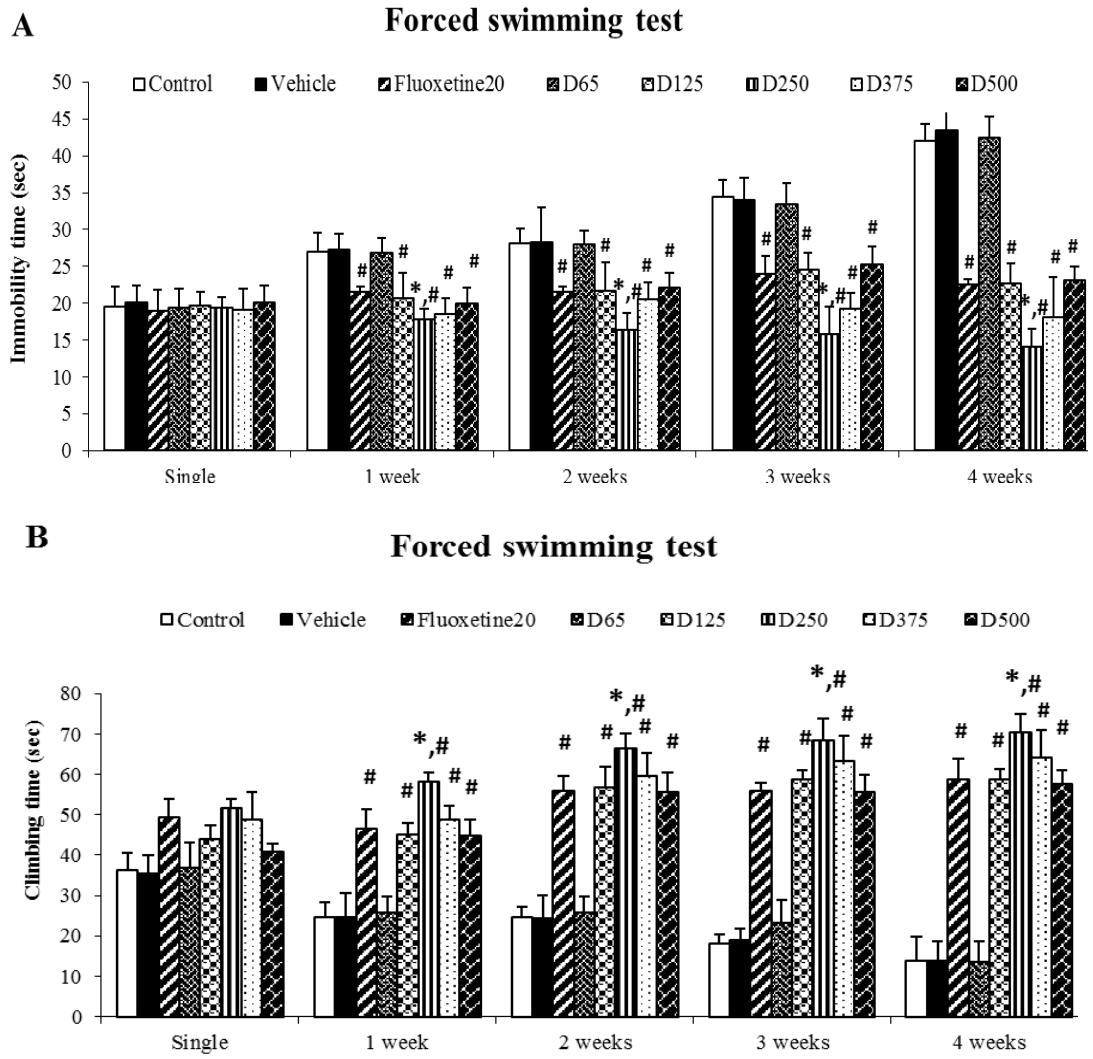


Figure 18 The anti-depressant effect of *A. graveolens* on immobility time (A) and climbing time (B) of mice subjected to forced swimming test. Control: no treatment group, Vehicle: DW-treated group, Fluoxetine: 20 mg/kg BW fluoxetine-treated group, D65: 65 mg/kg *A. graveolens*-treated group, D125: 125 mg/kg *A. graveolens*-treated group, D250: 250 mg/kg *A. graveolens*-treated group, D375: 375 mg/kg *A. graveolens*-treated group, D500: 500 mg/kg *A. graveolens*-treated group. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Fluoxetine-treated group, $p<0.05$.

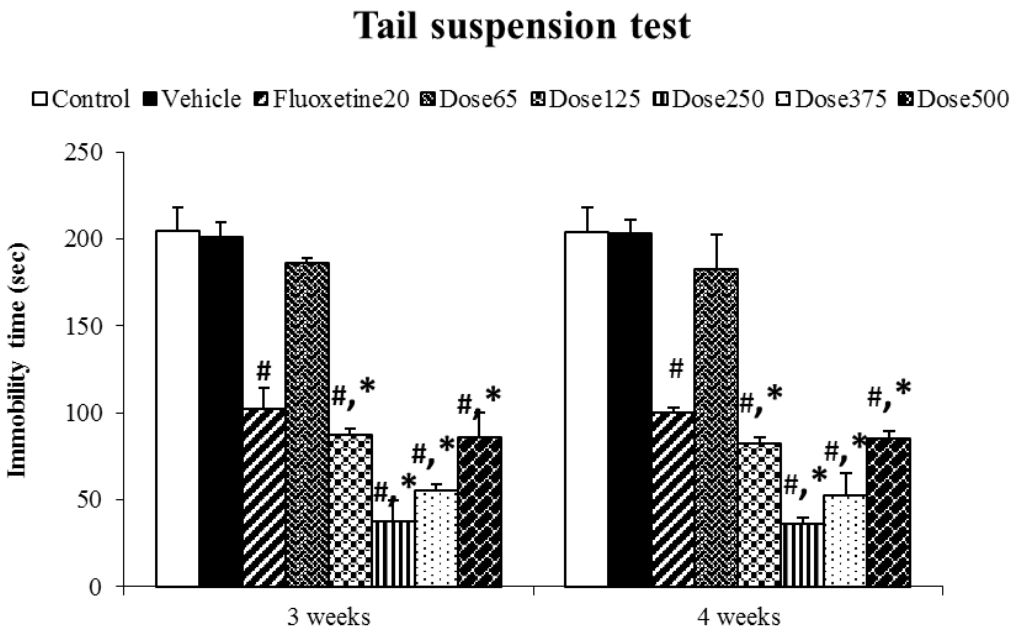


Figure 19 The anti-depressant effect of *A. graveolens* on immobility time of mice subjected to tail suspension test. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Fluoxetine-treated group, $p<0.05$.

-Effect of *A. graveolens* to suppress monoamine oxidase-type A (MAO-A) activity

The effect of *A. graveolens* on brain MAO-A was shown in Figure 20. The administration of *A. graveolens* (125, 250, 375 and 500 mg/kg) markedly decreased MAO-A activity when compared to the vehicle ($p<0.05$) in the cerebral cortex and striatum areas. Peak effect was found in the 125 mg/kg *A. graveolens* treatment group.

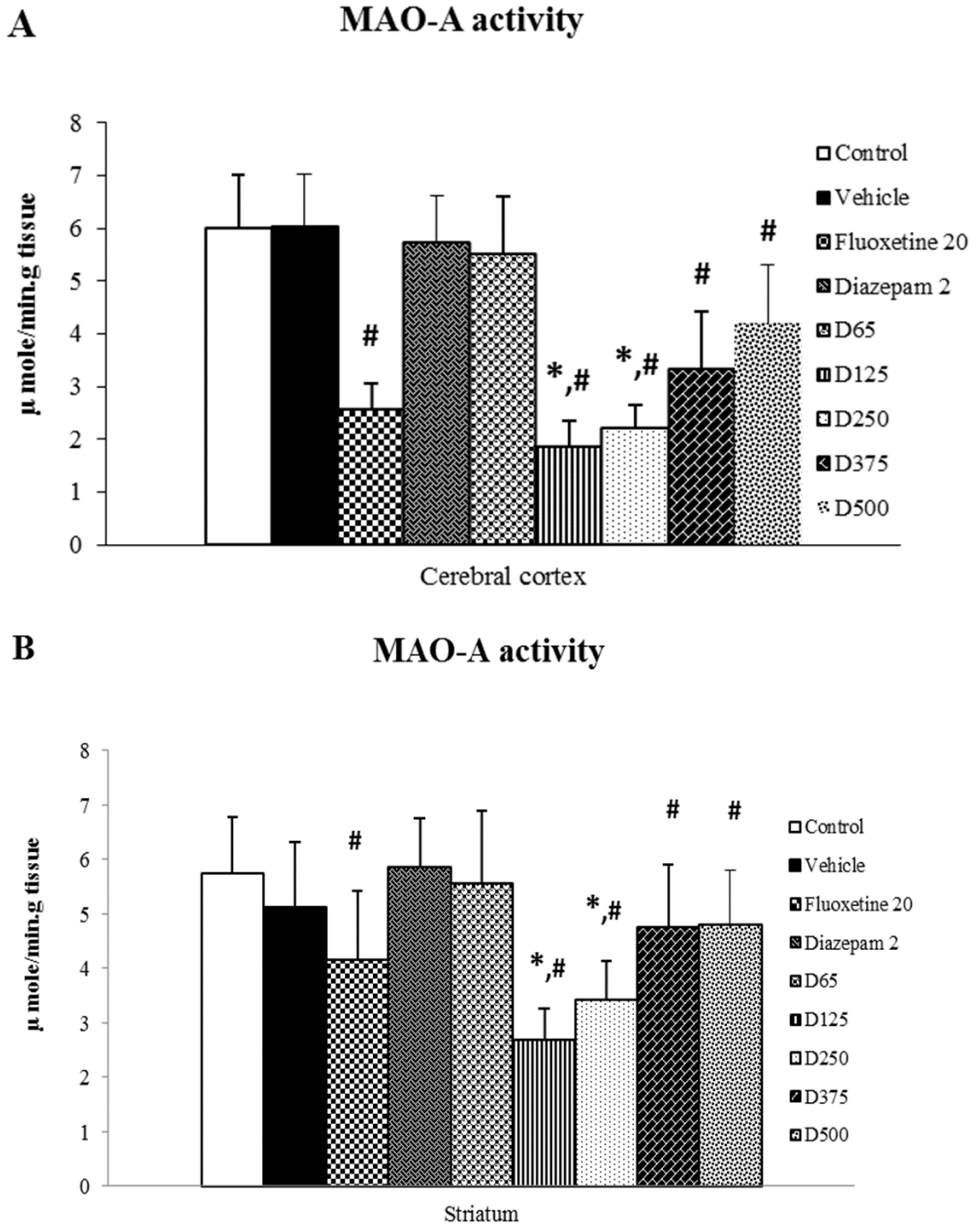


Figure 20 The effect of *A. graveolens* on MAO-A activity assay in cerebral cortex (A) and striatum (B). Data were represented as mean ± S.D., n=8 each, # vs. control group, $p<0.05$; * vs. Fluoxetine-treated group, $p<0.05$.

3.1.3 Cognitive enhancing effect

The effect of *A. graveolens* on spatial memory, determined by using escape latency and retention time in morris water maze test as indices and were demonstrated in Figures 21. In addition, the

effect of *A. graveolens* on non-forced recognition memory, was determined using object recognition test and were demonstrated in Figures 22. The results showed that vehicle did not produce significant changes in both tests. Donepezil, a standard drug for dementia treatment which was used as a positive control in this study, significantly decreased escape latencies whereas increased retention time in morris water maze test ($p<0.05$ all). Moreover, it was shown to increases novel exploration time or discrimination index in object recognition test ($p<0.05$ all). *A. graveolens* of 125-500 mg/kg BW significantly decreased the escape latency in morris water maze test after 1-4 weeks of treatment ($p<0.05$ all). However, retention time significantly increased after 2-4 weeks of treatment ($p<0.05$ all). The *A. graveolens* treatment of 125-500 mg/kg BW increased the novel exploration time higher than those found after donepezil treatment ($p<0.05$ all). The peak effect was found in *A. graveolens* at dose of 250 mg/kg BW. The cognitive enhancing effect of *A. graveolens* on both cognitive tests also failed to shows a dose-dependent effect.

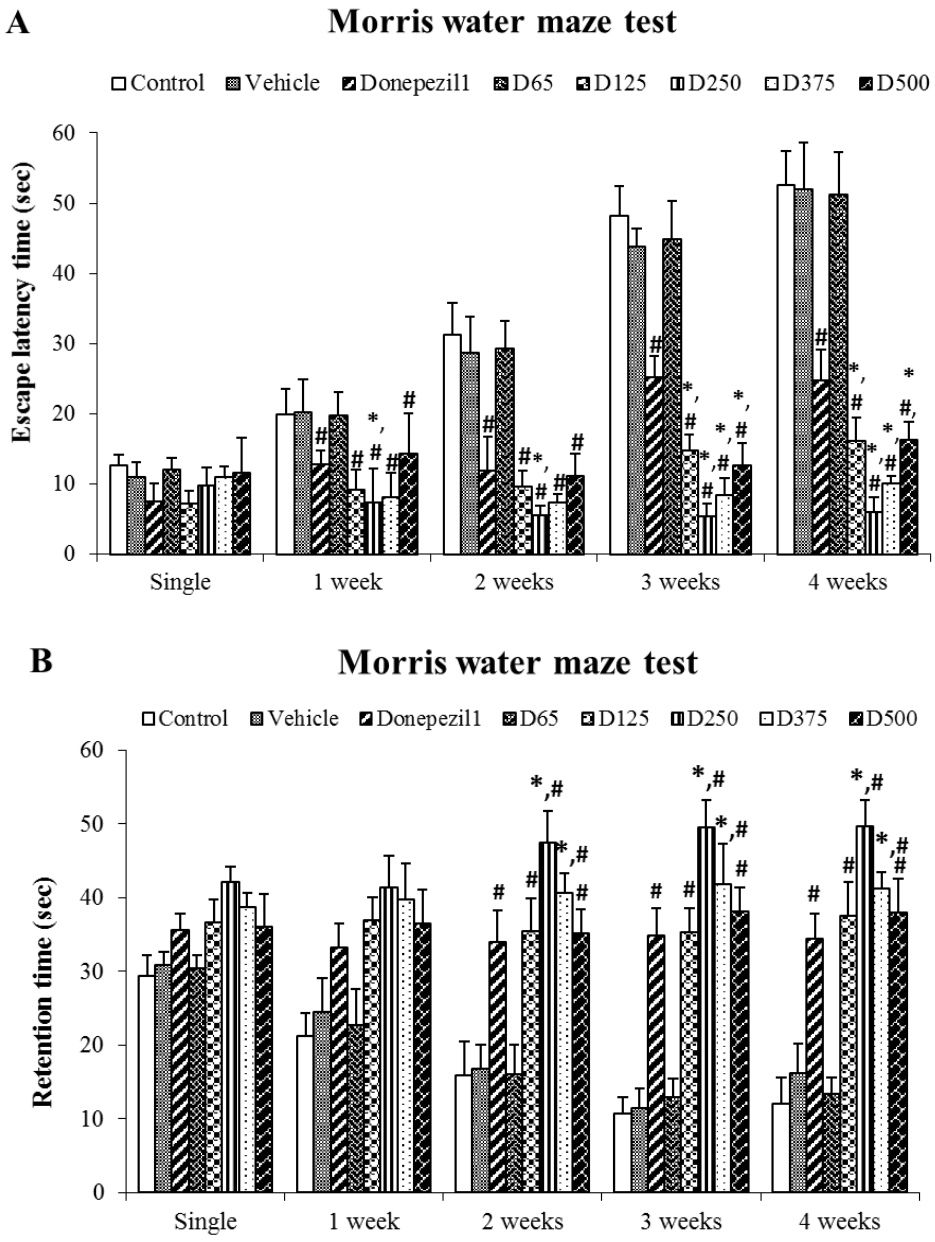


Figure 21 The spatial cognitive enhancing effect of *A. graveolens* on escape latency time (A) and retention time (B) of mice subjected to morris water maze test. Control: no treatment group, Vehicle: DW-treated group, Donepezil: 1 mg/kg BW donepezil-treated group, D65: 65 mg/kg *A. graveolens*-treated group, D125: 125 mg/kg *A. graveolens*-treated group, D250: 250 mg/kg *A. graveolens*-treated group, D375: 375 mg/kg *A. graveolens*-treated group, D500: 500 mg/kg *A. graveolens*-treated group. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Donepezil-treated group, $p<0.05$.

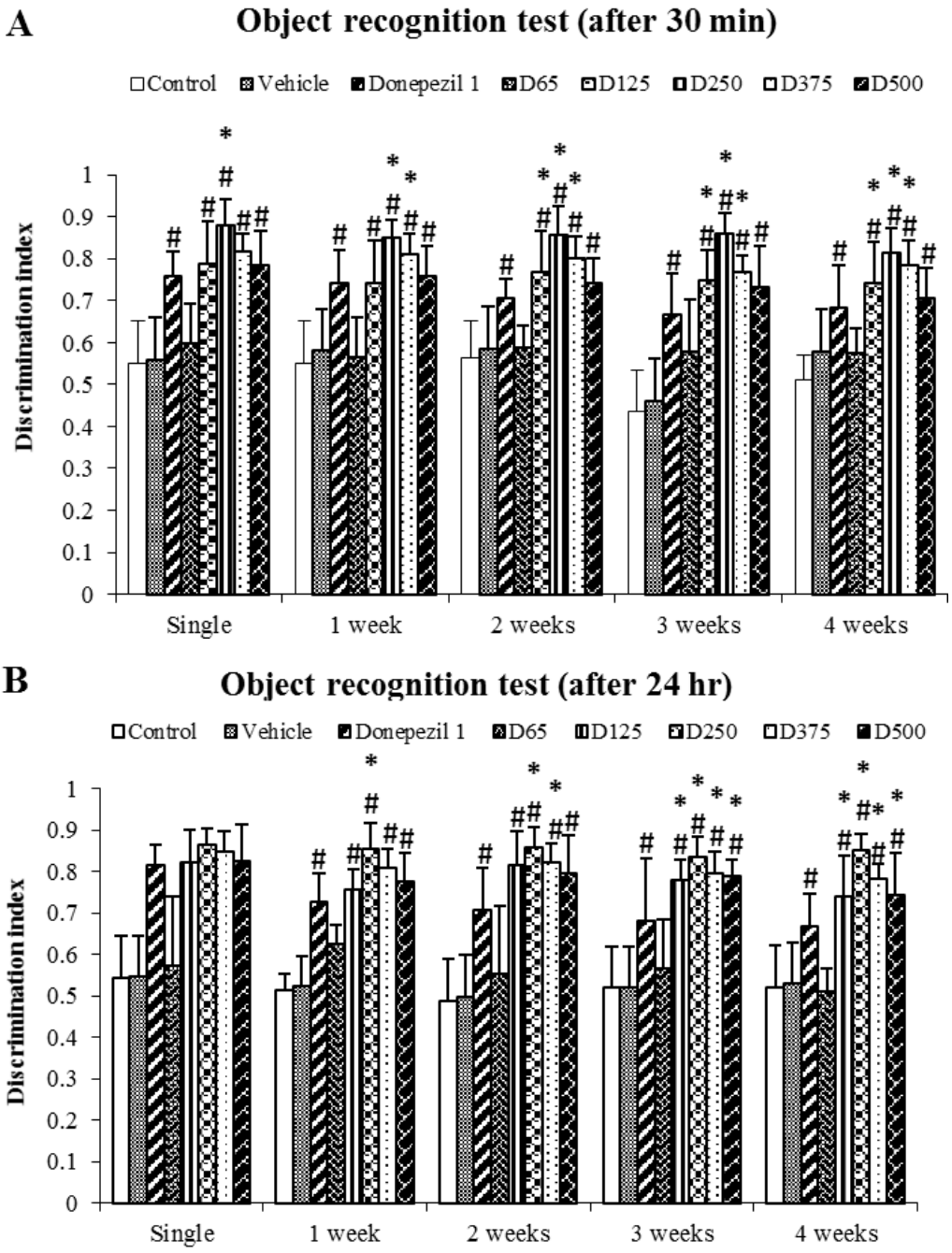


Figure 22 The non-spatial memory enhancing effect of *A. graveolens* on discrimination index after 30 min (A) and 24 h (B) of mice subjected to object recognition test. Data were represented as mean ± S.D., n=8 each, # vs. vehicle-treated group, $p<0.05$; * vs. Donepezil-treated group, $p<0.05$.

3.1.5 Effect of *A. graveolens* to reduce brain oxidative stress

In Table 4 showed that both positive control and all doses of *A. graveolens* used in this study significantly decreased the level of malondialdehyde (MDA), increased % inhibition of superoxide anion and activity of glutathaione peroxidase enzyme.

Table 4 The effect of *A. graveolens* on MDA level, % inhibition of $O_2^{\cdot -}$, and GPx activity in brain areas of healthy mice.

Groups	MDA level (nmol/mg protein)			% inhibition of O_2^{-}			GPx activity (Unit/g protein)		
	cortex	**hippocampus**	**striatum**	**cortex**	**hippocampus**	**striatum**	**cortex**	**hippocampus**	**striatum**
Control	5.06±0.62	4.10±0.31	3.73±0.36	10.93±1.01	14.36±1.27	12.43±0.94	8.45±1.71	9.46±0.96	8.87±0.68
Vehicle	4.82±0.44	4.02±0.18	3.62±0.59	10.85±1.72	14.21±1.88	12.82±0.71	9.09±1.27	10.73±0.88	8.54±2.04
Vit C 100	4.78±0.53	3.98±0.15	3.53±0.42	11.22±1.56	15.02±2.24	13.01±0.92	9.55±2.21	11.01±1.11	9.23±2.11
Fluoxetine 20	[a]3.18±0.31	-	[a]3.04±0.64	[a]15.30±0.69	-	[a]18.86±1.27	[a]16.13±1.06	-	[a]15.41±0.61
Donepezil 1	[a]3.04±0.70	[a]3.01±0.23	3.68±0.75	[a]15.82±1.22	[a]20.73±1.17	-	[a]15.92±1.81	[a]16.72±0.80	-
Diazepam 2	[a]3.10±0.51	-	[a]2.84±0.58	[a]15.19±0.95	-	[a]19.16±1.06	[a]15.73±0.90	-	[a]15.82±1.22
D65	4.95±0.25	4.15±0.12	-	10.90±1.92	14.15±0.63	13.43±0.96	10.19±0.81	9.58±1.31	9.83±0.93
D125	[a]3.13±0.11	[a]3.11±1.00	[a]2.93±0.33	[a,b,c]20.68±1.71	16.12±1.23	[a,b]29.68±1.08	[a]16.55±1.18	[a]13.53±1.18	[a,b]18.37±1.15
D250	[a]2.95±0.41	[a]2.95±0.43	[a]3.16±0.46	[a,b,c]27.60±1.28	[a,c]29.96±1.66	[a]21.90±0.73	[a,b,c]19.55±1.88	[a,c]18.96±0.94	[a]15.71±1.26
D375	[a]3.06±0.63	[a]3.23±0.61	[a]3.37±0.55	[a]16.94±1.04	[a]22.14±1.75	[a]20.88±0.88	[a]15.59±1.16	[a]13.06±1.81	[a]13.28±1.10
D500	[a]3.35±0.64	[a]3.31±1.06	3.56±0.45	[a]14.77±0.85	16.55±1.09	[a]18.00±0.83	[a]14.43±2.04	12.13±0.95	[a]12.10±1.22

Data were represented as mean ± S.D. of three replicates, n=8 each, [a] vs. vehicle-treated group, $p<0.05$; [b] vs. Fluoxetine-treated group, $p<0.05$; **[c]** vs. Donepezil-treated group, $p<0.05$; **[d]** vs. Diazepam-treated group, $p<0.05$

3.1.6 Effect of *A. graveolens* on the number of living neuron in cerebral cortex, hippocampus, and striatum areas.

We also determined the effect of *A. graveolens* on neuron densities in various sub regions of hippocampus and cortex, the brain area which played an important role on learning and memory and striatum, a key role in emotional state. Our data did not show significant changes in neuron densities in any regions of hippocampus, cortex, and striatum after the administration of vehicle as shown in Table 5-7. It was found that the administration of *A. graveolens* significantly increased neuron densities in all sub regions of hippocampus and cortex especially at dose of 250 mg/kg BW and striatum at dose of 125 mg/kg BW respectively.

Table 5 The effect of *A. graveolens* on the number of living neuron of cerebral cortex (cell number/0.15 mm^2)

Groups	Cerebral cortex			
	Frontal	**Parietal**	**Temporal**	**Occipital**
Control	63.20±7.58	60.05±8.41	55.30±9.98	50.55±6.21
Vehicle	60.25±7.85	64.85±11.27	51.65±8.81	46.30±8.09
Vit C 100	57.70±7.29	58.80±6.71	47.70±5.58	43.75±4.24
Fluoxetine 20	56.90±5.30	[a]56.20±5.01	52.70±6.34	41.50±4.17
Donepezil 1	[a]68.80±3.94	[a]68.10±4.31	[a]63.50±9.03	[a]59.00±8.49
Diazepam 2	59.70±4.81	55.70±4.97	47.00±3.92	48.70±4.03
D65	50.32±7.92	51.36±6.87	48.04±7.16	41.28±7.55
D125	65.05±5.19	62.15±5.62	57.15±10.31	[a]52.65±6.04
D250	[a,b,c,d]78.15±6.77	[a,b,c,d]74.80±9.66	[a,b,c,d]70.30±7.17	[a,b,c,d]68.40±6.17
D375	[a]70.80±7.97	[a]65.85±5.23	[a]60.00±6.93	[a]57.10±6.34
D500	54.75±4.60	55.15±5.45	51.70±5.89	45.35±4.86

Data were represented as mean ± S.D. of three replicates, n=8 each, [a] vs. vehicle-treated group, $p<0.05$; [b] vs. Fluoxetine-treated group, $p<0.05$; [c] vs. Donepezil-treated group, $p<0.05$; [d] vs. Diazepam-treated group, $p<0.05$.

Table 6 The effect of *A. graveolens* on the number of living neuron of hippocampus brain areas (cell number/0.15 mm^2)

Groups	Hippocampus			
	CA1	**CA2**	**CA3**	**DG**
Control	85.75±16.68	89.05 ±9.78	60.70±8.94	11.10±13.69
Vehicle	90.35±18.36	86.95±8.05	55.85±7.50	93.95±12.44
Vit C 100	90.85±16.86	71.15±7.48	56.05±4.52	90.70±7.49
Donepezil 1	[a]120.80±11.96	[a]101.60±7.62	[a]67.90±5.09	[a]103.00±9.59
D65	87.36±12.27	71.44±7.28	49.08±6.74	84.52±6.79
D125	100.35±10.49	82.20±7.75	57.30±6.51	100.35±7.86
D250	[a,b]136.95±8.53	[a,b]110.15±12.68	[a,b]79.60±6.36	[a,b]114.40±9.34
D375	[a]98.10±10.65	[a]92.00±8.48	[a]63.45±8.26	[a]91.40±12.52
D500	83.40±5.79	79.95±6.70	56.60±6.73	90.75±8.88

Data were represented as mean ± S.D. of three replicates, n=8 each, [a] vs. vehicle-treated group, $p<0.05$, [b] vs. Donepezil-treated group, $p<0.05$.

Table 7 The effect of *A. graveolens* on the number of living neuron of striatum (cell number/0.15 mm^2)

Groups	Striatum
Control	83.30±19.33
Vehicle	90.55±20.58
Vit C 100	92.68±15.43
Fluoxetine 20	[a]123.40±13.53
Diazepam 2	[a]125.00±11.04
D65	88.08±10.15
D125	[a,b,c]132.30±6.31
D250	[sa]116.55±7.50
D375	[a]106.01±11.55
D500	87.33±6.53

Data were represented as mean ± S.D. of three replicates, n=8 each, [a] vs. vehicle-treated group, $p<0.05$, [b] vs. Fluoxetine-treated group, $p<0.05$; [c] vs. Diazepam-treated group, $p<0.05$

3.2 Neuroprotective effect of *A. graveolens*

3.2.1 The cognitive deficit condition induced by scopolamine

3.2.1.1 Effect of *A. graveolens* on spatial memory

Figure 23 showed the vehicle treatment with scopolamine significantly prolong escape latency time than control group suggesting the spatial memory deficit. Co-administration of *A. graveolens*

with scopolamine could reverse memory deficit condition particularly 250 mg/kg BW.

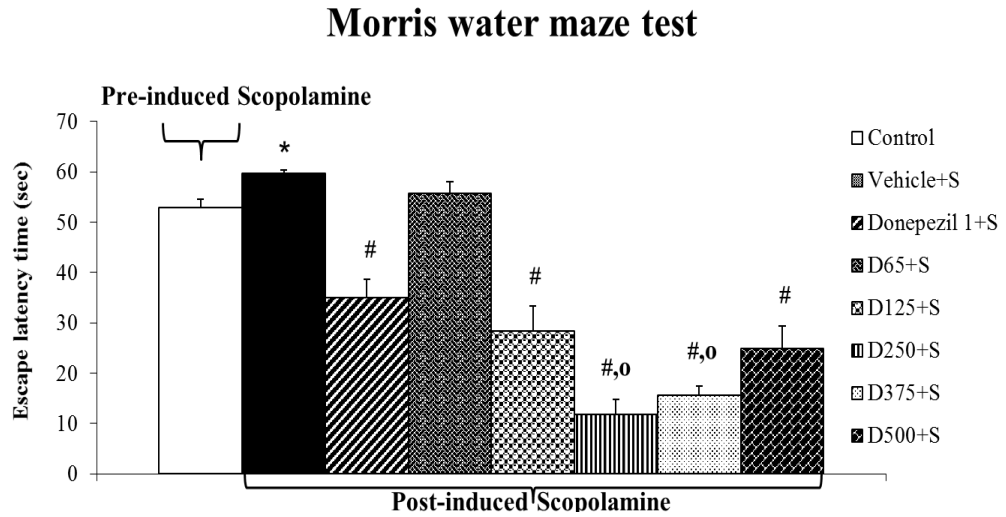


Figure 23 Effect of *A. graveolens* on escape latency time of mice subjected to Morris water maze test. Control: pre-induced scopolamine control group, Vehicle+S: a scopolamine-treated vehicle group, Donepezil 1+S: 1 mg/kg BW Donepezil plus scopolamine-treated group, D65+S: 65 mg/kg *A. graveolens* plus scopolamine-treated group, D125+S: 125 mg/kg *A. graveolens* plus scopolamine-treated group, D250+S: 250 mg/kg *A. graveolens* plus scopolamine-treated group, D375+S: 375 mg/kg *A. graveolens* plus scopolamine-treated group, D500+S: 500 mg/kg *A. graveolens* plus scopolamine-treated group. Data were represented as mean ± S.D. of three replicates, n=8 each, *-p<0.05 compared with control group; #-p<0.001 compared with vehicle+S treated group; ^{o}-p<0.05 compared with Donepezil+S treated group.

3.2.1.2 Effect of *A. graveolens* on non-spatial memory.

During the experimental period, vehicle treated group plus scopolamine revealed prominent decrease in the discrimination index indicating memory deficit. Co-administration of *A. graveolens* and scopolamine significantly increased the discrimination index in a dose-

dependent manner ranging from 250 to 500 mg/kg BW as depicted in figure 24.

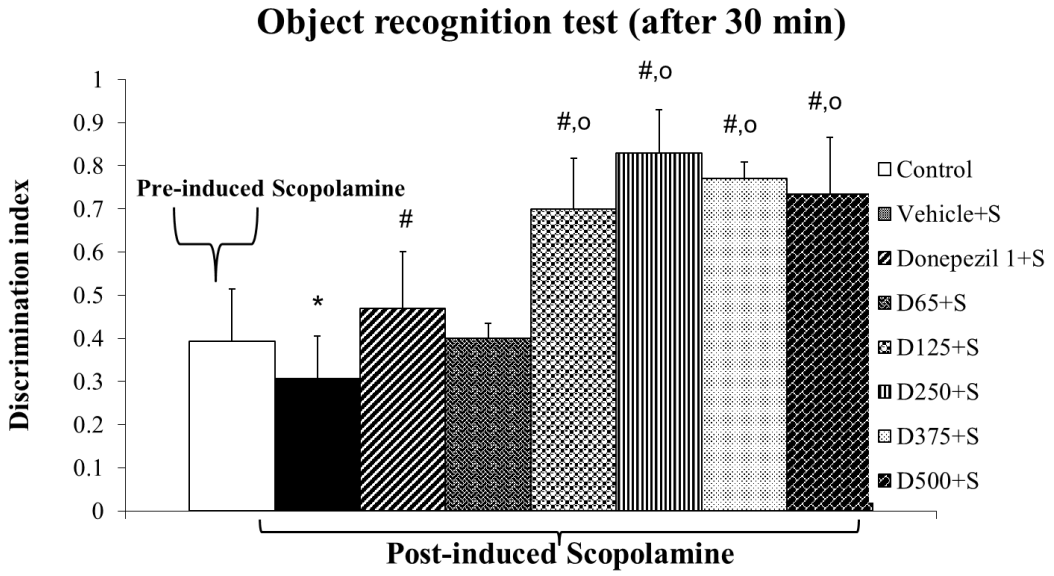


Figure 24 Effect of *A. graveolens* on discrimination index of mice subjected to Object recognition test. Data were represented as mean ± S.D. of three replicates, n=8 each, * $p<0.05$ compared with control group; #-$p<0.001$ compared with vehicle+S treated group; ^{o}-$p<0.05$ compared with Donepezil 1+S treated group.

3.2.1.3 Effect of *A. graveolens* on the alteration of acetylcholinesterase (AChE) activity

Acetylcholinesterase (AChE) is a key enzyme to degrade acetylcholine as acetyl and choline for recycling neurotransmitter. In the present study, we examined the acetylcholinesterase activity in hippocampus and cerebral cortex isolated from mice brains to ensure whether the efficacy of *A. graveolens* in behavioral test (Morris water maze and object recognition tests) is related to AChE alteration (Figure 25). The AChE activity in hippocampus and cerebral cortex was remarkably decrease in donepezil (1 mg/kg BW) and/or *A. graveolens*

(125, 250, 375, and 500 mg/kg BW)-treated animals ($p < 0.001$). Interestingly, *A. graveolens* at dose of 250 mg/kg BW treatment showed significant decreased AChE activity than positive control drug in both brain regions ($p < 0.001$).

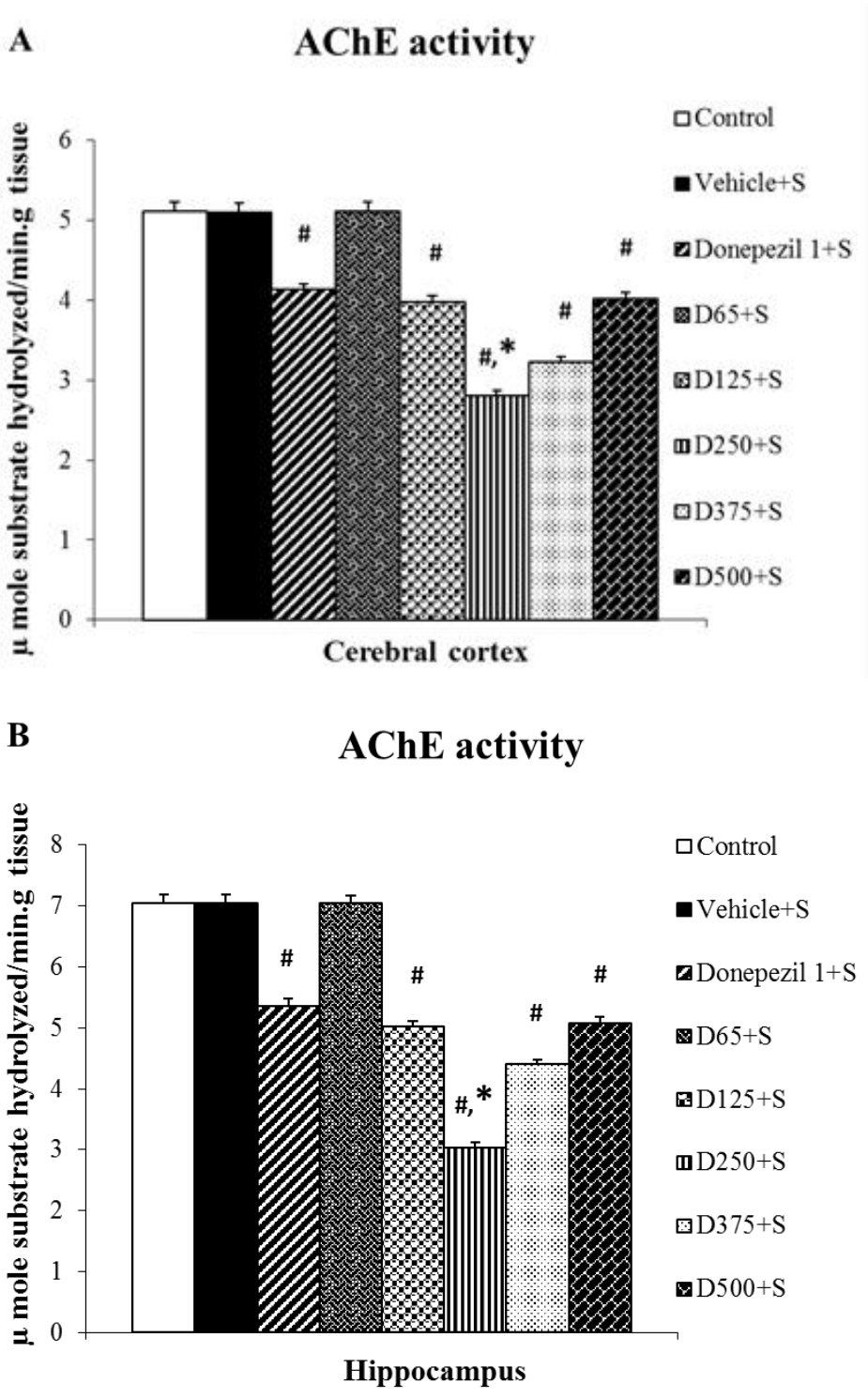


Figure 25 Effect of *A. graveolens* on AChE enzyme activity in cerebral cortex treated with scopolamine(A) and hippocampus treated with scopolamine (B). Data were represented as mean ± S.D. of three replicates, n=8 each, *-p<0.001 compared with Donepezil+S treated group; #-p<0.001 compared with vehicle+S treated group.

3.2.2 The motor deficit condition induced by MPTP

3.2.2.1 Effect of *A. graveolens* on resting tremor and swimming scores in MPTP-induced mice

To evaluate the severity of PD model, we used to test the resting tremor score and swim scale in mice after 1, 3 and 7 days of MPTP induction as displayed in Figure 26. The results displayed that the MPTP-treated group increased the resting tremor and decreased swimming scores than control group significantly. Only *A. graveolens* at dose of 375 mg/kg BW decreased resting tremor and increased swimming scores all duration of treatment compared with MPTP-treated group ($p<0.05$). In addition, after 7 days of MPTP administration the extract of 375 mg/kg BW showed reduced of resting tremor scores and improve swimming score than tidomet plus 25 mg/kg BW-treated group significantly ($p<0.05$)

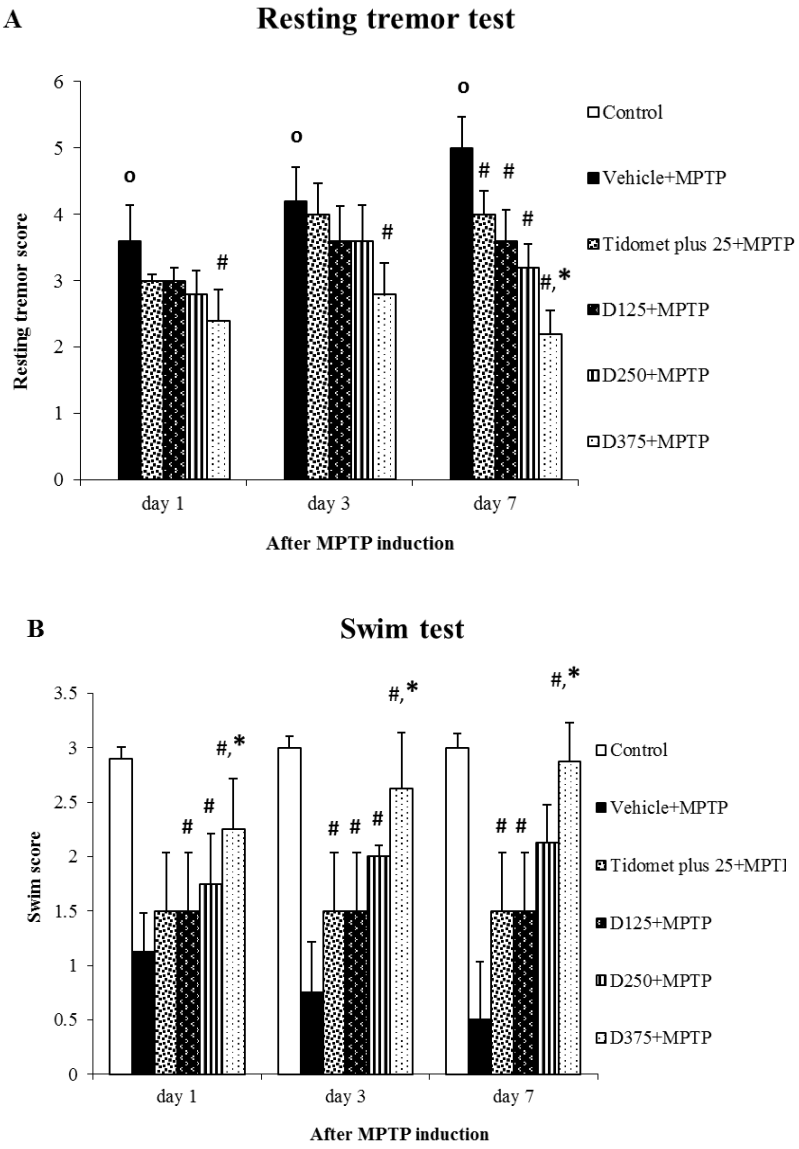


Figure 26 Effect of *A. graveolens* on resting tremor score (A) and swim score (B) after 1, 3, and 7 days of MPTP administration. Control: no treatment group, Vehicle+MPTP: NSS-treated group plus MPTP, Tidomet plus25+MPTP: 25 mg/kg BW tidomet plus-treated group plus MPTP, D125+MPTP: 125 mg/kg *A. graveolens*-treated group plus MPTP, D250+MPTP: 250 mg/kg *A. graveolens*-treated group plus MPTP, D375+MPTP: 375 mg/kg *A. graveolens*-treated group plus MPTP. Each data column represents the mean ± S.D., n = 8/group, ^{o}-p<0.01 compared with control group; #-p<0.05 compared with vehicle-treated group; *-p<0.05 compared with tidomet plus-treated group.

3.2.2.2 Effect of *A. graveolens* on retention time of rotarod test in MPTP model.

To study the coordination, we determined the retention time of rotarod apparatus in mice after 1, 3 and 7 days of MPTP treatment (Figure 27). The results displayed that the MPTP-treated group decreased the retention time than control group significantly. Only *A. graveolens* at dose of 375 mg/kg BW increased retention time in all duration of treatment when compared with MPTP-treated group ($p<0.05$). In addition, after 7 days of MPTP administration the extract of 375 mg/kg BW showed increased retention time than tidomet plus 25 mg/kg BW-treated group significantly ($p<0.05$).

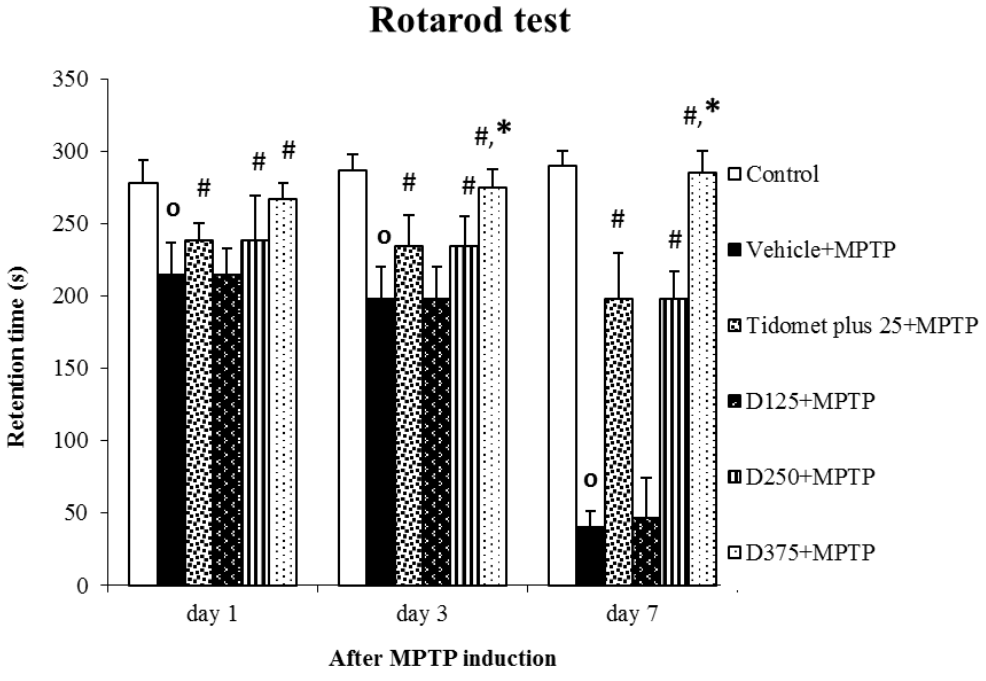


Figure 27 Effect of *A. graveolens* on retention time of rotarod apparatus after 1, 3, and 7 days of MPTP administration. Each data column represents the mean ± S.D., n = 8/group, ^{o}-$p<0.001$ compared with control group; #-$p<0.05$ compared with vehicle-treated group; *-$p<0.05$ compared with tidomet plus-treated group.

3.2.2.3 Effect of *A. graveolens* on the foot fault time of grid walk test in MPTP-treated mice

To examine motor and coordination skills, the time of foot fault in grid walk test was tested after 1st, 3rd and 7th day of MPTP induction (Figure 28). The crude extract at the dose of 250 and 375 mg/kg BW-treated group for all duration showed significantly reduced number of foot fault compared to vehicle-treated group (p<0.05), interestingly the *A. graveolens* of 375 mg/kg BW after 3 and 7 days of MPTP injection improved significantly the number of foot faults compared with the tidomet plus -treated groups (p<0.05).

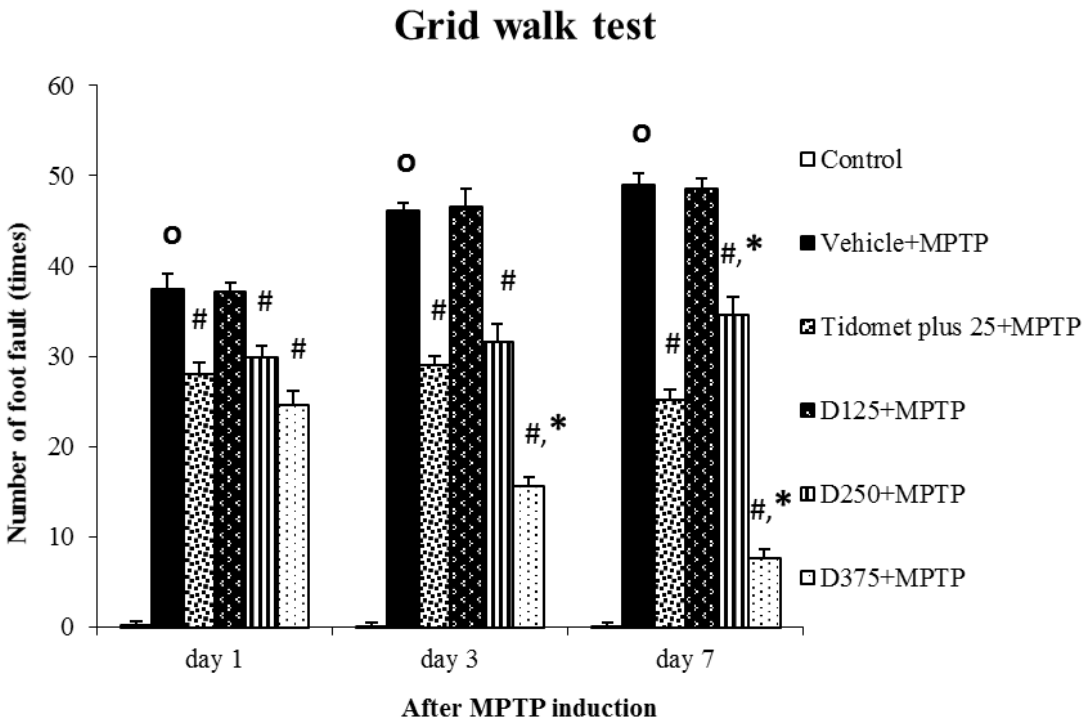


Figure 28 Effect of *A. graveolens* on number of foot fault time of grid walk test after 1, 3, and 7 days of MPTP treatment. Each data column displays the mean ± S.D., n = 8/group, **o**-p<0.01 compared with control group; #-p<0.05 compared with vehicle-treated group; *-p<0.05 compared with tidomet plus-treated group.

3.2.2.4 Effect of *A. graveolens* on the time of forepaws touch of drag test in MPTP-induced mice

The number of forepaws touch in drag test was determined for balance ability on 1st, 3rd and 7th day after MPTP injection (Figure 29). There was significant difference between the control and vehicle-treated mice that mean our PD model was induced successfully (p<0.001). The *A. graveolens* at the dose of 250 and 375 mg/kg BW treated group for all duration exhibited significantly increased number of forepaws touch compared to vehicle-treated group (p<0.05), interestingly the *A. graveolens* of 375 mg/kg BW after 3 and 7 days of MPTP treatment displayed the highest of forepaws touch time when compared to the vehicle and tidomet plus-treated groups respectively (p<0.05).

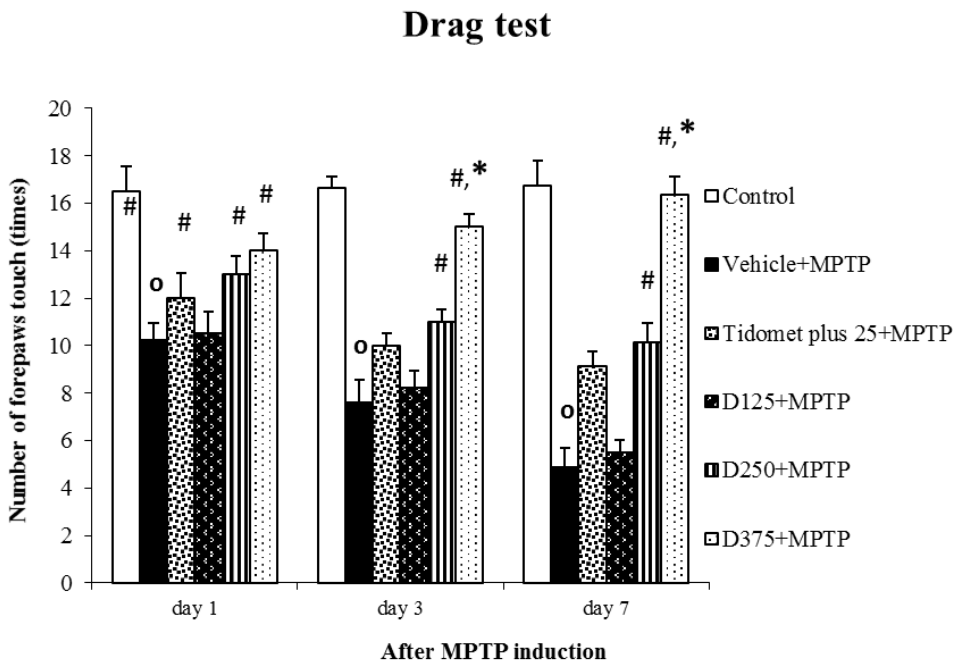


Figure 29 Effect of the extract of *A. graveolens* on forepaws touch time of drag test after 1, 3, and 7 days of MPTP treatment. Each data column displays the mean ± S.D., n = 8/group, °-p<0.001 compared with control group; #-p<0.05 compared with vehicle-treated group; *-p<0.05 compared with tidomet plus-treated group.

3.2.2.5 Effect of *A. graveolens* on latency time and numbers of foot fault of narrow beam test in MPTP-treated mice

The latency time of narrow beam walk test was analyzed for testing motor and coordination while the time of foot fault for determining balance after MPTP injection for 1, 3, and 7 days (Figure 30). The vehicle-treated group increased latency time and number of foot error than control group significantly. The 250 and 375 mg/kg BW extract-treated groups significantly reduced latency time and number of foot slip error compared to vehicle-treated group on 3 and 7 days after MPTP injection. Especially, the *A. graveolens* 375 mg/kg BW after 7 day of MPTP treatment reduced the number of foot error when compared with the vehicle and tidomet plus-treated groups significantly.

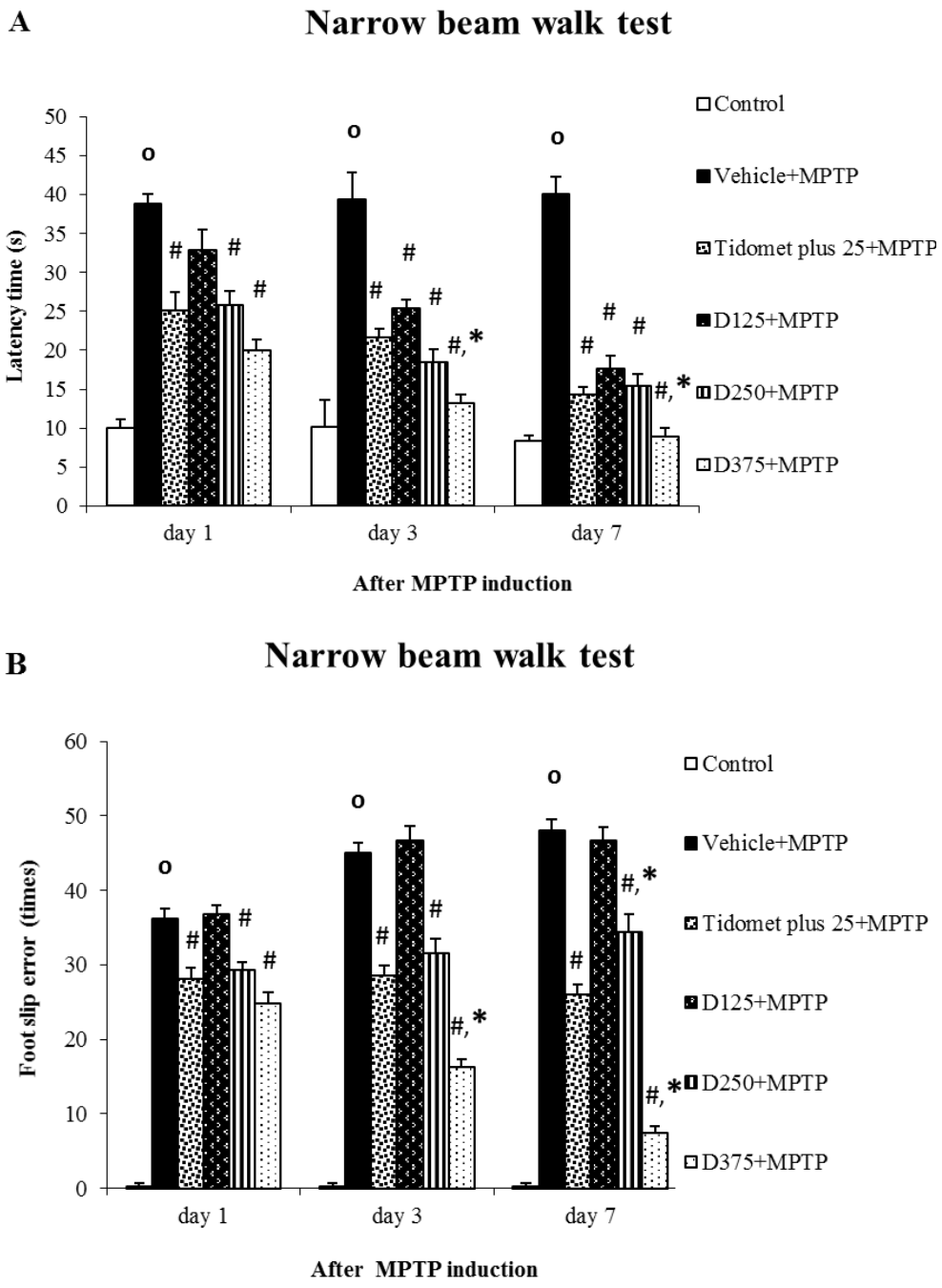


Figure 30 Effect of *A. graveolens* on latency time (A) and foot slip error (B) of narrow beam test after 1, 3, and 7 days of MPTP induction. Each data column represents the mean ± S.D., n = 8/group, **º**-$p<0.01$ compared with control group; #-$p<0.05$ compared with vehicle-treated group; *-$p<0.05$ compared with tidomet plus-treated group.

3.2.2.6 Effect of *A. graveolens* on MDA level, % inhibition of $O_2^{\cdot-}$, GPx, and MAO-A, B activities in cerebral cortex and striatum areas of mice after MPTP treatment

To elucidate the mechanisms of the protective effects of *A. graveolens*, we analyzed the oxidative stress parameters of the GPx activities, MDA contents, % inhibition of $O_2^{\cdot-}$ whereas the related neurotransmitter pathway of MAO-A,B activities were also tested in the cortex and striatum of every group of mice. The results of these biochemical parameters were represented in Table 8. The MDA content and MAO-A,B activities in the striatum of MPTP-treated mice were significantly increased while the % inhibition of $O_2^{\cdot-}$ and GPx activity of vehicle+MPTP treatment group were reduced compared with those other groups (p<0.05). However, this increase in MDA level and MAO-A,B activities whereas the decrease in % inhibition of $O_2^{\cdot-}$ and GPx activity were significantly ameliorated when mice received *A. graveolens* treatment at dose of 250 and 375 mg/kg BW administration after MPTP induction (p<0.05). In addition, *A. graveolens* 375 mg/kg BW treatment largely attenuated this changes when compared to tidomet plus 25+MPTP-induced group (p<0.05).

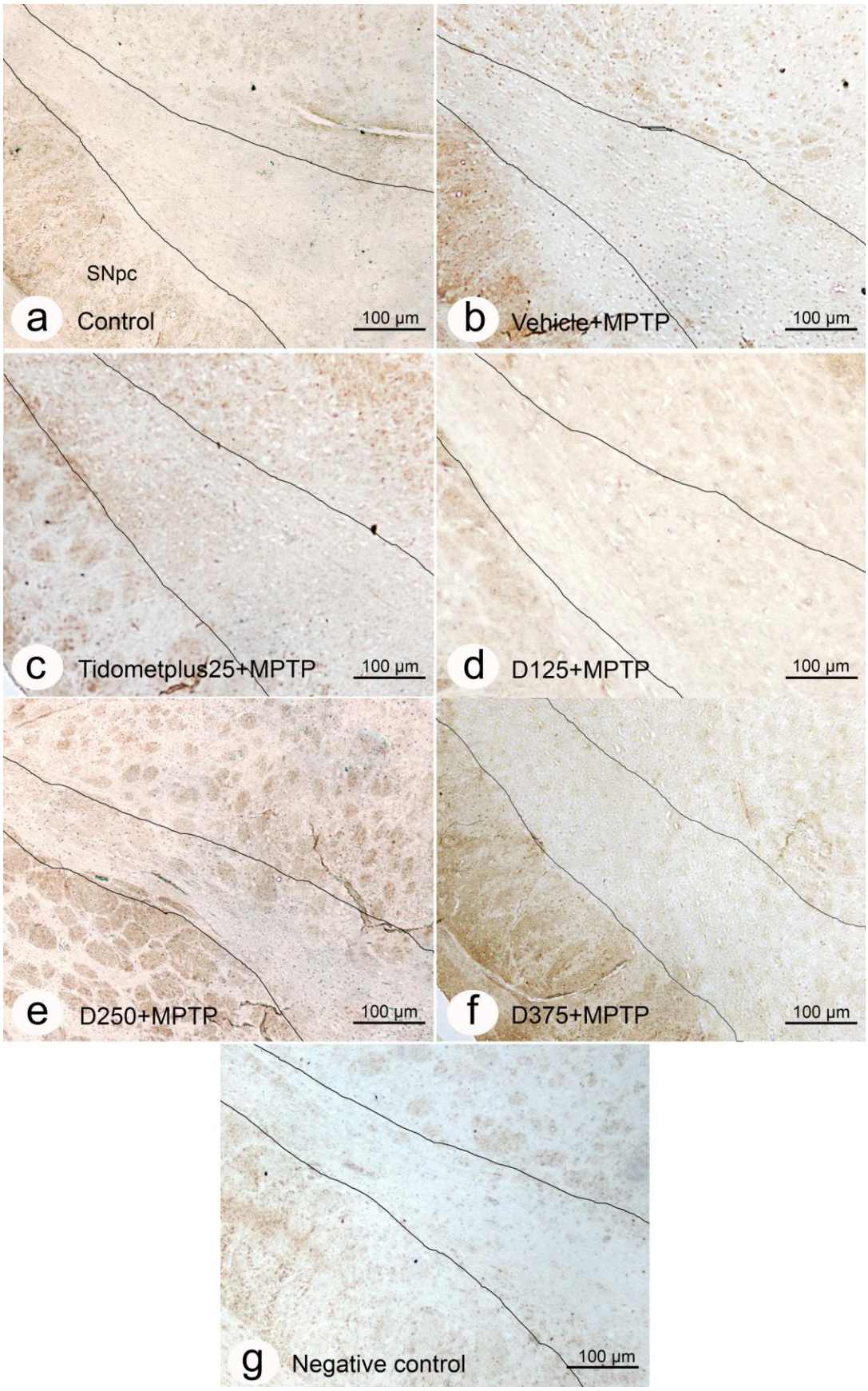
Table 8 The effect of *A. graveolens* on MDA level, % inhibition of $O_2^{\cdot-}$, GPx activity , and MAO-A,B activities of cerebral cortex and striatum areas of mice after MPTP treatment.

Groups	MDA level (µmol/mg protein)		% inhibition of O_2^-		GPx activity (Unit/g protein)		MAO-A activity (µmole/min.g tissue)		MAO-B activity (µmole/min.g tissue)	
	cortex	**striatum**	**cortex**	**striatum**	**cortex**	**striatum**	**cortex**	**striatum**	**cortex**	**striatum**
Control	2.22±0.26	1.53±0.56	16.48±2.14	19.87±1.99	18.45±1.70	20.87±1.68	3.00±1.01	2.73±1.05	5.01±1.01	4.73±1.05
Vehicle+MPTP	[a]6.34±0.41	[a]5.96±0.22	[a]5.16±1.46	[a]8.32±1.10	[a]5.09±2.27	[a]6.54±2.04	[a]9.02±1.00	[a]10.13±1.18	[a]15.00±1.04	[a]17.13±1.18
Tidomet plus 25+MPTP	[b]2.81±0.48	[b]1.70±0.13	[b]13.08±1.95	[b]11.87±2.03	[b]15.92±0.81	[b]13.41±1.61	[b]5.46±0.67	[a]6.16±1.25	[b]10.46±0.67	[b]8.16±1.25
D125+MPTP	2.93±0.41	4.77±0.15	10.85±1.55	9.98±2.55	6.19±1.81	7.83±0.93	8.51±1.19	9.56±1.34	11.51±1.19	10.56±1.34
D250+MPTP	[b]2.12±0.43	[b]3.75±0.12	[b]12.05±1.99	[b]14.04±2.26	[b]10.55±1.18	[b]12.37±1.15	[b]5.86±0.68	[b]6.70±0.57	[b]10.86±0.68	[b]8.70±0.57
D375+MPTP	[b]2.01±0.35	[b]0.17±0.19	[b]15.75±1.13	[b,c]18.14±1.26	[b]16.54±1.16	[b,c]18.71±1.26	[b]4.21±0.52	[b,c]3.42±0.72	[b,c]6.21±0.53	[b,c]4.42±0.72

Data were represented as mean ± S.D. of three replicates, n=8 each, [a] *vs.* control group, $p<0.01$; [b] *vs.* vehicle+MPTP-treated group, $p<0.05$; [c] *vs.*Tidomet plus 25+MPTP-treated group, $p<0.05$.

3.2.2.7 Effect of *A. graveolens* on MPTP-increased of Ionized binding adaptor molecule-1 (Iba-1) positive cell immunoreactivity in SNpc.

Ionized binding adaptor molecule-1 (Iba-1) represents a specific neuroinflammatory marker of activated microglia cells. To analyze whether the MPTP treatment was related to microglial activation, we determined the Iba-1 immunopositive cells in SNpc brain slides of mice. The immunostaining revealed only a few faintly immunoreactive microglial cells in the SNpc of control mice (Figure 31A, a). In acute MPTP treatment, numerous marked immunoreactive Iba-1-positive activated microglias were observed 24 h after the last injection of MPTP (Figure 31A, b), similar to treatment with *A. graveolens* at dose of 125 mg/kg BW (Figure 31A, d). Conversely, *A. graveolens* at dose of 250-375 mg/kg BW expressed Iba-1 positive cells significantly (Figure 31A, e-f) which were consistent with the result of the number of Iba-1 positive cells as shown in Figure 31B, respectively.


SNpc
a Control
100 µm
b Vehicle+MPTP
100 µm
c Tidometplus25+MPTP
100 µm
d D125+MPTP
100 µm
e D250+MPTP
100 µm
f D375+MPTP
100 µm
g Negative control
100 µm

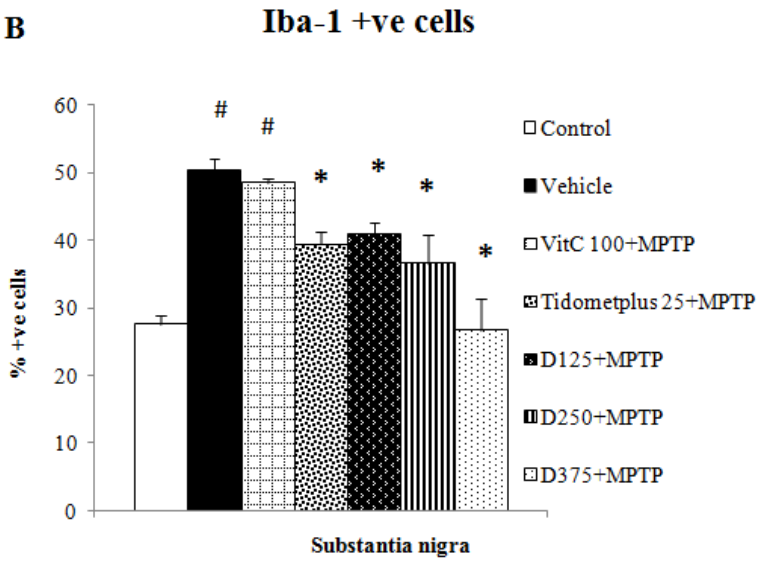
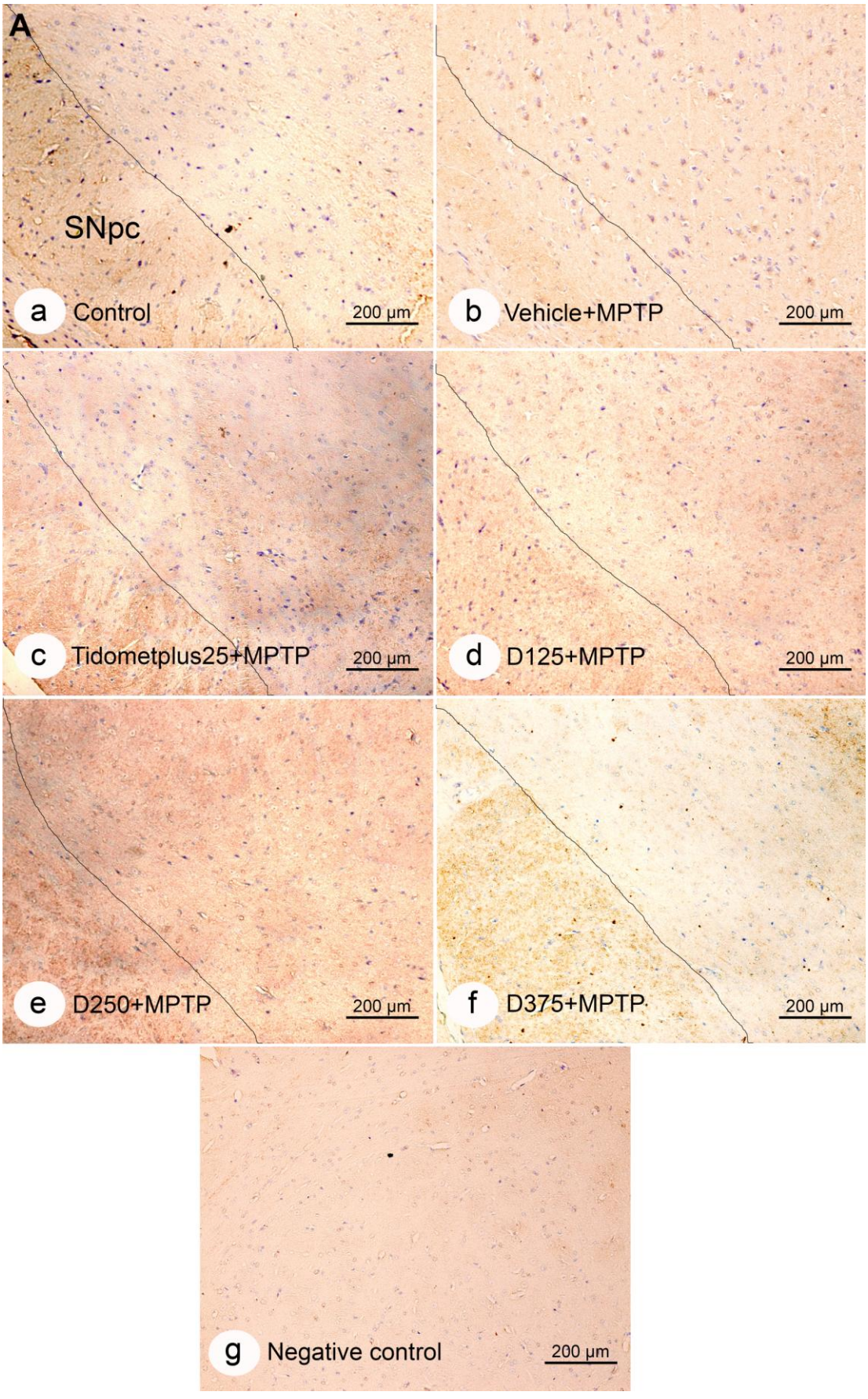


Figure 31 Representative immunostaining and semi-quantitative result of Iba-1. (A) Representative immunostaining of Iba-1 positive activated microglia cells in mice after administration of MPTP in substantia nigra; control group (a), MPTP+vehicle (NSS) (b), MPTP+tidomet plus (25 mg/kg BW) (c), MPTP+*A. graveolens* (125 mg/kg BW) (d), MPTP+*A. graveolens* (250 mg/kg BW) (e), MPTP+*A. graveolens* (375 mg/kg BW) (f), and negative control without anti-Iba-1 antibody (g). (B) Semi-quantitative result showed the expression of Iba-1 in substantia nigra of mice after administration of MPTP. Mean ± S.D., n=4/group, ^{o}-p<0.01 compared with control group; #-p<0.05 compared with MPTP/vehicle treatment group, *-p<0.05 compared to tidomet plus+MPTP treatment group.

3.2.2.8 Effect of *A. graveolens* on MPTP-reduced of tyrosine hydroxylase positive cell immunoreactivity in substantia nigra.

Tyrosine hydroxylase is rate limiting step enzyme of DA biosynthesis of dopaminergic neuron, we focused on the TH positive cell expression in mice brain slides especially SNpc area. The representative microphotographs of TH immunostaining in the substantia nigra were shown in the Figure 32A. The TH positive cells of

DA neuron were simply obvious in the substantia nigra area of control group (Figure 32A, a) in contrast, mice that received NSS-only treatment with MPTP administration showed a remarkable deprivation of TH-immunopositive cells, and only 25.01 ± 4.26% of the TH positive neurons in the Substantia nigra pars compacta compared with the negative staining cells (Figure 32A, b). Interestingly, *A. graveolens* at the dose of 125, 250 and 375 mg/kg BW+MPTP-treated groups could ameliorate TH immunopositive staining cells to 27.19 ± 6.16%, 35.73 ± 4.27%, 45.08 ± 4.27 %, respectively compared to NSS+MPTP-treated group ($p<0.05$, Figure 32A, c-d). Moreover, the *A. graveolens* at the dose of 375 mg/kg BW showed increase the number of positive cells compared with vehicle+MPTP and tidomet plus25+MPTP treatment groups ($p<0.05$, Figure 32A, e) which were coherent with the result of the quantitative number of TH immunopositive cells in Figure 32B.


A
SNpc
a Control
200 μm
b Vehicle+MPTP
200 μm
c Tidometplus25+MPTP
200 μm
d D125+MPTP
200 μm
e D250+MPTP
200 μm
f D375+MPTP
200 μm
g Negative control
200 μm

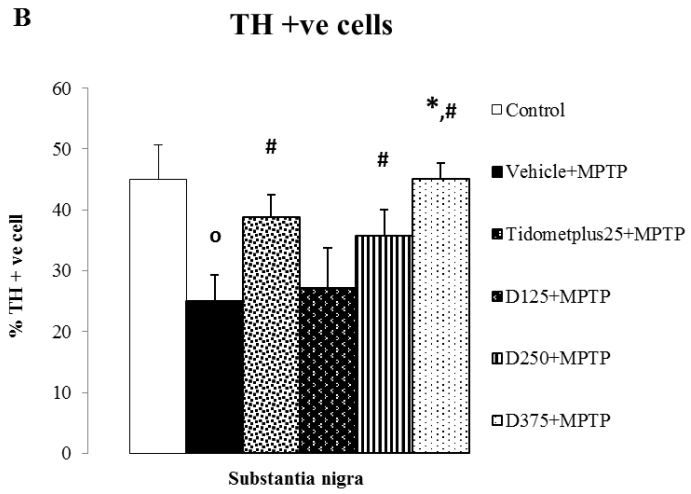


Figure 32 Effect of *A. graveolens* on MPTP-induced decrease of TH immunostaining in the substantia nigra of mice. (A) Representative microphotographs showing control group (a), MPTP+vehicle (NSS) (b), MPTP+tidomet plus (25 mg/kg BW) (c), MPTP+*A. graveolens* (125 mg/kg BW) (d), MPTP+ *A. graveolens* (250 mg/kg BW) (e), MPTP+*A. graveolens* (375 mg/kg BW) (f), and negative control without anti-TH antibody (g). (B) The effect of *A. graveolens* on the number of TH immunostaining neurons in the substantia nigra after MPTP induction. The TH positive neurons are expressed as mean ± S.D., n=4/group, ^{o}-p<0.01 compared with control group; #-p<0.05 compared with MPTP/vehicle treatment group, *-p<0.05 compared to tidomet plus+MPTP treatment group.

CHAPTER 4

DISCUSSIONS

Exploring promising herbal medicines in order to treatment of a range of psychiatric diseases and cognitive impairments have been focused over the past three decades. The research combined the testing of various phytochemicals with preclinical and clinical trials. Such an example was found in extract from *Apium graveolens* L., a traditional Chinese herbal medicine that has been commonly used for diabetes mellitus, hypertension, anti-gout, and birth control. However, anxiolytic activities, the anti-depressant, and cognitive enhancing effect of this plant still remaining unclear.

First of all, there were no effects of any of the doses *A. graveolens* associated with all the spontaneous motor behaviours of the open field tests as showed in Figure 12. However, each animal was examined only once in each of the behavioral tests. If we used the same mice to analyze locomotion in the open-field test and then examined their immobility time in the forced swimming test, the results between the forced swimming and tail suspension tests may be different. Therefore, to confirm the reliability of the behavioral tests, both the tail suspension and forced swimming tests were done after performing and not with the open-field analysis. The effects of *A. gravelolens* on the open field activity of the mice in our study showed no significant differences and implied that the model for testing depression and cognition had been established successfully.

The behavioral screening for anxiolytic effect in healthy mice was performed by using dark-light box, hole broad test, sleep analysis-induced by Pentobarbital, elevated plus maze, and open field test which are the common screening examination for anxiolytic or anxiogenic activities (Lister et al., 1987; Takeda et al., 1998; Choleris et al., 2001; Bourin and Hascoët, 2003; Kuribara et al., 2003). Our results showed that *A. graveolens* crude extract ranging from 125-500 mg/kg BW displayed the anxiolytic effect after 2 weeks of oral administration significantly in all behavioral tests ($p<0.05$ all). Remarkably, 125 mg/kg BW of *A. graveolens* exhibited peak effect as shown in Figure 13-17.

There are many groups of drug therapy in anxiety disorders and obsessive compulsive disorders patients (Sayed et al., 2014), the first choice of drug is selective serotonin reuptake inhibitors (SSRIs) and the second alternatives such as benzodazepines like diazepam, monoamine oxidase inhibitors (MAOIs). However, the adverse effects of these drugs have been reported continuously. Therefore, to find the novel drug without or fewer side effects may propose to be the goal target of anxiety disorders. Our study expressed that *A. graveolens* at dose ranging from 125-250 mg/kg BW showed the inhibiting MAO-A activity in both cerebral cortex and striatum. Especially, 125-250 mg/Kg BW as demonstrated in Figure 20. The proposed active pure compound which possessed the anxiolytic effect of *A. graveolens* might be L-tryptophan due to it was the compound for biosynthesis of serotonin (Momin and Nair, 2001; Momin and Nair, 2002).

The screening for pharmacologically antidepressant-like activity, was determined by the forced swimming and tail suspension tests which are the most common and reliable tests for evaluating the

determination in an inescapable situation and the anti-depression effect was tested by the reduced immobility time (Morris, 1984; Steru et al., 1985). In figure 18-19, our results showed that fluoxetine at a dose of 20 mg/kg BW exhibited antidepressant-like activity in both the forced swimming and tail suspension tests, while the *A. graveolens.* crude extract at a dose of 125-500 mg/kg BW decreased this behaviors at all treatment times with the same pattern that occurred after fluoxetine treatment of single dose until 4 weeks of administration ($p<0.05$ all). Nevertheless, the decline of the immobility time was not dose-dependent in both behavioral experiments.

The role of monoamines such as epinephrine (NE), 5-hydroxytryptamine (5-HT), and Dopamine (DA) in the central nervous system theory of depression hypothesized that the monoamine levels were low, but there was no evidence on the mechanism for the deprivation of monoamine (Schildkraut, 1965; Coppen, 1967; Delgado and Moreno, 2000; Savegnago et al., 2007; Yi et al., 2010). Hence, the recent effective strategies for controlling depression are to increase the brain serotonin and/or noradrenaline neurotransmitters (Delgado and Moreno, 2000; Leonard, 2001; Xia et al., 2007). MAO-A is an enzyme that metabolizes monoamine neurotransmitters so an elevation of the level of the MAO-A enzyme in all brain regions may be the primary cause of monoamine depletion in depression. The results in Figure 20 showed that *A. graveolens.* extract might exhibit its antidepressant-like effects via regulating the serotonergic system and this was confirmed by the remarkable decrease of the MAO-A activity in mice brain by the *A. graveolens* extract at doses of 125, 250, 375 and 500 mg/kg BW-treated groups when compared to the vehicle ($p<0.05$) in the mice brain. The

maximal effect was shown in the 125 mg/kg *A. graveolens*-treated group. Thus, the proposed active pure compound which possessed the anti-depressant effect of *A. graveolens* might be also L-tryptophan because it was the initiating substance for biosynthesis of serotonin (Momin and Nair, 2001; Momin and Nair, 2002)

For cognitive enhancing effect, the morris water maze and object recognition tests are generally usual for evaluation of spatial and non-spatial memory determination respectively (Morris, 1984; Antunes and Biala, 2012). The results showed that the donepezil-treated group decreased the escape latencies whereas there was an increase in the retention time in the morris water maze test and the novel exploration time in the object recognition test ($p<0.05$ all). It was of interest that, the *A. graveolens* of 125-500 mg/kg BW significantly reduced the escape latency of the morris water maze test after 1-4 weeks of treatment ($p<0.05$ all). Moreover, the retention time was significantly elevated after 2-4 weeks of treatment ($p<0.05$ all). The *A. graveolens* treatment with 125-500 mg/kg BW improved the novel exploration time higher than those found after donepezil treatment ($p<0.05$ all) as displayed in Figure 21-22. According to the study of the pure compound, *L-3-n*-Butylphthalide from *A. graveolens* seeds, stems and leaves ameliorated cognitive dysfunction, enhanced long-term spatial memory, a decrease of β-amyloid deposition, and a controlled production of amyloid precursor protein (APP) in transgenic Alzheimer's disease (AD) mice (Peng et al., 2010). Thus, the *A. graveolens* possessed a cognitive enhancing effect in both the spatial and non-spatial cognition tests.

Donepezil has been reported to downregulate the AChE activity by elevating the endogenous ACh level in the autonomic nervous

system (Kasa et al., 1995). In this study, we found that the *A. graveolens* fed at 125-500 mg/kg BW demonstrated suppression of the activity of AChE in both the cortex and hippocampus brain tissues as demonstrated in Figure 25. From the effects of this extract we inferred that *A. graveolens* administration may protect or promote the central cholinergic systems that were similar to the result of the root extract of chinese celery that exhibited 100% inhibition of the AChE enzyme in an *in vitro* model (Szwajgier and Borowiec, 2012). On the other hand, the methanolic leaf extract of this plant showed an inhibition of AChE activity of only 4.7% (Gholamhoseinian et al., 2009). Thus, the proposed active pure compounds which possessed the cognitive enhancing effect of *A. graveolens* might be *L-3-n*-Butylphthalide and α-tocopherol (Ching and Mohamed, 2001; Momin and Nair, 2001; Momin and Nair, 2002).

The most notable finding was that the peak effect of the methanolic extract of whole *A. graveolens* showed different concentrations for different effects (125 mg/kg for its anxiolytic-like and antidepressant-like effects and 250 mg/kg for its cognitive enhancing effect). These effects might indicate that the methanolic extract of *A. graveolens* contained a number of active ingredient that affected difference pathways. Moreover, a high concentration of the methanolic extract of whole *A. graveolens* (500 mg/kg) had a lower effect at a low concentration (125 or 250 mg/kg). This finding is probably due to the reduction of gastrointestinal absorption which is consistent with Al-Howiriny and Baananou and colleagues who found that a high concentration of celery extract decreased the function of the gastrointestinal tract (Al-Howiriny et al., 2010; Baananou et al., 2012).

Nonetheless, further investigations to elucidate the mechanisms underlying the cognitive enhancing effects of *A. graveolens* in animals are still required. Therefore, we set up to study the neuroprotective effect against scopolamine induced-cognitive impairment.

The present study further examined the effects of *A. graveolens* on memory deficit of AD model using scopolamine. The scopolamine-treated mice were examined for cognitive function using morris water maze and object recognition test. The vehicle plus scopolamine (3 mg/kg BW, i.p.) group displayed significant increase of the escape latency time ($p<0.001$) and decrease discrimination index ($p<0.001$) indicating successful scopolamine-induced memory impairment. Donepezil, a member of AChE inhibitors, has been used as a standard drug for AD treatment to reverse cognitive deficit (Čolović et al., 2013) and used as a positive control in this study. In Figure 23-24, *A. graveolens* at dose of 125, 250, 375 and 500 mg/kg BW demonstrated the ameliorative effects against scopolamine-induced memory impairment in C57BL/6 mouse model. The *A. graveolens*-treated mice spent shorter escape latency time but higher discrimination index than vehicle plus scopolamine groups. Apparently, the mice received 250 mg/kg BW *A. graveolens* exhibited maximal effect when compared to the other groups. This is consistent with our previous data showed that 500 mg/kg BW have low protective effect when compare to 250 mg/kg BW.

A. graveolens significantly reversed memory impairment in scopolamine-treated mice implying that it may improve cognitive function. Previous research showed that *A. graveolens* seeds, stems and leaves containing the pure compound of *L-3-n*-butylphthalide ameliorated cognitive impairment, promoted long-term spatial memory, decreased

amyloid-β and regulated amyloid precursor protein (APP) production in transgenic AD mice (Peng et al., 2010).

To determine the effect of *A. graveolens* on brain AChE activities. In the *A. graveolens* at dose of 125, 250, 375 and 500 mg/kg BW treated group demonstrated a significant decreased AChE activity as shown in Figure 25. Concurrently the 250 mg/kg BW *A. graveolens* administration denoted maximal responsiveness in accordance with the behavioral tests. The brain AChE activities were directly associated with duration of escape latency time implies that a higher AChE activity causing an increased in escape latency time. From these results, we found that the improvement of memory deficit by *A. graveolens* is presumably due to its inhibiting effect on AChE activity in the cerebral cortex and hippocampus. This finding agrees well with Dhingra and Kumar (2012) reported the relationship between AChE activity and escape latency of morris water maze test (Dhingra and Kumar, 2012). In addition, The water extract of celery roots can inhibit AChE enzyme *in vitro* (Swajgier and Borowiec, 2012). Attempt has been made on the methanolic extract of celery stems reported to prevent amyloid-β induced neuronal PC12 cells death (Park et al., 2009).

The theory proposed that the cognitive disturbance, anxiety and depressive disorders were associated with superfluous free radical production of reactive-oxygen species (ROS) in the living cell (Michel et al., 2012). The generation of ROS can cause tissue and cell injury, and even result in cell death. Another main target of the reactive free radical was membrane lipids by starting the self-perpetuating procedure of lipid peroxide, which disturbs the membrane integrity and function. Enzymatic endogenous antioxidants included superoxide dismutase (SOD), catalase

(CAT) or GPx that can scavenge ROS and free radicals or stop their generation. Hence, we determined free radical production (MDA level and % inhibition of O_2^-) and the radical scavenging enzyme activity of GPx. In table 4, both the positive control and all doses of *A. graveolens* used in this study significantly decreased the level of MDA, increased the % inhibition of the superoxide anion and activity of GPx enzyme which was consistent with many previous studies, the seed extract of this herb displayed about 23% inhibition of free radicals by using the di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical and a combination with orange peel oil that enhanced the effect to decrease lipid peroxide by about 74.6% (Wei and Shibamoto, 2007). The pure active ingredient,5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-l benzopyran-4-one and 2,3-dihydro-6-hidroxy-5-benzofuran carboxylic acid suppressed the activity of xanthine oxidase (XO) by around 85.44% (Iswantini et al., 2012). Apigenin and *DL-3-n*-Butylphthalide, the pure compounds inhibited the generation of free radicals and the dysfunction of a mitochondrial *in vitro* model by hydrogen peroxide treatment (Huang et al., 2010). The high dosage of the pure compound, luteolin inhibited the lipopolysaccharide (LPS), tumor necrosis factor-α, nitric oxide production and superoxide expression in an *in vitro* model (Chen et al., 2008).

We confirmed the effect of *A. graveolens* on the density of neuron in the cortex, striatum and hippocampus brain areas. Our data in table 5-7 showed significant increase in the neuron densities in many regions of the hippocampus, striatum and cortex after the administration of *A. graveolens* especially at a dose of 125 and 250 mg/kg BW. The previous published research had proposed that the pure compound that had been extracted from *A. graveloens* fruit, apigenin enhanced mature

neuron differentiation both *in vitro* and *in vivo* (Taupin, 2009), and an unfractionated water extract of *A. graveolens* promoted differentiation in an *in vitro* model of neuronal stem cells including both neurons and neuroglial cells like the astrocytes and oligodendrocytes (Tie-Qiao et al., 2006) was consistent with the previous study using a methanolic stem extract of *A. graveloens* that prevented PC12 neuronal cells death-induced by *β*-amyloid (Park et al., 2009).

Our results proposed that *A. graveolens* possessed anxiolytic and anti-depressant which were directly related to dopamine neurotransmitter. Therefore, we were interested in neurodegenerative disease like Parkinson's disease which showed severely the decrease of DA in SNpc. In addition, the high dosage of the pure compound *in vitro*, luteolin can inhibit the lipopolysaccharide (LPS), tumor necrosis factor-α, nitric oxide production and superoxide expression which were associated with neuroinflammation of PD model (Chen et al., 2008).

To determine the neuroprotective effect of *A. graveolens* on Parkinson-like symptoms mice. Parkinson's disease (PD) is the second most common neurodegenerative disorder which effected many neurons cells, especially dopaminergic neurons in the midbrain (Dragicevic et al., 2015). The severe loss of about 50% of DA neurons is optimal model for drugs screening in PD (Ghorayeb et al., 2002). There was strong directly correlation between motor deterioration and DA deprivation in parkinson-induced mice (Henderson et al., 2003; Haobam et al., 2005).

The therapeutic drug for PD by L-DOPA has been reported the adverse effects after taking for a long duration consist of dyskinesia, sleep disturbance, and depression (Finley, 2001). Thus, the combination of this drug with other drugs to increase the bio-availability or retention time

is still being the therapeutic approach (Nagatsu and Sawada, 2006). Tidomet plus is combination of levodopa and carbidopa to inhibit peripheral elimination of L-DOPA. Furthermore, the previous research found that levodopa may be neurotoxin itself and promote the nigrostriatal projections degeneration (Fahn et al., 2004) and the continued treatment of DA that leading to the intracellular-mediated oxidative stress accumulation (Caudle et al., 2008).

We used the MPTP to specifically and selectively induce the dopaminergic neurons death in nigrostriatal pathway of parkinsonism mice models due to closely resemble imitating the behavioral manifestation and neurochemical mechanisms of PD, including mitochondrial dysfunction, apoptosis, and oxidative stress (Schmidt and Ferger, 2001; Schober, 2004). In this study, the MPTP model is administered as an acute or a subacute establishment following the method of Sedelis (Sedelis et al., 2001) which can induce the deprivation of dopamine and its metabolites in striatum (Miville-Godbout et al., 2016). By the way, its neurological toxic effect can be reversible (Franke et al., 2016) and experimental animals that received acute or sub-acute dosage of MPTP did not always show the impairment of motor function. Thus, we need to determine whether the consecutive treatment of MPTP in mice was successful or not by using the behavioral tests of motor and coordination (rotarod apparatus and latency time of narrow traverse), balance (drag, grid walk, and foot slip error), and severity of parkinsonian mice (resting tremor and swim scale). Our results suggested that untreated PD group significantly exhibited the deteriorations of motor and coordination, balance and also severely Parkinsonism scale compared to control group without MPTP induction ($p<0.05$) implied that the PD

model was successful induced. Treatment with *A. graveolens* extract at dose of 250 and 375 mg/kg BW in PD mice ameliorated all of behavioral abnormalities when compared with vehicle+MPTP induction groups (p<0.05). Especially, *A. graveolens* extract at dose of 375 mg/kg BW showed the peak effect of behavioral improvement as compared to tidomet plus 25+MPTP treatment groups (p<0.05) as exhibited in Figure 26-30.

The oxidative stress has been important implicated in the PD pathogenesis. Therefore, we measured the MDAs level, GPx activity and % inhibition of $O_2^{\cdot -}$ in cerebral cortex and striatum brain areas which were involved to normal motor control and movement. The results in Table 8 exhibited that *A. graveolens* crude extract of both 250 and 375 mg/kg BW+MPTP administration groups can alleviate all of oxidative stress parameters when compared with NSS+MPTP-induced group (p<0.05). Remarkably, the highest dose of extract displayed the strong effect to improve all parameters compared to tidomet plus 25+MPTP-treated groups (p<0.05). Taken together, the results of this present study indicated that *A. graveolens* might possess the antioxidant effect to inhibit these oxidative stress pathways which was consistent to our previous study found that the *A. graveolens* extract contains a high content of flavonoid and phenolic compounds which showed total peroxide level and oxidative stress index decrease. Our previous research also showed that *A. graveolens* has antioxidant effect via reducing the MDA level while elevating the GPx activity and % inhibition of $O_2^{\cdot -}$ in healthy mice (Tanasawet et al., 2016).

There was well known that the enzymatic catabolism of dopamine by mitochondrial enzyme MAO results in the production of its

metabolize and hydrogen peroxide, resulting in highly hydroxyl radicals deposition through the Fenton reaction (Chiueh et al., 1992). The reduced degradation of dopamine could inhibit the MAO dependent mechanism of dopamine elimination in this research was the target. Treatment with *A. graveolens* extract at dose of 250 and 375 mg/kg BW-treated groups showed a decrease of both MAO-A,B activities compared to vehicle+MPTP induction groups of both cortex and striatum brain areas ($p<0.05$) as demonstrated in Table 8. On the other hand, the 375 mg/kg BW+MPTP treatment groups exhibited significant difference of MAO-A, B activities to tidomet plus 25+MPTP-treated group ($p<0.05$) so the extract may protect the dopaminergic neurons against the inhibition of these enzyme activities. According to our study, we suggested that *A. graveolens* possesses the capability to reduce MAO-A activity associated with anxiolytic effect in non-lesion mice (Tanasawet et al., 2016).

The neuroinflammatory marker of Iba-1 was determined to elucidate whether the microglia cells was activated (Figure 31A). The vehicle plus MPTP-treated group apparently exhibited Iba-1 immunopositive cell when compared to control group ($p<0.05$) that mean we can use MPTP to successfully induce PD model. Remarkably, *A. graveolens* of 375 mg/kg BW-treated group significantly reduced Iba-1 positive cells expression compared to vehicle plus MPTP treated group ($p<0.05$) which is consistency with the result of quantitative analysis of Iba-1 as expressed in Figure 31B.

Finally, to confirm the immunohistological analysis of TH as displayed in Figure 32A. The marked loss of TH immunoreactivity in SNpc from 7 day after the acute MPTP injection (15 mg/kg, i.p.) in untreated group (Figure 32A, b) compared to control group without MPTP

treatment (Figure 32A, a). Treatment with *A. graveolens* ranging from 125-375 mg/kg BW in Parkinson brain mice displayed gradually increased TH positive cells respectively (Figure 32A, c-e) compared with NSS+MPTP treatment. These results were also coherent with the quantitative number of TH positive immunostaining cells as represented in Figure 32B. *A. graveolens* might possess the neuroprotective effect of the dopaminergic neurons in MPTP mice model. Therefore, the proposed active pure compound of *A. graveolens* which can attenuate the motor deficit condition-induced by MPTP might be Luteolin (Anthony and Dweck, 2009).

The further study, to extensively determine the possible underlying mechanism of *A. graveolens* in neuroinflammatory pathway and also a chronic model of Parkinson's disease still being required.

CHAPTER 5
CONCLUSIONS

Our present study showed that *A. graveolens* mediated a potent anxiolytic-like, antidepressant-like effects that involved the biochemical changes to decrease the MAO-A activity resulted in increase dopamine or serotonin which is the main neurotransmitter that related both anxiety and depression disorders. Moreover, the cognitive enhancing effect or neuroprotective effect induced by scopolamine that were associated with cholinergic pathway to decrease AChE activity, and antioxidant pathway that was related to a decrease of the MDA level, the % inhibition of the superoxide anion while it increased the GPx activity. Concerning to Parkinson's disease model, *A. graveolens* extract could ameliorate behavioral impairments through intervening to decrease oxidative stress parameters and the MAO-A, B activities resulted in protecting the dopaminergic neuron. Therefore, this plant extract can be an alternative and valuable source for anti-Parkinsonian drug discovery but the further studies to elucidate the mechanisms are required.

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APPENDICES

APPENDIX A

PREPARATION OF PHOSPHATE BUFFER SALINE (PBS) SOLUTION

0.1 M phosphate buffer saline including KCl 0.8 g, KH_2PO_4 0.8 g, NaCl 32 g, and Na_2HPO_4 4.6 g.

First, KCl, KH_2PO_4, NaCl and Na_2HPO_4 were dissolved in 3.8 L of distilled water (DW) using stirrer and then adjust pH to 7.4 with NaOH solution. Finally, make the volume up to 4 L.

APPENDIX B

PREPARATION OF TISSUE SECTIONS FOR CRESYL VIOLET STAINING

Procedures:

1. Mice were transcardially perfused with 9% normal saline solution (NSS) until getting clear brain samples.

2. After the perfusion, the brain were separated and post fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS) over night at refrigerator 4 ^{o}C.

3. Tissues were rinsed with PBS and immersed in 30% sucrose solution to keep cryoprotection.

4. These specimens were frozen immediately with cryostat deep freezer at -22 ^{o}C. After freezing, 15 µm thick of samples are cut on cryostat.

5. Sections there were stored in PBS and picked up on slides triple coated with a 0.01% aqueous solution of a high molecular weight poly-L-lysine to receive ready slide for cresyl violet staining.

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APPENDIX C

PREPARATION OF TISSUE SECTIONS FOR IMMUNOHISTOCHEMISTRY (IHC) TECHNIQUE

Procedures:

1. Mice were transcardially perfused with 9% normal saline solution (NSS) until getting clear brain samples.

2. After the perfusion, the brain were separated and post fixed with 10% formaldehyde in 0.1 M phosphate buffer (PBS) over night at refrigerator 4 ^{o}C.

3. Tissues were washed with running tap water for 15-20 min.

4. Immersed in 1-3 xylene for 5 min each.

5. Dehydrate the slide sections in serial concentration of alcohol; absolute 95% and 70% alcohol approximate 3 min per each process.

5. Put the samples into 1-3 aqueous paraffin wax.

6. Prepare paraffin mold and block, embed centrally the tissue samples and aqueous paraffin wax into mold and block, and wait until it was cool and dry on ice.

7. Take off the mold and keep the paraffinized block in refrigerator at 4 ^{o}C overnight.

8. The paraffinized blocks on ice were on ice always and prepare the microtome apparatus for cutting slides as 4-5 µm thickness.

9. Pick up the cut tissue and put on the water bath at 15-20 ^{o}C. Be careful of tissue to be shrink or damaged.

10. Take the tissue samples on slide and let it dries on thermoregulator. The slides are ready to be stained of IHC analysis.

References:

Bancroft JD. Tissue Processing. In: Bancroft JD, Gamble M, editors. Theory and practice of histological techniques. Notthingham: Elsevier Health Sciences; 2008; 83-88.

APPENDIX D

CRESYL VIOLET STAINING FOR NISSL SUBSTANCE

Cresyl violet can be used to represent Nissl Substance. The principle of this technique is a basic acid-base reaction, where the cationic dyes bond with the anionic RNA of the Nissl substance and the DNA of cell nuclei.

Staining solutions contain of 0.5% g/ml aqueous cresyl violet solution and 10% acetic acid. And then mix 10% acetic 7 ml in 0.5% g/ml aqueous cresyl violet solution 100 ml and adjust pH ranging from 3.5-3.8. Keep the solution at room temperature for 24-48 h. The solution should be warm gently and filtered before used.

Procedures:

1. Immerse slides into xylene solution for 3 times, approximate 3-5 min each.

2. Dehydrate the sections in serial concentration of alcohol; absolute 95% and 70% alcohol approximate 3 min per each process.

3. Wash the sections in running tap water for 5 min.

4. Stain the sections in cresyl violet solution for 1-2 min or just dip and observe under light microscope to see the violet Nissl body´s staining.

5. Immerse the slides in a serial concentration of alcohol; 70%, 95% and absolute alcohol for 3 min per each process until the background is marked clearly.

6. Clear the sections in 1-3 xylene solution for 2-3 min.

7. Mount the slides and cover slipped with DPX per mount.

8. Determine and take the photo of the sections under 40x magnification light microscope (Olympus).

Results: Nissl body was shown as violet color.

Reference:

Paxions G, Chorles W. Cresyl Violet. In: Paxinos G, Chorles W, editors. The rat brain in stereotaxic coordinates. London: Academic Press; 1981: 9-17.

APPENDIX E

IMMUNOHISTOCHEMICAL STUDY OF TYROSINE HYDROXYLASE (TH) AND IONOZED BINDING ADATOR MOLECULE-1 (IBA-1) POSITIVE CELLS

Reagent and solutions:

1. 0.05 M PBS-T buffer pH 7.4.
2. 0.01 M PBS buffer pH 7.4.
3. 40% formic acid solution
4. 0.5% H_2O_2 in methanol
5. Primary antibody against Tyrosine hydroxylase (TH) and Ionized binding adaptor molecule-1 (Iba-1) dilution 1:200.
6. Secondary antibody-conjugated Horseradish peroxidase (HRP) dilution 1: 2000.
7. Diaminobenzedine (DAB) kit consists of 0.4% H_2O_2
8. 1% glycine in KPBS-BT
9. 0.1 % Bovine serum albumin (BSA)
10. Acetic acid solution

Procedures:

1. Pre-treatment with 40% formic acid solution for 0.5-1 h.
2. Antigen retrieval step was preceded by using acetic acid solution and then slides were heated in microwave for 1-2 min. Wait until slides were cool at room temperature.
3. Inhibit endogenous peroxidase activity by incubating in 0.5% H_2O_2 in methanol for 30 min.

4. Wash slides in running tap water for 5 min then wash slides again in distilled water (DW) for 3 min.

5. Rinse slides in PBS and PBS-T for 5 min per each process.

6. Immerse in 1 % glycine in PBS for 10 min.

7. Rinse slides again in PBS and PBS-T for 5 min per each process.

8. Remove excess buffer, then apply the 0.1% BSA in PBS-T to the sections and incubate in moist chamber for 1 h. in order to minimize background staining.

9. Take off excess BSA.

10. Incubate sections in rabbit primary antibody against TH or Iba-1 diluted 1:200 in PBS-T at room temperature overnight. (This step is omitted in control slider)

11. Wash out excess antiserum and slides in PBS-T and PBS for three 5 min changes.

12. Drain off excess buffer and incubate slides with 120 µl for working solution of secondary HRP conjugated- mouse antibody to rabbit at incubator, 37 ^{o}C for 2 h.

13. Wash slides in PBS-T and PBS for three 5 min changes.

14. React for peroxidase activity in DAB kit for 10-15 min at room temperature.

15. Observe and recheck under light microscope to see the brown color of positive cells.

16. Wash in running tap water, let dry and mount sections in DPX per mount.

17. Take photo of the TH and Iba-1 immonostaining photographs under 20X magnification of light microscope (Olympus).

Reference:

Ahmad M, Saleem S, Ahmad AS, Ansari MA, Yousuf S, Hoda MN, et al. Neuroprotective effects of Withania somnifera on 6-hydroxydopamine induced Parkinsonism in rats. Hum Exp Toxicol 2015; 24(3): 137-47.

APPENDIX F

PREPARATION OF HOMOGENATED TISSUE

After the last oral gavage of treatment, all mice were anesthetized with intraperitoneal (i.p.) injection of pentobarbital sodium (nembutal®) at dose of 50 mg/kg BW. Their brains were separated and kept cool on ice. Then these tissues were homogenized and kept at freezer -80 ^{o}C.

Reference:

Marzel P. General principle and procedure for drug metabolism is *vitro*. In: La Du BN Mandel HG, Way EL. Editors. Fundamentals of drug metabolism and drug disposition. New York: Krieger Publishing Company; 1979: 527-52.

APPENDIX G

DETERMINATION OF LIPID PEROXIDE CONTENTS

Reagents:

1. 8.1% SDS (sodium dodecyl sulfate).

2. 20% acetic acid solution adjusts to pH 3.5 by using NaOH.

3. 0.8% TBA (thiobarbituric acid).

4. 1,1,3,3-tetramethoxy propane (TMP) was used as an external standard, and the level of lipid peroxide was expressed as nmol of malondialdehyde (MDA).

Procedures:

1. Add the following substances in the table into the series of glass tubes with screw capped.

	Blank (ml)	Standard (ml)	Unkown (ml)
Sample (1:30)	-	-	0.2
8.1% SDS	0.2	0.2	0.2
20% Acetic acid (pH 3.5)	1.5	1.5	1.5
0.8% TBAs	1.5	1.5	1.5
TMP stock standard	-	0.2	-
Distilled water	0.8	0.6	0.6

2. Heated the tubes in the water-bath at 95-100 ^{o}C for 60 min.

3. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) are added and shaken vigorously.

4. After centrifugation at 4,000 rpm for 10 min, the organic layer is taken and its absorbance at 532 nm is measured.

5. The content of lipid peroxide is expressed is terms of nmol MDA/100 mg protein.

Calibration curve of TMP:

1. Prepare a serial TMP standard in water in the following concentrations: 2.0 nmol/0.2 ml, 4.0 nmol/0.2 ml, 6.0 nmol/0.2 ml, 8.0 nmol/0.2 ml, 1.0 nmol/ 0.2 ml.

2. Do the procedure as in step 2.

3. Determine the absorbance at 532 nm. The O.D. was plotted against concentration of MDA which expressed as nmol MDA/100 mg protein of brain tissue.

Reference:

Ohkawa H, Ohishi N, Yaki K. Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-8.

APPENDIX H

DETERMINATION OF ACETYL CHOLINESTARASE (ACHE) ACTIVITY

Solutions:

1. 0.1 M phosphate buffer saline (PBS), pH 8.0
2. 0.075 M acetylthiocholine iodide (ATCld)
3. 0.01 M dithiobisnitrobenzoate (DTNB)

Prepare brain homogenates:

A. Weight a sample (about 30 mg) and add 1 ml of PBS/30 mg tissue (30 mg/ml). Homogenize until the brain is uniformly dispersed in the buffer. Place the tube on ice always.

B. Assay

1. Turn on the spectrophotometer, set at 412 nm and warm up for at least 15 min before reading.
2. Label the assay wells for triplicate number of each in program.
3. Add 100 µL of homogenated sample to each well.
4. Mix 50 µL DTNB to the well, shake by shaker and place it for 5 min. This allows the solution to reach room temperature.
5. Add 10 µL ATCld quickly and mix as well on shaker vigorously.
6. Immediately take readings at 30 s, 60 s, 2 min, and 3 min.

7. Repeat this procedure for the other sample homogenates. Run the control through the same procedure except do not add substance (ATCld) but add 10 µL PBS instead.

C. Calculate of the rate of the reaction:

1. Graph the data for the different brain regions-change in absorbance/min against time.

Calculate the rate of color change per minutes for each reading and average the rates between each run for each brain region, calculate the rate of the reaction according to the following equation:

$R = \Delta A/(1.36*10^4) \times 1\ (200/3320)\ Co = 1.22(10^{-3})\ A/Co$

R= rate, in moles substrate hydrolyzed/min. g tissue

ΔA = change in absorbance/min.

Co= original concentration of tissue (mg/ml) 200/3320 are volume corrections 1.36 (10^4) is the extinction coefficient of the yellow product.

2. Analyze and make a graph to show the enzyme activity of each brain region.

References:

Robertson RT, Holunann CF, Bruce JL, Oyle JTC. Neonatal enucleation reduces specific activity of acetylcholinesterace in developing rat visual cortex. Devel Brain Res 1988; 39: 298-302.

Thompson RF, Freeman WH. The Brain: A Neuroscience Primer. New York, 1993; 13.

APPENDIX I

DETERMINATION OF GLUTATHIONE PEROXIDASE ACTIVITY

Reagents:

A. 50 mM Sodium Phosphate Buffer with 0.4 mM EDTA, pH 7 at 25 ^{O}C. Prepare in deionized (DI) water using Sodium Phosphate, Monobasic, Anhydrous and Ethylenediaminetetraacetic Acid (ETDA), Tetrasodium Salt.

B. 1 mM Sodium Azide Solution (Buffer w/ Azide). Prepare Sodium Azide in reagent A.

C. β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced form (β-NADPH). Use 5 mg vial of β-NADPH (Reduced Form).

D. Glutathione Reductase Enzyme Solution (GR) was prepared freshly before use; a solution contains 100 units/ml of GR in cold DI water.

E. 200 mM Glutathione, Reduced (GSH). Prepare in DI water using GSH.

F. 10 mM Sodium Phosphate Buffer with 1mM Dithiothreitol, pH 7 (Buffer w/DTT). Prepare in DI water using Sodium Phosphate, Monobasic, Anhydrous and DL-Dithiothreitol.

G. Standard Glutathione Peroxidase Enzyme Solution (Std GPx). Immediately before use, prepare a solution containing 1.5, 2, 2.5,

3, and 5 units/ml of Glutathione Peroxidase in cold buffer w/DTT (Reagent F).

H. 0.042% (w/w) Hydrogen Peroxide (H_2O_2). Prepare in deionized water using Hydrogen Peroxide, 30% (w/w) Solution.

Prepare brain homogenates:

Weight a brain sample and diluted in the cold buffer w/DTT (Reagent F, 25 mg brain tissue/ml). Homogenize this solution until the brain is uniformly dispersed in the buffer.

Procedures:

1. Prepare a reaction cocktail by pipetting the following reagents into 5 mg vial of Reagent C (β-NADPH)

B. Buffer w/Azide 23 ml

D. GR 0.25 ml

E. GSH 0.125 ml

2. Mix and adjust to pH 7, 25 ^{o}C.

3. Pipette (in µl) the following reagents into tube and put into 96 wells plate:

	Cocktail	Buffer w/DTT	DI water	Std GPx	Sample
Blank	-	-	200	-	-
Test 1	200	3.4	-	-	-
Test 2 GPx	200	-	-	3.4	-
Test 2 Sample	200	-	-	-	3.4

4. Add by inversion and see the absorbance, A_{340nm} of the reaction cocktail until constant using a spectrophotometer.

5. Mix (in micrometers) $H_2 O_2$ (Reagent H):

	H_2O_2
Test 1	3.4
Test 2 Std GPx	3.4
Test 2 Sample	3.4

Then, suddenly mix by inversion and record the reduced value in A_{340nm} for 3 min. Receive the rate of change ($\Delta A_{340nm/}$min) for both Test 1 (No reaction) and Test 2 (Reaction).

6. Calculate Diff ΔA_{340nm}/min as following:

Diff $\Delta A_{340nm/}$min= ($\Delta A_{340nm/}$min Test 2) - (ΔA_{340nm}/min Test 1)

7. Draw the GPx standard curve by plotting Diff ΔA_{340nm}/min against Std GPx (units/ml) and examine the linear equation of the curve.

8. Calculate the activity of GPx in samples using Diff ΔA_{340nm}/min and the linear equation of the standard curve.

9. Report and graph the enzyme activity and it is expressed in units/mg protein.

Reference:

Wendel A. Enzymatic Basis of Detoxication Glutathione Peroxidase. In W.B. Jakoby Edn. Academic Press, New York, 1980; 2: 333-53.

APPENDIX J

MEASUREMENT OF MONOAMINE OXIDASE-A,B (MAO-A,B) ACTIVITIES

Peroxidase-linked assay of MAO-A, B activities protocol:

1. Homogenates of brains were prepared 1:10 (w/v) in ice-cold potassium phosphate buffer (0.2 M, pH 7.6).

2. Incubate with 500 µM tyramine plus 500 nM pargyline or 2.5 mM tyramine plus 500 nM clorgyline in order to inhibit the MAO-B or A activities, respectively.

3. The chromogenic solution contains of vanillicacid (1 mM), 4-aminoantipyrine (500 mM), and peroxidase (4 U/ ml) in potassium phosphate buffer (0.2 M, pH 7.6) were prepared on a daily basis and kept at 4 C until used.

4. The wells contains 50 µl of tissue homogenate, 50 µl chromogenic solution, and 200 µl 100 mM of amine substrate, prepared in potassium phosphate buffer.

5. Reaction mixtures were warmed at 37 ^{o}C.

6. Read at 498 nm of spectrophotometer.

Reference:

Holt A., Sharman D.F., Baker G.B., Palcic M.M. A continuous spectrophotometric assay for monoamine oxidase and related enzymes in tissue homogenates. Anal Biochem 1997; 244(2): 384-92.

APPENDIX K

THE PRINCIPLES OF ANIMAL CARE OUTLINED BY FACULTY OF SCIENCE, PRINCE OF SONGKLA UNIVERSITY

PRINCE OF SONGKLA UNIVERSITY
15 Karnjanawanij Road, Hat Yai, Songkhla 90110, Thailand
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MOE0521.11/ 682 Ref.09/2013

May 27 , 2013

This is to certify that the research project entitled " Study the potential effect of *Apium Graveolens L.* to protect against neurodegeneration in Parkinson's like symptoms induced by MPTP in mice " which was conducted by Dr. Pennapa Chonpathompikunlert, Faculty of Science , Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

Kitja Sawangjaroen.

Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University

VITAE

Name Phetcharat Boonruamkaew
Student ID 5510230033
Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor degree (Physical therapy)	Walailak University	2009
Master degree (Anatomy)	Mahidol University	2011

Scholarship Awards during Enrolment

2011-2014	Prince of Songkla University Graduate Studies Grant
2012-2013	The Graduate thesis scholarship of Prince of Songkla University
2013	The Full grant support to participate in IBRO-APRC Associate School of Neuroscience at Brain research center, Department of Zoology, Banarus Hindu University, Varanasi, India
2013-2015	The Brand's Brain Research Centre from Cerebos Pacific Limited
2014	The overseas Ph.D. scholarships of graduate school of PSU
2015	The Full grant support to participate in IBRO-APRC Advanced School of Neuroscience at Brain Research Institute Monash, Jeffrey Cheah School of Medicine and Health Sciences, Monash University, Malaysia
2015-2016	The Graduate research scholarship of National Research Council of Thailand (NRCT)
2016	The Full grant support to participate in IBRO-APRC School of Neuroscience at Medical school of Tarbiat Moderes University, Tehran, Iran

Work - Position and Address

Lecturer at department of Physical therapy, Faculty of Medicine, Prince of Songkla University, Kho-hong, Hat Yai, Songkhla, Thailand 90112

Lists of Publication and Proceeding

1. Boonruamkaew P, Chonpathompikunlert P, Nagasaki Y. Redox nanoparticle therapeutics for acetaminophen-induced hepatotoxicity in mice. Oxidative Medicine and Cellular Longevity; Harmful and Beneficial Role of ROS 2016; 4984597: 1-10.
2. Tanasawet S, Boonruamkaew P, Sukketsiri W, Chonpathompikunlert P. Anxiolytic and free radical scavenging potential of Chinese celery (*Apium graveolens*) extract in mice. Asian Pacific Journal of Tropical Biomedicine 2016; Accepted 23th February 2016 (In press).
3. Boonruamkaew P, Sukketsiri W, Tanasawet S, Chonpathompikunlert P. Ameliorative Effect of *Apium graveolens* L. on Scopolamine-Induced Amnesia Mice. At the 1st International Conference on Herbal and Traditional Medicine (HTM), Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand 2015 January 28-30th. (Special Issue at Isan Journal of Pharmaceutical Sciences; Volume 10 (Supplement); January 2015; p 82-93.
4. Boonruamkaew P, Sukketsiri W, Tanasawet S, Chonpathompikunlert P. An *Apium Graveolens* extract influences the mood and cognition in healthy mice. Acta Pharmaceutica Sinica 2016 (Submitted).
5. Boonruamkaew P, Sroyraya M, Sukketsiri W, Chonpathompikunlert P. The ameliorative effect of *Apium graveolens* L. related to antioxidant and neurochemical activities on MPTP-induced Parkinson-like symptoms mice. Journal of Biomedical Science 2016 (Submitted).