Evaluation of the Pharmacological Effects and the Underlying Mechanisms of Selected Chinese Herbs on Dementia

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Abstract of thesis entitled:

Evaluation of the Pharmacological Effects and the Underlying Mechanisms of Selected Chinese Herbs on Dementia

Submitted by **NG Chun Fai**

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Chinese herbal medicines (CM) have long been used for treatment of neurological disorder, such as headache and convulsions. CM has been considered as one of the alternative countermeasures for dementia. In this context, we aimed to screen and investigate the pharmacological effects of aqueous extracts of selected Chinese Herbs using neuronal-like cell and different animal models of dementia. Based on literature review, four Chinese herbs which are generally used for neuroprotection as folk medicine, has been selected, including Chuanxiong Rhizoma (CX), Polygalae Radix (YZ), Gastrodiae Rhizoma (TM) and Uncariae Ramulus cum Uncis (GT).

For *in vitro* screening studies, neuronal pheochromocytoma cell, PC-12, was

used as a screening platform for the protective effects of selected CM against dementia. The aqueous crude extracts of CX, YZ and TM were non-cytotoxic up to 1000μg/ml and GT was non-cytotoxic up to 500μg/ml, which determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Besides, our data demonstrated that all selected CM could abolish $A\beta_{25-35}$-induced toxicity significantly, especially GR and CR. Both of them were able to reduce apoptosis and ROS production induced by $A\beta_{25-35}$. Among selected CM, TM demonstrated the highest efficacy and therefore was selected for further mechanistic study.

For the *in vitro* mechanistic studies, the effect of TM on Aβ-induced toxicity was examined by MTT assay in rat pheochromocytoma PC-12 cells. Besides, annexin V/PI double staining and caspase-3 activity were used to detect apoptosis; while reactive oxidative species (ROS) production was detected with fluorescence probe DCFH-DA by flow cytometry. Activity of anti-oxidative enzymes, catalase, superoxide dismutase and glutathione peroxidase were also detected. The expression of caspase-3, tau and phosphorylation tau was determined by Western blotting In PC-12 cells, treatment of TM extract could abolish Aβ-induced toxicity, reduce apoptosis and reduce caspase-3 expression and activity. Tianma extract also significantly suppressed Aβ42-induced ROS production, restored superoxide

dismutase activity and promoted catalase and glutathione peroxidase activity dose dependently. TM treatment can also reduce the phosphorylation of tau.

On the other hand, the glutamate-induced excitotoxicity and the acetylcholinesterase activity were investigated using rat primary cortical neurons. Our results showed that TM effectively inhibited Aβ-induced activation of acetylcholinesterase. However, TM was not effective against excitotoxicity.

The effects of aqueous extract of TM on neurological phenotypes (reduction in lifespan, locomotion and degeneration of ommatidia) of transgenic *Drosophila* model of AD, which express in their neurons, were evaluated. Our results showed that the lifespan and the locomotor activities of the Aβ42-expressing *Drosophila* were significantly increased, and degeneration of ommatidia was reduced when treated with TM.

The effects of aqueous extract of TM on other type of dementia were assessed by traumatic brain injured rat. Intake of our extract did not affect the rats' weight. Moreover, our results demonstrated that TM treatment significantly improved the locomotor activities of the brain injured rat on rotarod. The results

demonstrated that TM protects the rats against neurodegeneration induced by trauma.

In conclusion, our study evaluated the aqueous extract of four Chinese herbs for their potential protective effects on beta-amyloid induced toxicity. Among them, TM demonstrated the highest efficacy and further studied. Our data suggested that TM possesses neuroprotective effect, through anti-apoptotic and anti-oxidative mechanisms, which might explain for its protective effects against Aβ-induced neurodegeneration in *Drosophila* and traumatic brain injury in rats. The study provided pharmacological evidences for the usage of Tianma as a potential treatment of dementia.

摘要

在傳統中醫藥中，一直有中藥材用於治療神經系統疾病，如頭痛和抽搐。而在現代醫療中，中藥材一直被視爲尋找治療癡呆症藥物的寶庫。在本文中，我們的目的在於篩選和研究有潛力的中藥水提物，在類神經元樣細胞和不同的動物模型平台上研究它們在癡呆症上的藥理作用。由文獻中，我們選定了四種民間常用的補腦或益智的中藥材，當中包括川芎、遠志、天麻和鉤藤。

對於細胞模型的篩選研究中，我們選用了類神經細胞的大鼠嗜鉻細胞瘤細胞(PC-12)作爲研究平台。川芎、遠志和天麻的水提物在 1000μg/ml 和鉤藤的水提物在 500μg/ml 都對細胞沒有毒性。此外，我們的研究發現四種水提物都可以有效減低 β-澱粉樣蛋白引起的毒性，而當中以川芎和天麻尤爲有效。此外，它們都能減少 β-澱粉樣蛋白引起的細胞凋亡和氧化作用。在選定四種中藥材中，天麻表現出最高的功效，因此被選定爲進一步的機理研究對象。

天麻在 PC-12 細胞平台上，對 β-澱粉樣蛋白引起的毒性的起了保護作用。在抗細胞凋亡方面，膜聯蛋白 V /碘化丙啶雙染色法測定了天麻能減少 β-澱粉樣蛋白引起的細胞凋亡。此外，天麻能減低 caspase-3 的表達和活性。在抗氧化方面，我們使用 DCFH-DA 來測定細胞內的氧自由基，結果發現天麻能減少活性氧的產生。另一方面，天麻能激活細胞內的過氧化氫酶、超氧化物

歧化酶、穀胱甘肽過氧化物酶，這類酶能夠分解細胞內的活性氧，從而引致減少氧自由基的結果。此外，天麻還可以降低 tau 蛋白的磷酸化。這幾方面都能夠降低 β-澱粉樣蛋白引起的毒性。

另一方面，我們使用大鼠原代皮層神經元作爲平台，測試了天麻對抗谷氨酸誘導的興奮性毒性及乙酰膽鹼酯酶的活性進行了研究。我們的研究發現，天麻有效地抑制 β-澱粉樣蛋白激活的乙酰膽鹼酯酶。然而，天麻對抗谷氨酸誘導的興奮性毒性並沒有效果。

在動物模型上，我們用了類老人癡呆症的轉基因果蠅模型，β-澱粉樣蛋白在它們神經元的表達引起了壽命減少、運動性退化和複眼退化的傷害。而我們的研究發現，天麻水提物能顯著延長帶病果蠅的壽命、延緩 β-澱粉樣蛋白引起的運動性退化及複眼退化。

我們也使用了創傷性腦損傷大鼠作爲平台，研究天麻在其他類型的癡呆症上的作用。實驗證明，攝取天麻水提物並不會影響老鼠的體重。最重要的是，我們的研究發現天麻水提物能顯著提高腦創傷大鼠在 Rotarod 上運動性。結果表明，天麻能減輕大鼠在腦創傷後引起的神經退化。

總結而言，我們的研究評估四種中藥材的水提物在保護細胞抗衡 β-澱粉樣蛋白引起的毒性。其中，天麻表現出最高的療效。在進一步的研究中，天麻通過抗細胞凋亡和抗氧化的機制，表現出顯著的神經保護作用，這或許可以解釋其在類老人癡呆症果蠅和腦創傷大鼠上的保護作用。這一項藥理研究證明，天麻有潛成進一步研究成爲治療老年癡呆症的藥物。

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List of Abbreviations

6-OHDA	6-hydroxydopamine
Aβ	Beta-amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ADP	Adenosine diphosphate
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
AUC	Area under the curve
BACE	β-secretase
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
CAT	Catalase
CAM	Complementary and alternative medicine
CCI	Controlled cortical impact
CDK5	Cyclin-dependent kinase 5
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CT	Computed tomography
CUHK	The Chinese University of Hong Kong
CV	Cardiovascular system
CyO	*Curly of Oster*
DCF	2',7'-dichlorodihydrofluorescein
DCFH	2′,7′-dichlorodihydrofluorescin
$DCFH_2$-DA	2′,7′-dichlorofluorescin diacetate
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
Elav	*Embryonic lethal abnormal vision*
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FD&C	Federal Food, Drug, and Cosmetic Act

FITC	Fluorescein isothiocyanate
FPI	Fluid percussion injury
GABA	γ-aminobutyric acid
GI	Gastrointestinal system
GMR	*Glass Multiple Reporter*
GPx	Glutathione peroxidase
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HS	Horse serum
ICM	Institute of Chinese Medicine
IgG	Immunoglobulin G
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MAP2	Microtubule-associated protein 2
MARK	Microtubule affinity-regulating kinase
MCAO	Middle cerebral artery occlusion
MRI	Magnetic resonance imaging
MTT	3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide
NAChR	Neuronal nicotinic acetylcholine receptor
NF-κB	Nuclear factor kappa-B
NFT	Neurofibrillary tangle
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
OGD	Oxygen-glucose deprivation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PHF	Paired helical filament
PI	Propidium iodide
PiB	Pittsburgh compound B
PNS	Peripheral nervous system

PS1	Presenilin 1 / γ-secretase
pTau	Phosphorylated tau
Resp	Respiratory system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SD rat	Sprague Dawley rat
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOD	Superoxide dismutase
TBE buffer	Tris/Borate/EDTA buffer
TBI	Traumatic brain injury
TBS	Tris-buffered saline
TCM	Traditional Chinese Medicine
TLC	Thin-layer chromatography
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-β
TTBS	Tris-buffered saline with 1% Tween-20
UAS	Upstream activating sequence
UV light	Ultraviolet light

Abbreviations of herbal samples used in this study

CX	Chuanxiong Rhizoma	川芎
GT	Uncariae Ramulus cum Uncis	鉤藤
TM	Gastrodiae Rhizoma	天麻
YZ	Polygalae Radix	遠志

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Chapter 1
Introduction

1.1 Introduction to Dementia and Alzheimer's Disease

1.1.1 Dementia

Dementia is a decline of cognitive ability such as thinking, reasoning, memory or language ability, which is more rapid than normal aging. Instead of a disease, dementia is a group of symptoms that appear among a group of diseases. It is more prominent in older populations. But in rare cause, dementia happens before 65. The patients suffer from declining function that affect their daily life, to the late stage that affects the activities of daily living like food intake, personal hygiene, excretion and dressing.

Most type of dementia is non-reversible, meaning there is no cure or attenuation for the progression of the condition. In people of older age, the risk for dementia rise with the age. Alzheimer's disease, vascular dementia, Huntington's disease, multiple sclerosis, infections affecting brain, such as HIV/AIDS and Lyme disease, Parkinson's disease, Pick's disease, and progressive supranuclear palsy can also be the cause of dementia. However, there are also causes that are reversible with treatments, such as brain injury or tumour. Within the long list of causes of dementia, the most common type of dementia is Alzheimer's disease (Alzheimer's Association., 2013).

In 2010, Alzheimer's disease and other dementias affect 35.6 million of people worldwide, which contribute to about 0.5% of the global population. The

global economics impact was up to US$604 billion, which is much higher than cancer or heart diseases. From the forecasting of Alzheimer's Disease International, the number would increase by 85% by 2030 (Figure 1.1) (Alzheimer's Disease International.).

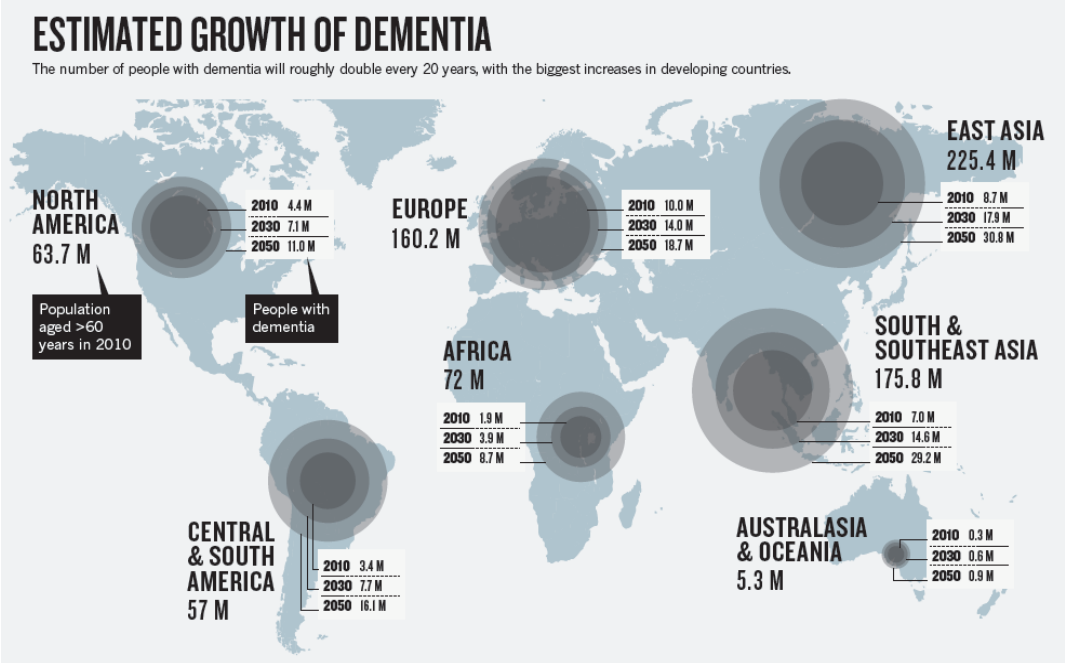


Figure 1.1 The Prevalence and Growth of Dementia (Adopted from (Abbott, 2011))

Living standard increase in developing countries, like China and India, leads to a prediction of exponential increase in prevalence of dementias in the future (Abbott, 2011). The reason behind is the longer lifespan due to better living. As the main risk factor for dementia is age, an increased number of aged populations would lead to a larger dementia population. The increase in people with dementia means an increased need of costly health care for dementia patients, which pose a

large economical impact to the countries and contribute to the large economical impact increase that we mentioned.

1.1.2 Excitotoxicity

Among the various diseases that cause dementia, there is a common destructive metabolic pathway called excitotoxicity (Dodd*, et al.*, 1994). This topic was widely investigated and the mechanism is now known. The influx of calcium ion into neuronal cells and other deleterious events, due to the excessive excitation of a neuronal amino acid receptor, *N*-methyl-D-aspartate (NMDA) receptor, damages the neurons and causes the neurological disorders. The detailed excitotoxic events will be discussed below.

In normal brain, glutamate acts as a neurotransmitter, is responsible for the communication at the synapse between neurons. Among the amino acid neurotransmitter, glutamate and aspartate are the major excitatory ones, while γ-aminobutyric acid (GABA) and glycine are the major inhibitory ones (Dodd*, et al.*, 1994). The excitation of glutamate receptors by glutamate lead to the influx of calcium ions into the postsynaptic spine. The excitation produced by glutamate is rapid (Lynch, 2004). However, excitation by glutamate is stable, relatively long lasting. Long-term potentiation (LTP) is the increase in the magnitude of a post-synaptic response by glutamate and is produced in hippocampus (Teyler and DiScenna, 1987), while long-term depression (LTD) is the lowering of transmission efficacy occurs following a low-frequency repetitive stimulation by glutamate and is produced in the cerebellum (Ito, 1989). Among them, LTP is

believed to be the process underlies the memory formation. Therefore, glutamate is important for the normal brain function. Normally, extracellular glutamate is rapidly removed by glial cells and neurons to maintain a low and non-toxic concentration of glutamate in long-term.

However, when there is accumulation of glutamate and excessive glutamate excitation, the event turns into a devastating one. Glutamate acts on NMDA receptor, which consists of a channel that is permeable to calcium, sodium and potassium ions when excited (Barger, 2004, Dong*, et al.*, 2009). The result is the influx in calcium which normally responsible for synaptic plasticity. The excessive glutamate excitation causes influx of calcium ions and depolarization. The depolarization will activate the voltage-dependent calcium channels, which allow further and excessive influx of calcium ions. The accumulation of excessive intracellular calcium activates a series of enzymes, including protein kinase C, nitric oxide synthase, calcium/cadmodulin-dependent protein kinase II, phosphatases, phospholipases, proteases, and endonucleases, etc (Nicotera*, et al.*, 1992). Protein kinase C is the enzyme that controls the proliferation and apoptosis of cells, with different functions by different isotypes (Musashi*, et al.*, 2000). Nitric oxide synthase is important for the vasodilation and inflammatory response. However, overactivation of nitric oxide synthase generates excessive nitric oxide, which will react with superoxides to form peroxynitrite and cause oxidative damage to the neurons. Extensive phospholipase A_2 activation by calcium ions can lead to breakdown of the cell membrane (Chien*, et al.*, 1979) and generate arachidonic acid. Arachidonic acid can prevent the glutamate uptake by cells and

further prolong the glutamate-induced excitotoxicity, forming a vicious cycle. Excessive calcium activation of protease (calpains) can lead to the breakdown of cytoskeleton of neurons. Calcium-activated endonuclease can cleave DNA and cause DNA fragmentation, which is a hallmark of apoptosis. The activation of these enzymes can directly damage the cellular structure and induce apoptosis of the neurons (Nicotera*, et al.*, 1992). The extensive death of neurons would cause memory impairment and dementia.

1.1.3 Cholinergic Deficits

Among various types of neurotransmission mechanism, the cholinergic system was the first neurotransmission system found (Tansey, 2006). Acetylcholine (ACh) was the neurotransmitter of the cholinergic system. The main receptors that recognize ACh are cholinergic muscarinic and neuronal nicotinic acetylcholine receptors (NAChRs). ACh and NAChRs are responsible for the communication at the synapse between neurons, and are widely expressed in the peripheral ganglia and in various areas of the brain. The action of NAChRs affects various physiological functions and cognitive functions such as learning and memory (Hasselmo, 2006).

During normal aging, cholinergic transmission will be slowly diminished, forming the gradual decline of physiological functions and cognitive functions in elderly. However, in neurodegenerative disorders such as Alzheimer's disease, the dysfunction of cholinergic system is found. The dysfunction is mainly due to reduced in expression of NAChRs, and the inhibition of α4β2 subtype and α7

NAChRs during Alzheimer's disease. The NAChRs are important for the survival of neurons duringe aging. Thus, the decline in number of functional NAChRs may contribute to the neuronal death, and leading to the neurodegeneration (Francis*, et al.*, 1999).

1.1.4 Alzheimer's Disease

1.1.4.1 Background

Alzheimer's disease (AD) was firstly identified in 1906 by a German physician, Dr. Alois Alzheimer (National Institutes of Health, 2013). He identified a collection of brain cell abnormalities as a disease. In one of his dead patients, who presented with long-term severe memory problems, confusion and difficulty understanding questions, the brain autopsy revealed dense deposits surrounding the nerve cells (which are the senile plaques composed of amyloid in modern science). In nerve cells, Dr. Alzheimer observed twisted bands of fibers (which are the neurofibrillary tangles composed of defective tau protein). Nowadays, these two observations are still definitive diagnostic signs in autopsy for Alzheimer's disease. Therefore, the disease was named after him, as Alzheimer's disease (AD).

Best-known symptom of AD is the declining of ability to remember new information. However, there are 10 warning signs of alzheimer's disease according to Alzheimer's Association (http://www.alz.org/alzheimers_disease_10_signs_of_alzheimers.asp)

i. Memory loss that disrupts daily life

ii. Challenges in planning or solving problems

iii. Difficulty in completing familiar tasks at home, at work or at leisure

iv. Confusion with time or place

v. Trouble understanding visual images and spatial relationships

vi. New problems with words in speaking or writing

vii. Misplacing things and losing the ability to retrace steps

viii. Decreased or poor judgment

ix. Withdrawal from work or social activities

x. Changes in mood and personality

As AD progress, the above symptoms would appear, sooner or later. In late stage of AD, the patient would be unable to perform activities of daily living, like dressing, eating, showering or using the washroom. In the final stage of the disease, the patients become bedbound, unable to speak or understand others' speeches, unable to recognize people with no exception for the most important ones and requires whole-day care. Falls and infections were the main cause of death for AD patients, due to their incapacitation. In rare cases, the diseases were able to progress to a point that affects the centers of the brain that control breathing and cause death.

1.1.4.2 Pathogenesis of Alzheimer's Disease

The pathogenesis of AD mainly involves 2 proteins – beta-amyloid protein and tau protein.

1.1.4.2.1 Beta-amyloid Protein

Beta-amyloid protein is believed to be the central of Alzheimer's disease. Although the exact mechanism of the disease is unknown, the devastative effect of beta-amyloid protein is quite clear. Beta-amyloid protein was originated from amyloid precursor protein (APP). APP is a single transmembrane protein with large extracellular N-terminal region (the ectodomain) and a short intracellular C-terminal region. It has isoforms containing 751 or 770 amino acids expressed in various cell types, and 695 amino acids expressed neurons (Mattson, 1997). APP was found to function as an extracellular matrix molecule, a regulator of axonal outgrowth, and a kind of autocrine trophic factor. However, the deletion of APP gene in mice did not cause early death or major defects. Only minor cerebral gliosis, changes in locomotor function was detected (Zheng*, et al.*, 1995). The findings may due to the expression of its homolog, amyloid precursor-like protein-1 and -2.

Normally, the major secretory fragment was cleaved by α-secretase, forming a soluble fragment APPs-α and a C-terminal fragment with 83 amino acids (LaFerla*, et al.*, 2007). The C-terminal fragment was still embedded in the membrane and was further cleaved by γ-secretase, forming a harmless fragment p3 and an APP intracellular domain that released to the cytoplasm (Haass*, et al.*, 1993).

In Alzheimer's disease condition, APP was cleaved by β-secretase instead,

forming a soluble fragment APPs-β and a C-terminal fragment with 99 amino acids. The C-terminal fragment was again further cleaved by γ-secretase, forming β-amyloid protein and an APP intracellular domain that released to the cytoplasm. β-amyloid protein consist of 2 forms – with 40 or 42 amino acids, due to the different cleavage of γ-secretase (LaFerla*, et al.*, 2007). Both forms of β-amyloid protein would be secreted from the neurons and self-aggregated into oligomers or assemble into fibrils seeds, protofibrils, fibrils and amyloid plaques (Figure 1.2) (Schnabel, 2011). The β-amyloid protein oligomers accumulate in synapses and block the long-term potentiation, and cause memory impairment (Hardy and Selkoe, 2002). The β-amyloid plaque can also induce the apoptotic cascade in synapses (Mattson*, et al.*, 1999).

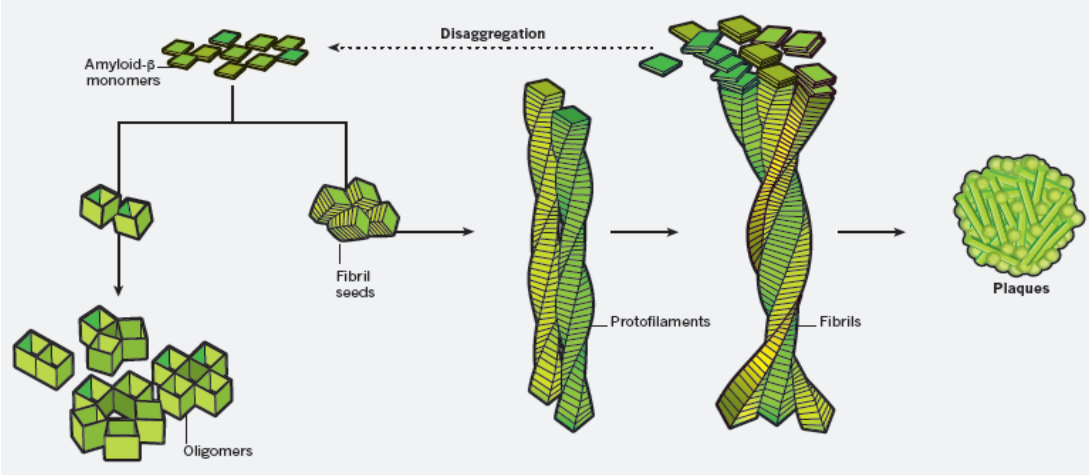


Figure 1.2 The aggregation of β-amyloid protein (Schnabel, 2011)

β-amyloid protein also leads to the generation of reactive oxygen species, because of the generation of hydrogen peroxide during the aggregation of β-amyloid protein. The generation of reactive oxygen species causes lipid

peroxidation. Lipid peroxidation inhibits the function of ion-motive ATPases, glucose and glutamate transporters, and also GTP-binding proteins. These actions cause toxic effect to cells and increase neurons' vulnerability to oxidative stress, and excitotoxicity (Mattson*, et al.*, 1992).

1.1.4.2.2 Tau Protein

Tau protein is a microtubule-associated protein that mainly expressed in the axons of the neurons. Normally, it functions to stabilize the microtubule, hence maintaining the cytoskeleton of the neurons. Tau protein has 6 isoforms in brain, which is contributed by alternative splicing. Each of them would have 3 to 4 tubulin binding domain, which help the binding of tau to the microtubules to serve its function (Avila*, et al.*, 2004, Buee*, et al.*, 2000).

In Alzheimer's disease condition, hyperphosphorylation of tau by several kinases, including GSK-3, CDK5, and MARK, takes place (Mandelkow and Mandelkow, 1998, Takashima*, et al.*, 1993). The hyperphosphorylated tau would detach from the microtubules. Lossing tau as a stabilizer of the microtubules, the structure and function of cytoskeleton in the neuron would be adversely affected. The structural deformation and the impaired axonal transport lead to synaptic dysfunction and neurodegeneration.

On the other hand, as the cytosolic unbound tau accumulates, it may undergo misfolding and aggregation (Avila, 2000). The aggregation initiates as a small non-fibrillary tau deposits (pretangles). The pretangles undergo conformational

change to form paired helical filaments (PHFs), which is a two-stranded helix. Accumulated bundles of PHFs would aggregate to form neurofibrillary tangles (NFTs) (Ballatore*, et al.*, 2007). During the process, glycation of tau may occur. The glycated tau can generate reactive oxygen species (ROS) and damage the neurons. The large NFTs can disrupt cellular functions, like axonal transport. Moreover, NFTs can bind to and trap more tau and other essential intracellular proteins. Thus, the tauopathies were trapped in the vicious cycle and the loss of essential intracellular proteins would lead to neurodegeneration.

1.1.4.2.3 Inflammatory Response to Amyloid Plaques and Apoptotic Neurons

Microglias are macrophage-like cells inside the brain, which are responsible to fight against brain injury and infections (Liu and Hong, 2003). During AD condition, they are activated by amyloid plaques and apoptotic neurons and produce inflammatory response. The microglias produce ROS, inflammatory cytokines (e.g. Tumor necrosis factor-α (TNF-α) and Tumor necrosis factor-β(TNF-β)) and toxin (Solito and Sastre, 2012). The non-specific immune response also takes place, which the complement system produces anaphylatoxins and recruits other inflammatory cell (Arumugam*, et al.*, 2004). The inflammation worsens the neurodegeneration process and leads to further progression of AD.

1.1.4.2.4 Death of Neuron and Memory Loss

The death of neurons firstly occurs in hippocampus, leading to the loss of episodic memory. That causes the AD patients to loss their recently acquired

memories about their own experience. Neurons in anterior and inferolateral temporal lobes, and the frontal lobes were also degenerating in AD pathology, leading to the semantic memory loss (Gold and Budson, 2008). The loss of semantic memory would present as the inability to name the common items as this memory system is responsible for the storage of general conceptual and factual knowledge. As the degeneration spread, it would affect the connectivity between neurons in frontal and parietal cortical region, causing the loss of working memory. The patient would demonstrate an inability to concentrate or pay attention, as working memory is responsible for storing information for seconds for further information reasoning and comprehension. Therefore, the early Alzheimer's disease symptoms were caused by these memory losses.

In later stage of disease, the degeneration of neuron would spread throughout the brain. Memory losses of other types, like procedural memory, take place. These memory losses lead to a more severe and extensive symptoms of late stage AD.

1.1.4.3 Current Western Diagnosis, Management and Medication

1.1.4.3.1 Diagnosis

Although the pathology of AD is known to be related to beta-amyloid plaque and misfolded tau protein, we cannot obtain these pathological evidences in potential AD patient and make the diagnosis. From diagnostic and statistical manual of mental disorders (2004) (American Psychiatric Association. and American Psychiatric Association. Task Force on DSM-IV., 1994), diagnosis of

AD could be done by a diagnosed dementia, with the exclusion of other causes.

Diagnosis of dementia can be achieved by several criterions as followings:

i. Memory impairment (impaired learning ability and forgetting newly learnt things). The learning ability can be tested by asking the patient to learn words. The ability to register the word to memory is assessed by asking the patient to repeat the word. The ability to retain and recall the memory is assessed by asking the patient for the word after a few minutes. The recognition ability is assessed by asking the patient to recognize the word in a list. The long term memory can be tested by asking the patient to recall his/her personal information. Failing to do these indicates memory impairment.

ii. Impaired language function (aphasia). Language function can be tested by asking the patient to name the items in the room, or his/her body parts, to follow simple orders (point at one item in the room then another) or to repeat short phrases.

iii. Difficulties in executing motor activities with normal motor function (apraxia). Motor activities can be tested by asking the patient to execute simple activities (brushing teeth, combing hair or stacking blocks)

iv. Impaired visual or touch recognition with normal sensory function (agnosia). Recognition ability can be tested by asking the patient to recognize objects (pen, table) or his/her family.

v. Impaired executive function (ability to think, plan, start and stop complex behaviour). The ability can be tested by asking the patient to find similarities

or differences between words, counting 1 to 10 or a to z, etc.

The above criterions must be severe enough to affect the social life and working of the patient. The decline must also be faster than normal aging when comparing with the previous functioning of the patient, in order for he/she to have diagnosed to be dementia. The diagnosis must also exclude delirium, which is an acute decline in cognitive ability that may not involve neurodegeneration.

After the diagnosis of dementia, the diagnosis of Alzheimer's disease must include a gradual but continuous decline in cognitive ability. In addition, the cognitive defects are not due to other nervous system conditions (Parkinson's disease, Huntington's disease, cerebrovascular disease or brain tumour), dementia caused by systemic disorders (vitamin B12 deficiency, HIV infection or hypothyroidism), persisting alcohol or drug abuse or other psychiatric conditions, like Schizophrenia or major depression.

By computed tomography (CT) or magnetic resonance imaging (MRI), brain atrophy, with wider cortical sulci and larger cerebral ventricles, would be observed in most cases. This observation is not specific for Alzheimer's disease. But it is good for excluding dementia by stroke or tumours (http://www.adni-info.org/ADNIStudyProcedures/MRIScans.aspx).

In 2004, researchers at the University of Pittsburgh, Pennsylvania, USA, had developed an analog of beta-amyloid binding thioflavin T, named as Pittsburgh

compound B (PiB). PiB can tag beta-amyloid plaque in living human brain, which can be detected by positron emission tomography (PET) (Figure 1.3) (Klunk*, et al.*, 2004). This technology can be used to detect the presence of beta-amyloid plaque in suspected AD patient, which may be applied as a diagnostic tool in the near future.

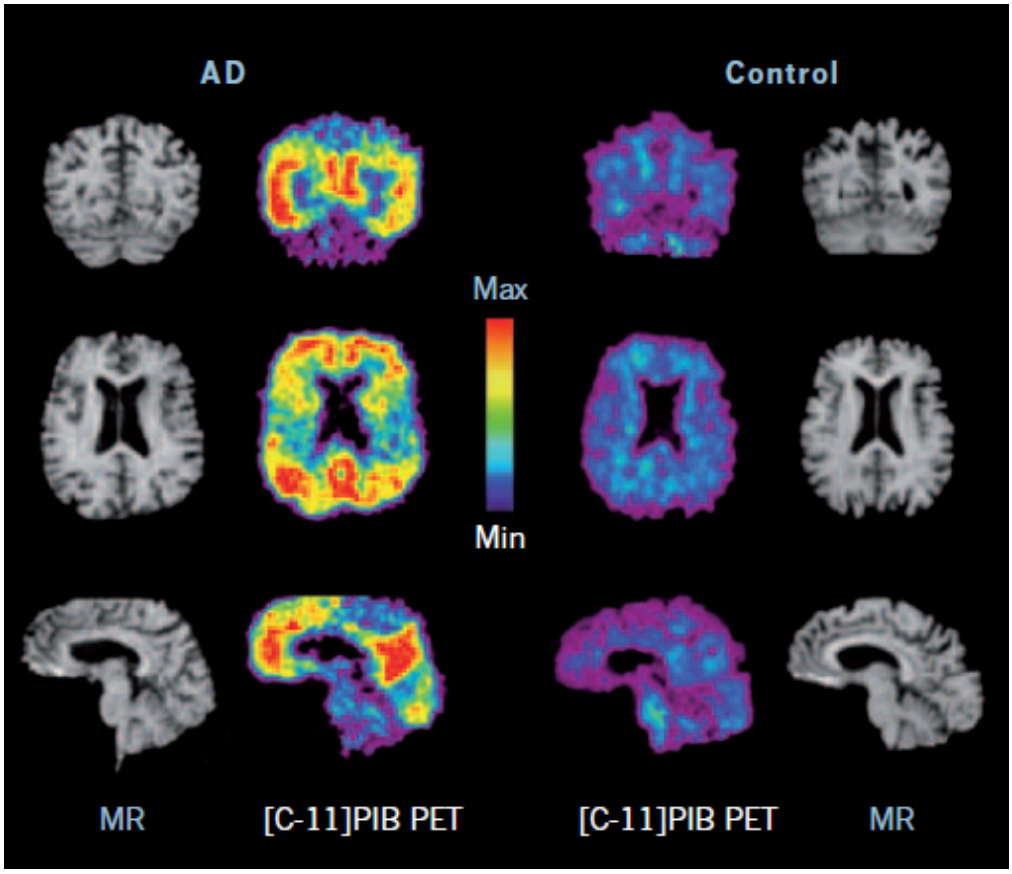


Figure 1.3 Pittsburgh compound B (PiB) lights up amyloid plaques in positron emission tomography (PET) images of the human brain (Williams, 2011).

1.1.4.3.2 Management of Alzheimer's disease

After the diagnosis of the disease, medical, physical and psychological

managements of the patients and their families are important to optimize their quality of life. Here are some managements (modified from http://www.alz.org/professionals_and_researchers_14298.asp)

i. Encourage them that life does not end with a diagnosis of Alzheimer's disease, due to the fact that patients' and families' attitude greatly affect the progression of the disease.
ii. Make the patients and families know about details for the changes in cognition and behavior. Knowing those information help them plan and ready to cope with the situation that may come.
iii. Address safety issues, including the home environment, driving and wandering. As falling and accident at home are the often cause of death for Alzheimer's patient, modification of home environment is necessary to accommodate the changes of the patients in cognition and behavior.
iv. Understand approaches to keeping the patient independent as long as possible, as the self-esteem of the patients are also important and activity may help slowing the cognitive decline (Deweerdt, 2011).

1.1.4.3.3 Current Medication

There is no cure for Alzheimer's disease. However, drugs are available to slow down the neurodegeneration and thus the progression of the disease. The main focus of current treatment is to reduce the speed of decline in cognitive ability. The two currently used groups of drugs are cholinesterase inhibitors and *N*-methyl d-aspartate (NMDA) receptor antagonists used to treat cholinergic

deficits and excitotoxicity.

a) Cholinesterase Inhibitors

Cholinesterase is the enzyme responsible for the breakdown of acetylcholine (ACh), which is the neurotransmitter in central nervous system (CNS) and peripheral nervous system (PNS). It is important in CNS for memory. Cholinergic deficit leads to memory impairment as mentioned above. In Alzheimer's disease patients (Winblad*, et al.*, 1985), the concentration of ACh can drop by 90%, which certainly cause impairment in memory and other brain functions. Cholinesterase inhibitors are found to retain the level of ACh, and therefore improve the brain functions. These inhibitors show significant effect on slowing down the disease at the mild stage. The approved ACh inhibitors are donepezil (Aricept®), rivastigmine (Exelon®), or galantamine (Razadyne®) (Table 1.1). Since ACh have functions in other systems, the general side effects of these drugs are related to the increase in ACh level, such as bradycardia, hypotension, and bronchoconstriction, etc (Table 1.1).

b) NMDA Receptor Antagonists

As mentioned above, excessive excitation of NMDA receptor by NMDA or glutamate causes damage on nerve cells. The process is called excitotoxicty. NMDA receptor antagonist can prevent the binding of NMDA or glutamate to the receptor and prevent excitotoxicty. Therefore, the damage and death of nerve cells are reduced and cognitive ability is improved. The approved NMDA receptor antagonist is memantine (Namenda®) (Table 1.1) (Reisberg*, et al.*, 2003).

Table 1.1 Overview of Approved Drugs for Alzheimer's Disease Treatment

Name	Indications	Mechanism	Side Effects
Donepezil (Aricept®)	Mild to severe Alzheimer's disease	Reversible inhibition of acetylcholinesterase	CV: Vasodilation, atrial fibrillation, hot flashes, hypotension GI: Fecal incontinence, gastrointestinal bleeding, bloating, epigastric pain CNS: Delusions, tremor, irritability, paresthesia, aggression, vertigo, ataxia, increased libido, restlessness, abnormal crying, nervousness, aphasia Resp: Dyspnea, sore throat, bronchitis
Rivastigmine (Exelon®)	Mild to moderate Alzheimer's disease	Reversible inhibition of acetylcholinesterase	GI: Constipation, gastritis CNS: Tremor
Galantamine (Razadyne®)	Mild to moderate Alzheimer's disease	Reversible inhibition of acetylcholinesterase	GI: Flatulence CNS: Aggression, lethargy, dysgeusia, hypersomnia

Memantine (Namenda®)	Moderate to severe Alzheimer's disease	Low to moderate affinity uncompetitive blocking of NMDA receptor	CV: Cardiac failure Blood: Anemia CNS: Transient ischemic attack, cerebrovascular accident, vertigo, ataxia, hypokinesia, aggression Resp: Pneumonia

1.1.4.4 New Approach on Drug Development against Alzheimer's Disease

The traditional approach was actually not a specific treatment for Alzheimer's disease. And none of the current drug can stop the progression of AD. To deal with AD once and forever, researcher started to target at the root – the beta-amyloid plaque and aggregated tau.

1.1.4.4.1 Targeting Amyloid

Evidences suggested that beta-amyloid plaque is highly involved in the pathogenesis of AD (Hardy and Selkoe, 2002, Mattson, 2004). Therefore, therapeutic researches on this arm mainly focus on two processes – the beta-amyloid protein cleavage and aggregation of beta-amyloid protein into plaque.

As mentioned above, γ- and β-secretase cleave the amyloid precursor protein (APP) into beta-amyloid protein. If we inhibit γ- and β-secretase cleavage of APP, no beta-amyloid protein can be produced. However, γ-secretase is also involved in the cleavage of other proteins, including Notch, which controls the development and differentiation of cells and is vital. The γ-secretase inhibitor was even found to promote the neurodegeneration in clinical trial (Gravitz, 2011). β-secretase is more specific to APP. A recent report in Nature also showed that a mutation of APP on β-secretase binding site in Icelandic population reduce the chance of suffering from Alzheimer's disease (Callaway, 2012). However, the active site for the enzyme is large. Difficulties arise on creating an inhibitor that is large enough to block the active site, while small enough to pass through the blood-brain barrier.

But this is certainly a target for further drug development.

Another way to prevent the toxicity is to prevent the aggregation of beta-amyloid protein into plaque. Conventional way of research is to prevent the aggregation of beta-amyloid protein by chemicals, namely *scyllo*-inositol and PF-04494700, which were undergoing their clinical trials. There is a more revolutionary approach that use vaccines containing antibodies to bind with beta-amyloid protein, which were in their phase III trial (solanezumab and bapineuzumab) (Gravitz, 2011). The vaccines were effective on clearing the plaque. However, the cognitive degeneration was not recovered. The possible reason behind is controversial – either the damage done is in an irreversible level, or the beta-amyloid oligomer is also a neurotoxic agent. Without clear picture of the interaction between beta-amyloid protein and the pathogenesis events of the disease, the drug development on this target would be tough.

1.1.4.4.2 Aiming at Tau and Neurofibrillary Tangles (NFTs)

Another arm of research focus is on Tau, which was mentioned to be hyperphosphorylated in AD and aggregate inside neurons to form NFTs. The directions are to prevent the addition of phosphate group to Tau, mainly by inhibition of the enzyme. A drug that inhibits GSK-3, which is the enzyme responsible for the addition of the phosphate group to Tau, was undergoing clinical trial. The main difficulties of this arm are the little understanding on Taupathies, the essential role of Tau on normal cells, which makes total inhibition of Tau impossible, and the overwhelming focus on beta-amyloid protein, which

reduced funding and research manpower on this arm.

1.1.4.4.3 New Function of the Old Medication

Conventional treatments focus on slowing the degeneration of neurons in a non-specific manner. There are also researches on this old path, which find new ways to protect the neurons. It is known that nerve growth factor (NGF) is essential for the living of neurons. Gene therapy using an adenovirus to deliver the gene of NGF to the most affected part via surgery, named CERE-110, is the example of how new method born from the traditional logic (Mandel, 2010). Of course, there is also the try-and-error research on old neuroprotective drugs. Some success is seen on Dimebon, which is an old drug developed in 1983 (Table 1.2).

Although the disease was not well-understood, the above target provided good starting point for researchers to deal with the disease. From the complexity of AD, a multi-targeting drug, which functions like the cocktail therapy in AIDS, may give a better effect. Therefore, there is plenty of room and target that we can choose to study.

Table 1.2 Drugs undergoing Clinical Trials in 2011 (Gravitz, 2011)

Drug	**Trial status**	**Mode of action**	**Developer**
Bapineuzumab	Phase III, ongoing	Humanized monoclonal antibody to amyloid-β; targets the peptide's N-terminus	Pfizer/ Janssen
Solanezumab	Phase III, ongoing	Humanized monoclonal antibody to amyloid-β; targets the centre of the peptide	Eli Lilly
Intravenous immunoglobulin (IVIg)	Phase III, ongoing	Isolated from pooled human blood, believed to have anti-amyloid-β and anti-inflammatory properties	Baxter
Latrepirdine (Dimebon)	Phase III, ongoing	Thought to stabilize mitochrondria, thereby protecting neurons and preventing them from malfunctioning	Pfizer/ Medivation
Scyllo-inositol /ELND 005	Phase II completed, Phase III in planning	Prevents or inhibits amyloid-β aggregation	Elan
Methylthioninium chloride (Rember)	Phase II completed, Phase III in planning	Unclear; thought to inhibit tau aggregation, but may be acting as an anti-amyloid-β disaggregator	TauRx Pharmaceuticals

CERE-110	Phase II, ongoing	Adenovirus-aided delivery of a nerve growth factor gene that helps protect neurons; delivered via surgery	Ceregene
PBT2	Phase IIb in planning	Metal chelator, small molecule that inhibits tau hyperphosphorylation and amyloid-β aggregation	Prana Biotechnology
Davenutide/AL-108	Phase II completed	Microtubule stabilizer, preventing tau hyperphosphorylation and tangle formation	Allon
BMS-708163	Phase II, ongoing	Inhibits formation of γ-secretase, thereby inhibiting formation of amyloid-β	Bristol-Myers Squibb
PF-04494700/ TTP488	Phase II, ongoing	RAGE inhibitor, modulates glial activity and reduces amyloid-β plaque formation	Pfizer
Tideglusib/NP-12 (Nypta)	Phase II, ongoing	GSK-3 inhibitor, preventing tau hyperphosphorylation	Noscira

1.2 Introduction of Traditional Chinese Medicine

1.2.1 Dementia and Alzheimer's Disease in Traditional Chinese Medicine

Dementia was recorded in Traditional Chinese Medicine literature for a long history, mainly as impairment in thinking, memory and language, which is not congenital. It was named as "善忘、健忘、好忘、喜忘" in various literature. Below were the abstracts from the ancient records:

i. 《素問・四時刺逆從論》："秋刺經脈，血氣上逆，令人善忘"

ii. 《素問・五常政大論》："太陽司天，寒氣下臨，心氣上從……熱氣妄行……善忘，甚則心痛。"

iii. 《素問・調經論》："血並於下，氣並於上，亂而喜忘。"

iv. 《靈樞・大惑論》："上氣不足，下氣有餘，腸胃實而心肺虛。虛則營衛留於下，久之不以時上，故善忘也。"

v. 《傷寒論》："陽明證，其人喜忘者，必有蓄血，所以然者，本有久瘀血，故令喜忘。"

vi. 《肘後方》："療人心孔滑塞多忘喜誤。"

vii. 《太平聖惠方》："夫心者，精神之本，意智之根，常欲清虛，不欲昏昧，昏昧則氣濁，氣濁則神亂，心神亂則血脈不榮，氣血俱虛，精神離散，恒多憂慮，耳目不聰，故令心智不利而健忘也。"

viii. 《奇效良方》："且如健忘者，陡然而忘其事也，皆主於心脾二經。蓋心之官則思，脾之官亦主思，此由思慮過矣。傷於心則血耗散，神不守舍；傷於脾則胃氣衰憊，而慮愈深。二者皆令人事則卒然而遂忘也。蓋心主血，因血少不能養其真臟，或停飲而氣鬱以生痰，氣既滯，脾不得舒，是病皆由此作。"

These records pointed out that dementia were caused by the blocked circulation of blood and "qi" in heart. From modern point of view, the "heart" in Traditional Chinese Medicine may mean brain if it is related to thinking. So, these descriptions may mean the blockage of blood in brain, which is similar to the vascular dementia in modern western medicine.

On the other hand, name and pathology of AD is not yet established in classic Chinese Medicine. In modern Chinese Medicine point of view, it is similar to "呆病、文痴、善忘、鬱症、癲症".

From symptomatic approach, the pathology can be due to:

i. 肝腎虛

ii. 臟腑功能失調，氣血虧虛，腦髓失養

iii. 心肝火旺，神明被擾 (魏凱峰, 2001)

iv. 痰瘀阻滯，清竅失靈 (胡海燕, *et al.*, 2006)

Therefore, the treatment approach can be

i. Normalizing function of liver, heart, spleen and kidney

ii. Reinforcing the weakness of the body

iii. Tranquilize the mind

iv. Promote blood circulation

v. Clearing the sputum

The main Chinese Medicines that were investigated is based on these criteria. Ginkgo Semen (銀杏), Ginseng Radix et Rhizoma (人參), Lycii Fructus (枸杞子),

Poria (茯苓), Polygoni Multiflori Radix (何首烏), Rehmanniae Radix et Rhizoma (地黃), Acori Tatarionwii Rhizoma (石菖蒲), Cuscutae Semen (菟絲子), Curcumae Longae Rhizoma (薑黃), *Huperzia serrata* (千層塔), are examples of the widely investigated herbs for neuroprotection or Alzheimer's disease. Single active component was isolated from some of these herbs and are undergoing further investigations or clinical trials. These findings show the potential of Chinese Medicines in the treatment of dementia and Alzheimer's disease.

1.2.2 Traditional Chinese Medicines as an Complementary or Alternative Medicine

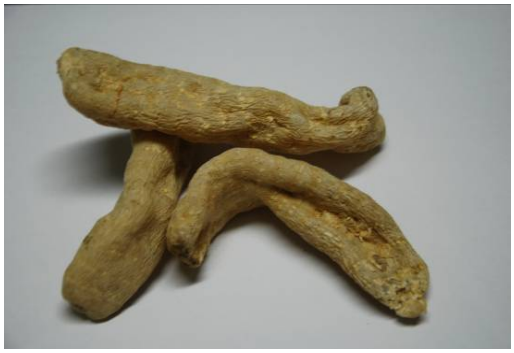
Complementary and alternative medicine (CAM) is a group of diverse medical and health care systems, practices, and products that are not generally considered to be part of conventional medicine. Complementary medicine is used together with conventional medicine, while alternative medicine is used to replace conventional medicine (National Center for Complementary and Alternative Medicine, 2008). Traditional Chinese Medicine (TCM) falls into the type of whole medical systems, which is defined as system that is built upon complete systems of theory and practice (National Center for Complementary and Alternative Medicine, 2009). Western medicine is effective mostly on specific disease, and acting through simple pathways. However, when the pathology is more complicated, Western medicine becomes ineffective and defective. The disappointment leads to the use of CAM (Leung*, et al.*, 2003).

In United States, an estimated 3.1 million US adults had used acupuncture in

the previous year. In the same study, natural product, like herbs, is the top alternative therapy used, with 17 percent of US adult using it (Bethesda, 2009). In China, Chinese medicine hospitals also provide diagnostic and treatment services in Western medicine (Leung*, et al.*, 2003). Some hospital of integrated therapy use TCM as treatment in the problem areas of Western medicine, such as vascular diseases, dermatitis and rheumatology. Despite of the widespread use of TCM, National Center for Complementary and Alternative Medicine stated that "Although TCM is used by the American public, scientific evidence of its effectiveness is, for the most part, limited." (National Center for Complementary and Alternative Medicine, 2009). Therefore, scientific researches were widely done to identify the efficacy and mechanisms of action of TCMs.

1.2.3 Introduction to the Selected Chinese Herbal Medicines

The selection of target Chinese herbal medicine was done according to Chinese medicine point of view. The selected Chinese herbal medicine should function as the one or more of approaches that listed in section 1.2.1, and should be used for brain-related disease in traditional use. After the selection, literature search was done to confirm the selected Chinese herbal medicine possess some neuroprotective effects in modern scientific research, but was not studied for their effect on treatment of Alzheimer's disease or related pathology. Based on these criteria, four Chinese herbal medicines were selected in our study, which are Tianma, Gouteng, Chuanxiong and Yuanzhi (Figure 1.4). Their traditional use in Chinese medicine practice and their biological functions in modern scientific studies would be discussed below.



Tianma

Gouteng

Chuanxiong

Yuanzhi

Figure 1.4 Photo of the selected Chinese herbal medicines

1.2.3.1 Tianma (Gastrodiae Rhizoma)

Gastrodia elata Blume., which is called Tianma (天麻) in Chinese, is widely used for treating head-related disease in China. Its rhizome is used. In the perspective of traditional Chinese medicine, Tianma is sweet, neither warm nor cool and belong to liver circulation. It has the function of tranquilizing the wind, stopping convulsion, normalizing the excessive "Yang" condition in liver, clearing the wind and circulation. It was firstly recorded in《神農本草經》as "久服益氣力，長陰，肥健，輕身，增年。". A better description of the modern use of Tianma

was recorded in《本草匯言》as "主頭風，頭痛，頭暈虛旋，癲癎強痙，四肢攣急，語言不順，一切中風，風痰。". It is used for treatment of convulsion and epilepsy. It can be used to treat numbness of limbs. It is also used for treatment of dizziness and headache, which is more relevant to neuroprotection (Lei*, et al.*, 1995). Traditionally, it is also commonly used for making a soup consisting of Gastrodiae Rhizoma, Chuanxiong Rhizoma, Angelicae Dahuricae Radix and head of fish to clear the wind from the head.

In scientific studies, Tianma also have various biological activities. Phenolic extract of Tianma and its active components, vanillin and p-hydroxybenzaldehyde, can protect neuron-like cell line from glumate-induced toxicity (Huang*, et al.*, 2006). Water and ethanolic extract of Tianma are proven to have anti-convulsive effect on kainic acid-treated epilepsy in rats (Hsieh*, et al.*, 2001). Its active components, vanillyl alcohol and 4-hydroxybenzaldehyde, also improved ferric chloride (Hsieh*, et al.*, 2000) and pentylenetetrazole- induced (Dai and Yu, 2002) epilepsy in rats, respectively. Water extract of Tianma was also found to have anti-depressant-like effect in rats (Chen*, et al.*, 2008). The methanolic extract of Tianma and its active components, gastrodin and p-hydroxybenzyl alcohol, improved scopolamine-induced memory loss (Wu*, et al.*, 1996). Tianma and its active components' anti-oxidative (Jung*, et al.*, 2007, Kim*, et al.*, 2011, Shin*, et al.*, 2011) and anti-inflammatory (Hsieh*, et al.*, 2005) effects may account for their function. But effect of water extract of Tianma on Alzheimer's disease and exictotoxicity was still unseen. This leaves a room for us to investigate and contribute to the full picture of neuro-function of Tianma.

1.2.3.2 Gouteng (Uncariae Ramulus cum Uncis)

Uncaria rhynchophylla, which is called Gouteng (鉤藤) in Chinese, is prescribed for treatment of cardiovascular or central nervous system disease in China. Its stem with hooks is used. In the perspective of traditional Chinese medicine, Gouteng is sweet, a bit cold and belong to liver and heart surroundings circulation. It has the function of tranquilizing the wind, stopping convulsion, clearing the heat and normalizing the condition in liver. It was firstly recorded in 《別錄》as "主小兒寒熱，十二驚癇。". A better description of the modern use of Gouteng was recorded in《本草綱目》as "鉤藤，手足厥陰經藥。足厥陰主風，手厥陰主火。驚癇眩運，皆肝風相火之病。鉤藤，通心包於肝木，風靜火息，則諸證自除。或云：入數寸於小麥中蒸熟，餵馬易肥。". It is mainly used for treatment of numbness and convulsions. It also has the function to treat headache and lightheadedness, which may be contributed by its anti-hypertensive effect (Lei*, et al.*, 1995). There is also a compound formula that consists of Gouteng and Tianma, which is used to treat hypertension. The main active component of Gouteng, rhynchophylline, is heat-labile. So, this herb should be extracted by heat for less than 20 minutes.

Scientific studies of Gouteng also proven to have anti-convulsive effect on kainic acid-treated epilepsy in rats (Hsieh*, et al.*, 1999). It also protects rat brain slices against N-methyl-D-aspartate-induced toxicity (Lee*, et al.*, 2003). Recent reports showed that ethanolic extract of Gouteng cotreatment can improve D-galactose-induced cognitive defects (Xian*, et al.*, 2011). Additionally, the

active components of Gouteng, rhynchophylline and isorhynchophylline, showed protective effect on beta-amyloid protein-induced toxicity (Xian*, et al.*, 2012). But still, the water extract of Gouteng was never studied in Alzheimer's disease-related field. This leaves room for the investigation.

1.2.3.3 Chuanxiong (Chuanxiong Rhizoma)

Ligusticum chuanxiong Hort., which is called Chuanxiong (川芎) in Chinese, is widely used as drug to promote circulation. Its rhizome is used. In the perspective of traditional Chinese medicine, Chuanxiong is spicy, warm and belong to liver, gallbladder and heart surrounding circulation. Its main function includes promoting blood and "qi" circulation, clearing the wind and painkilling. It was firstly recorded in《神農本草經》as "主中風入腦，頭痛，寒痹，筋攣急，金瘡，婦人血閉無子。", which describes the modern use of Chuanxiong. Chuanxiong was mainly used for pain caused by accumulation of blood and "qi". It is very famous for its function in obstetrics and gynaecology. Its functions vary from treating menstrual disorder and pain, to clearing accumulated blood clot after delivery. It also has cardiovascular effect on angina pectoris. The neuro-related functions include the treatment of ischemic stroke or headache. A compound formula of Chuanxiong and Tianma was also recorded in the Chinese Pharmacopeia 2010, which serves a similar function (Chinese Pharmacopoeia Commission, 2010).

Chuanxiong was the most commonly investigated herb among our four selected herbs. However, the researches were ranged from analgesic (Gao and Xu,

2010), anti-fibrotic (Wu, *et al.*, 2011), anti-inflammation (Or, *et al.*, 2011), anti-oxidation (Jiang, *et al.*, 2011, Ramalingam and Yong-Ki, 2010) to neuroprotection. In neuroprotection aspect, ethanolic and butanolic extracts of Chuanxiong were able to protect neuron-like cell line from serum deprivation-induced apoptosis. Its active component, tetramethylpyrazine, was able to improve kainite and scopolamine-induced neurotoxicity in rats.

1.2.3.4 Yuanzhi (Polygalae Radix)

Polygala tenuifolia Willd., which is called Yuanzhi (遠志) in Chinese, is widely used for treating suptum-related disease in China. Its root is used. In the perspective of traditional Chinese medicine, Yuanzhi is bitter and spicy, a bit warm and belong to heart, kidney and lung circulation. It has the function of tranquilizing the heart and the mind, clearing sputum, opening the mind and relieving the swelling. It was firstly recorded in《神農本草經》as "主咳逆傷中，補不足，除邪氣，利九竅，益智慧，耳目聰明，不忘，強志，倍力。". A better description of the modern use of Yuanzhi was recorded in《藥品化義》as "遠志，味辛重大雄，入心開竅，宣散之藥。凡痰涎伏心，壅塞心竅，致心氣實熱，爲昏聵神呆、語言蹇澀，爲睡臥不寧，爲恍惚驚怖，爲健忘，爲夢魘，爲小兒客忤，暫以豁痰利竅，使心氣開通，則神魂自寧也。". It is mainly used for treatment of insomnia or memory impairment due to nervous conditions, which is similar to dementia. It can be used to treat manic disorder due to sputum. Back to basic, it can also be used for coughing due to accumulation of sputum. It is also effective on relieving pox or swelling (Lei, *et al.*, 1995).

Scientific researches on Yuanzhi were mainly on neuro-related topics. Butanolic extract of Yuanzhi possessed protective effect on scopolamine-induced memory impairment in rat (Sun*, et al.*, 2007). Ethanolic extract of Yuanzhi protect rat primary neuron against neurotoxic agents (Park*, et al.*, 2002). The active component of Yuanzhi, tenuifolin and tenuigenin, can even inhibit the beta-amyloid protein secretion *in vitro* (Jia*, et al.*, 2004, Lv*, et al.*, 2009, Naito and Tohda, 2006). Therefore, Yuanzhi was chosen as the candidate to be investigated.

1.3 Objectives of the Present Study

The objective of the present study was to investigate the effects of the 4 selected Chinese medicines on dementia, in order to find potential herb as the complementary or alternative medicine for treatment of Alzheimer's disease and other types of dementia.

The main objectives include:

i. Extraction of selected TCM according to the standardized extraction protocol. Screening and determination of effects of the TCM extracts on reduction of beta-amyloid induced toxicity *in vitro*. The underlying anti-apoptotic and anti-oxidative effects would be determined.

ii. Investigation of potential anti-cholinesterase and anti-excitotoxic effect of the TCM extracts on primary cortical neurons, which are the traditional treatment approach of AD.

iii. Establishing the Alzheimer's disease *Drosophila* model to test the efficacy of selected TCM with prominent effect on *in vitro* system. Behavioural and phenotypical test would be focused. The effect can establish the relationship between the anti-beta-amyloid induced toxicity *in vitro* and the improvements in behaviours and phenotypes.

iv. Testing the abilities of effective TCM on other dementia model, such as traumatic brain injury model on rat, which explore the possibility of extending the use of the effective TCM on different neurodegenerative diseases.

1.3.1 Research Plan

In the present study, the Chinese herbal medicine would be extracted with water, as according to the traditional Chinese medicine extraction method. After that, the extract would be authenticated by thin layer chromatography. There will be 4 platforms used in the present study. The in vitro PC12 cell line platform will screen the most effective candidate against beta-amyloid-induced cytotoxicity, and neuroprotective mechanism of it would also be elucidated in aspects of apoptosis, oxidative stress and taupathies. After that, the effective candidate would be used on the beta-amyloid-expressong *Drosophila* platform. The in vivo effect of effective candidate against the beta-amyloid-induced neurodegeneration will be tested on this animal model. The shortened lifespan, impaired locomotion and the degeneration of ommatidia would reflect the degree of neurodegeneration, and the treatment of effective candidate should be able to reduce these defects. The effective candidate will also be investigated using the primary cortical neurons extracted from neonatal rats. The primary neurons have high proximity in properties with the neurons in human brain. Thus, the protective effect of the effective candidate would be validated by this platform. Furthermore, the effect of the effective candidate against excitotoxicity and acetylcholinesterase activity will be investigated, which both of them play an important role in the pathology of Alzherimer's disease. Lastly, the effect candidate will be used to treat the rat with traumatic brain injury, which is a model of other type of dementia. The neurodegeneration of the model would be assessed by their locomotor activity, as the motor region of the brain was seriously damaged by the trauma. With all the above platforms, the effect and action mechanisms of potential herb for treatment

of Alzheimer's disease and other types of dementia can possibly be elucidated (Figure 1.5).

TCM on Alzheimer's Disease and Other Types of Dementia

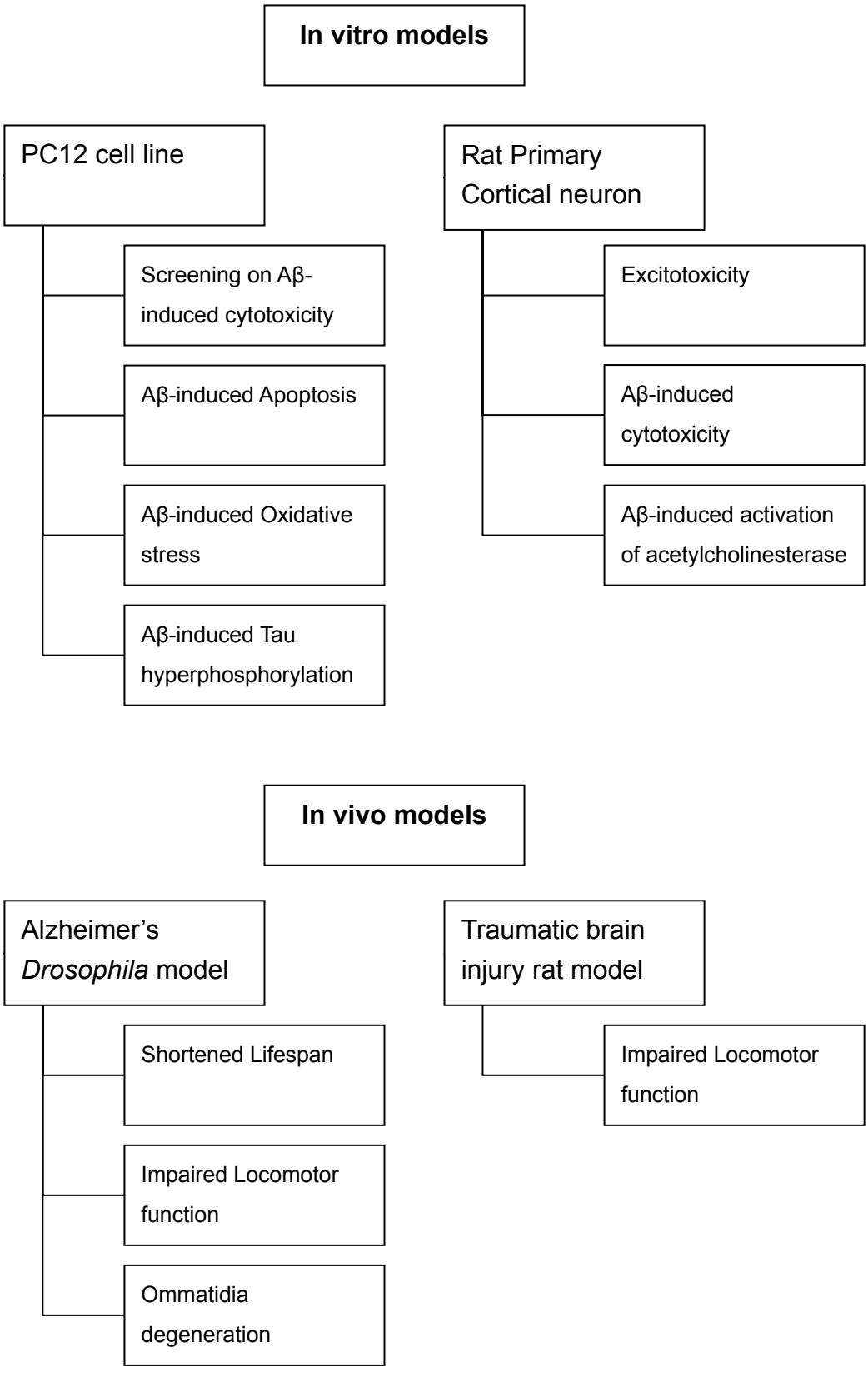


Figure 1.5 Research plan of the present study

Chapter 2
Herb Authentication and General Methodology

2.1 Source and Authentication of Raw Herbs

Dried rhizome of *Gastrodia elata* (Tianma, abbreviated as TM), dried hook-bearing stem branches of *Uncaria rhynchophylla* (Gouteng, abbreviated as GT), dried rhizome of *Ligusticum chuanxiong* (Chuanxiong, abbreviated as CX), and dried roots of *Polygala tenuifolia* (Yuanzhi, abbreviated as YZ) were purchased from the herbal wholesale market of Sheung Wan, Hong Kong. Thin-layer chromatography (TLC) was then utilized to identify the herbs by checking against reference herbs and their corresponding chemical markers, according to Chinese Pharmacopoeia 2010 (Chinese Pharmacopoeia Commission, 2010). Voucher specimens were deposited at the museum of Institute of Chinese Medicine (ICM), the Chinese University of Hong Kong (CUHK), with the following voucher numbers (Table 2.1):

Table 2.1 Voucher numbers of the herbs used in the study

Name of Herbs	Voucher Specimen Numbers
Gastrodiae Rhizoma (天麻)	2010-3294
Uncariae Ramulus cum Uncis (鉤藤)	2010-3295
Chuanxiong Rhizoma (川芎)	2010-3292
Polygalae Radix (遠志)	2010-3293

With the authenticated herbs, extraction was performed. Extraction was carried out as below:

- Raw herbs were washed gently with tap water to remove dust and other contaminants

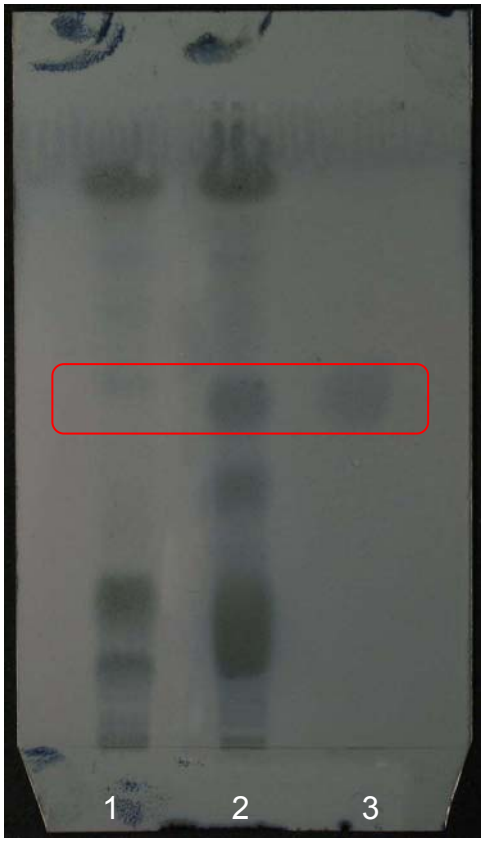
- The herbs were cut and 1 kg of each herb was weighed
- The herbs were extracted with water in 1:10 (w/v) under reflux for 1 hour, then the water extract was collected and the process was repeated for another hour
- The extracts were pooled and filtered
- The extract was concentrated using rotary evaporator under reduced pressure
- The concentrated extract was dried by lyophilization
- The dried powdered extract was stored in dry location

The extraction of GT was differed by the duration of first extraction shortened to 20 minutes, in order to preserve rhynchophylline . The extraction yield was listed in Table 2.2. The same batch of herbs and water extracts were used throughout the present study.

Table 2.2 Extraction yield of the herbs used in the study

Name of Herbs	Extraction Yield
Rhizoma Gastrodiae (天麻)	48.90 %
Ramulus Uncariae cum Uncis (鉤藤)	8.45 %
Rhizoma Chuanxiong (川芎)	37.73 %
Radix Polygalae (遠志)	34.16 %

Results below are the TLC authentication results for the four herbs. The herbs were extracted in methanol. Tianma was developed on the silica gel 60 F254 with developing system of ethyl acetate: methanol : water (7:3:1). The staining was done with 5% phosphomolybdic acid in 50% ethanol with heat. The photograph was taken under visible light (Figure 2.1). The TLC profile of our Tianma extract matched with the reference herb, and our Tianma extract contains the band representing gastrodin. Gouteng was developed with two methods. The first one was developed on the silica gel 60 F254 with developing system of chloroform : ethyl acetate (21:4) with 30% ammonia solution saturation. The staining was done with Dragendorff reagent, which was used for the detection of alkaloids. The photographs were taken under UV light at 254nm and 365nm (Figure 2.2). The second one was developed on the silica gel 60 F254 with developing system of chloroform : methanol : acetic acid (21:4: 4). The photos were taken under UV light at 254nm and 365nm. After that, the membrane was sprayed with 40% sulphuric acid and photographs were taken under UV light at 254nm and 365nm (Figure 2.3). The TLC profiles of our Gouteng extract from both methods matched with the reference herb. Chuanxiong was developed on the silica gel 60 F254 with developing system of n-hexane : ethyl acetate (3:1). The TLC profiles of our Chuanxiong extract matched with the reference herb. The photograph was taken under UV light at 254nm and 365nm (Figure 2.4). Yuanzhi was developed on the silica gel 60 F254 with developing system of chloroform : methanol : water (7:3:1). The photograph was taken under UV light at 254nm and 365nm (Figure 2.5). The TLC profiles of our Yuanzhi extract matched with the reference herb.



No	samples
1	Reference Herb
2	Tianma purchased
3	Gastrodin

Figure 2.1 TLC photo of Tianma taken under visible light (The band for gastrodin was indicated in red circle)

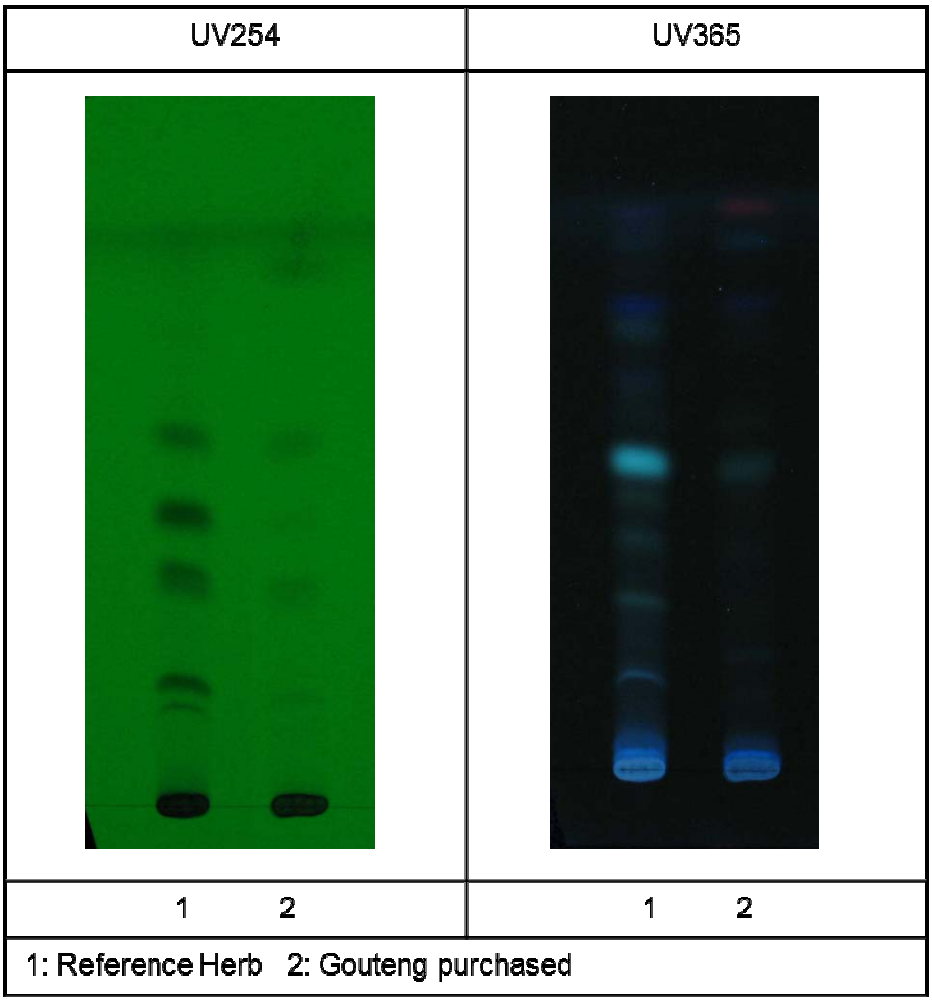


Figure 2.2 TLC photo of Gouteng stained with Dragendorff reagent taken under UV light

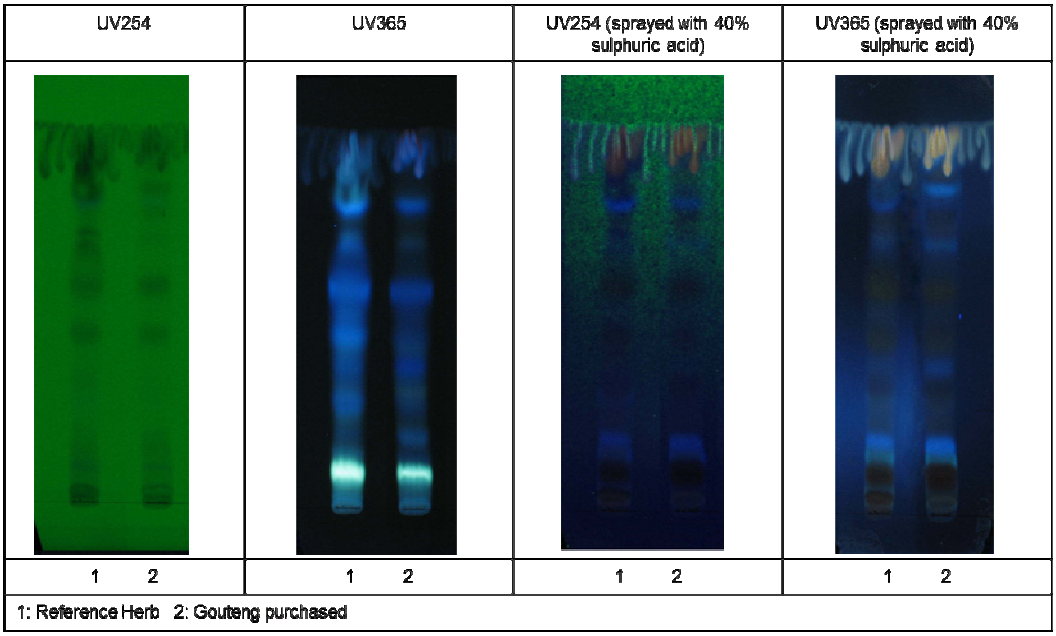


Figure 2.3 TLC photo of Gouteng taken under UV light

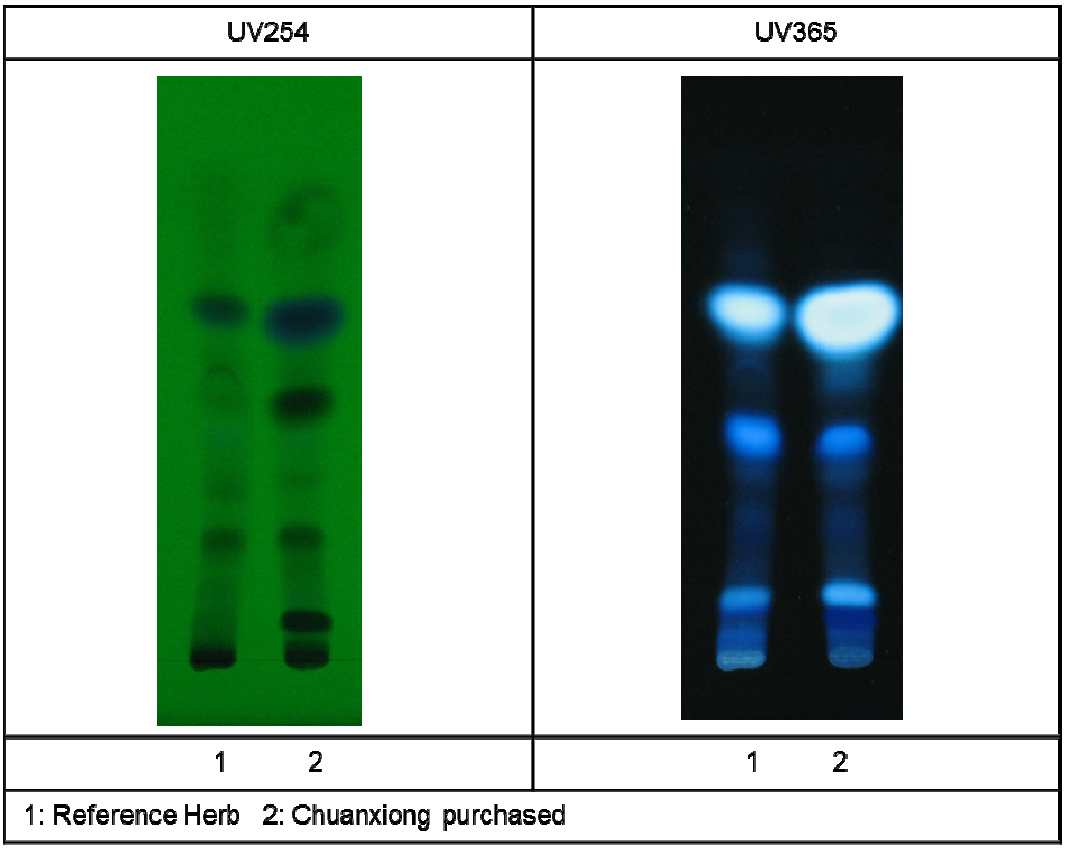


Figure 2.4 TLC photo of Chuanxiong taken under UV light

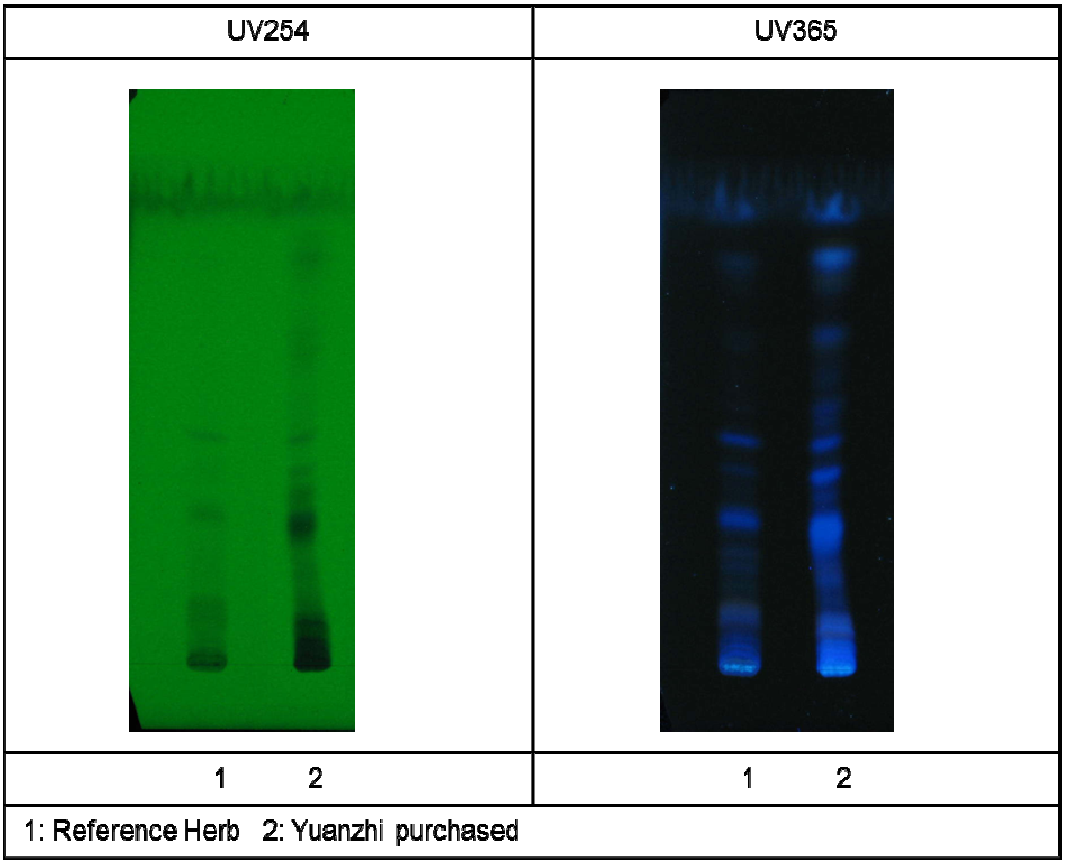


Figure 2.5 TLC photo of Yuanzhi taken under UV light

The quality control of Tianma was further determined by high-performance liquid chromatography (HPLC) quantification of gastrodin. The HP Agilent 1100 HPLC system consisted of an G1310A isocratic pump with solvent cabinet, G1328A manual injector, G1314A variable wavelength detector, G1316A thermostatted column compartment (Agilent Technologies, Santa Clara, CA, US), OptiPlex 760 desktop workstation (Dell, Round Rock, TX, US), and a Ultrasphere ODS C18 column (4.6mm×250 mm, 5μm, Beckman Coulter) (Figure 2.6). A mobile phase of acetonitrile-0.1% acetic acid (4.5:95.5) was applied, with a flow rate of 1.0 ml/min. The wavelength of the UV detector was set at 225 nm and column temperature was maintained at 33 ◦C. The HPLC profile of Tianma and gastrodin was shown in Figure 2.7A and Figure 2.7B respectively. The retention time was determined to be 5.5min. The linear curve showing the relationship between the area under the curve (AUC) of the HPLC profile of gastrodin standards and the concentration of gastrodin was plotted (Figure. 2.7C). From the graph, the concentration of gastrodin in our Tianma extract (1.00 mg/ml) was determined to be 22.26 μg/ml. The content of gastrodin is determined to be 2.226% in our extract, which is higher than the requirement (0.2%) in the Chinese Pharmacopeia 2010.

Figure 2.6 The HPLC system and the OptiPlex 760 workstation

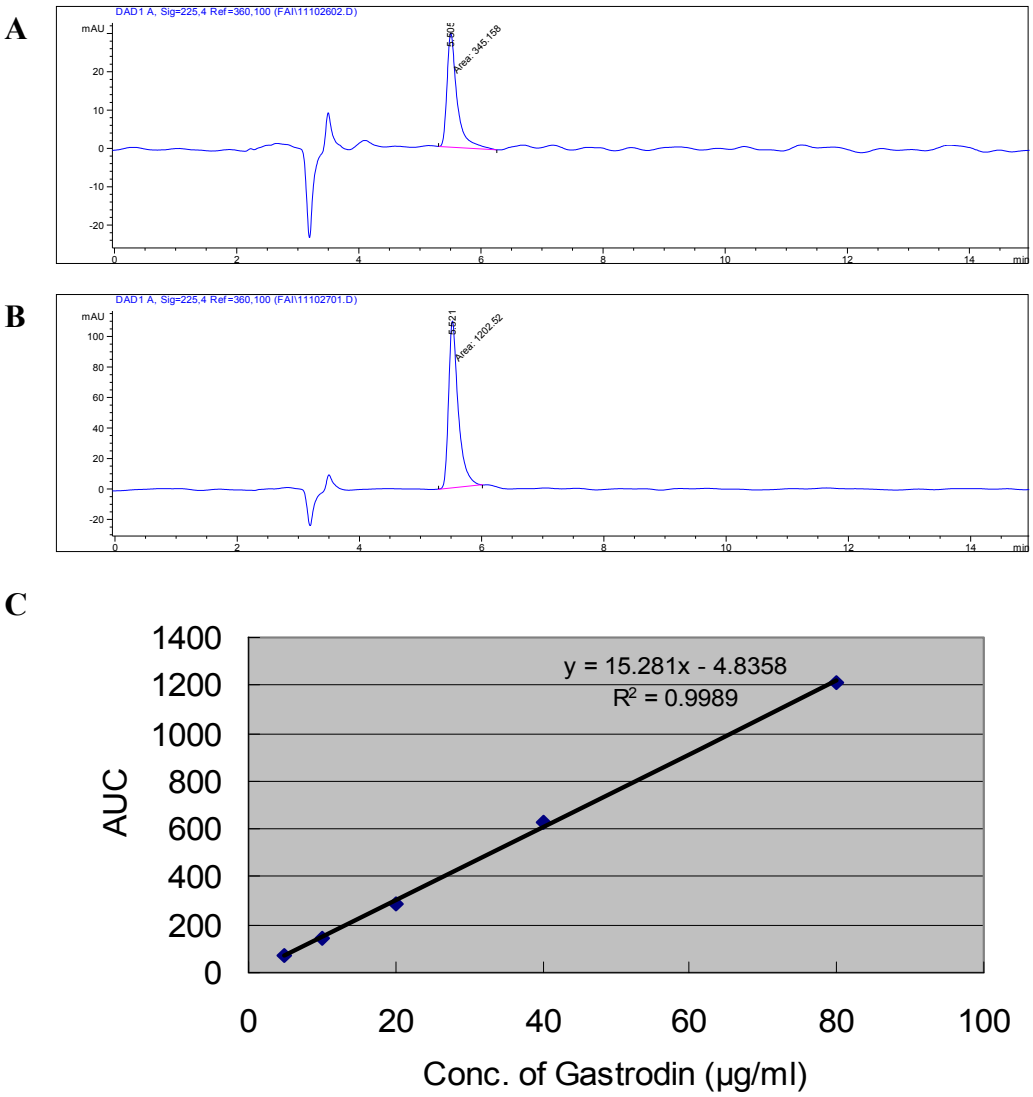


Figure 2.7 HPLC result on Tianma extract and gastrodin standard. (A) HPLC profile of Tianma extract. (B) HPLC profile of gastrodin. (C) Linear curve of AUC and concentration of gastrodin.

2.2 Materials

$A\beta_{25–35}$ peptide, 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT), caspase-3 assay kit and anti-β-actin antibody was purchased from Sigma-Aldrich (St Louis, MO, USA). RPMI medium 1640, neurobasal medium, B-27 serum-free supplement, GlutaMAX, fetal bovine serum (FBS), horse serum (HS) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Invitrogen (Carlsbad, CA, USA). Annexin V-FITC and propidium iodide were from BD Biosciences (San Jose, CA, USA). Superoxide dismutase and glutathione peroxidase assay kits were from Cayman Chemical (Ann Arbor, MI, USA). Catalase fluorometric detection kit was from Enzo Life Sciences (Faimingdale, NY, USA). PhosSTOP and cOmplete ULTRA tablets were from Roche (Penzberg, Germany). Antibodies anti-MAP2, anti-Bax, anti-Bcl-2, anti-cleaved caspase-3, anti-caspase-3, anti-rabbit IgG (HRP-linked) and anti-rabbit IgG (Alexa Fluor® 488 Conjugate) were from Cell Signaling Technology (Boston, MA, USA); and anti-Tau, anti-pTau, anti-mouse IgG (HRP-linked) and anti-goat IgG (HRP-linked) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immun-Star™ WesternC™ Chemiluminescence Kit and Precision Plus Protein Kaleidoscope standards were from Bio-Rads Laboratories (Hercules, CA, USA).

2.3 Ethical Approval

All animal studies were conducted under the license from Department of Health, Hong Kong Special Administrative Region of the People's Republic of China, and

the procedures were approved by the Animal Experimental Ethics Committee of the Chinese University of Hong Kong (Ref: 11/052/MIS-4, 12/086/MIS-1, 13/031/MIS).

2.4 General Methods

2.4.1 Cell Viability Assay

Treated cells were incubated with 30 μl of 5 mg/ml MTT (final concentration, 1.5 mg/ml) for 4 h. The supernatant then was removed and 100 μl of dimethyl sulfoxide was added. Plates were shaken for 10 min and determined with a microplate reader at 540nm. The optical density of control cell was 100% viability.

2.5 Statistical Analysis

The results of the normal group were compared with those of Aβ-induced control group and were analyzed by Student's t-test. The results of treatment groups were compared with Aβ-induced control group by one-way ANOVA (followed by post-hoc Dunnett test). Time point assays were compared by two-way ANOVA (followed by Bonferroni post-tests). The lifespan was analyzed by Log-Rank analysis and chi-square comparison.

All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., California, USA). The data were expressed as mean ± standard deviation (SD) for *in vitro* studies and mean ± standard error of the mean (SEM) for *in vivo* studies. A value of $p < 0.05$ was considered

statistically significant.

Chapter 3
In vitro Protective Effects of Selected TCM Extract against β-amyloid Peptide Fragment 25-35-induced Cytotoxicity in PC12 Cells

3.1 Introduction

Our screening assay and mechanistic study was performed on PC12 cell line. This i*n vitro* model provides a well-controlled, stable, simple, and high throughput platform for the early stage of drug development. By using the aggregated $A\beta_{25-35}$ to induce cell death, the inhibitory effects of our candidate herbs against Aβ-induced cytotoxicity would be assessed. After the preliminary screening, the most potent candidate would be selected for the further studies on anti-oxidation and anti-apoptosis.

A single cell clonal line which responds reversibly to nerve growth factor (NGF) was established from a transplantable rat adrenal pheochromocytoma in 1976 (Greene and Tischler, 1976). The cell line was named as PC12. By exposing to NGF, PC12 cells stop proliferation and produce long, branching neurites similar to those produced by sympathetic neurons in primary cell culture (Greene, 1978). Wthdrawal of NGF leads to the neuritis degeneration and restart mitosis again (Greene, 1978). PC12 cells synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine, but not epinephrine (Greene and Tischler, 1976). These properties resemble noradrenergic adrenal chromaffin cells and sympathetic neurons. Due to its similarity with sympathetic neurons and its reversible response to NGF, PC12 cells were widely used in study of neuronal function (Chuang*, et al.*, 2013), neuronal differentiation (Xu*, et al.*, 2012) and

neurodegeneration (Lee*, et al.*, 2012, Wang*, et al.*, 2013). In other studies, the short- and long-term cultured of PC12 cells have different responses to inducers of cytotoxicity, which the aged PC12 cells were less responsive (Romero*, et al.*, 2010). Therefore, only young PC12 cells were used in this study.

β-amyloid peptide fragment of 1-42 is cytotoxic to cultured cells after incubation and aggregation (Mattson, 1997). However, some of its fragments, such as 1-11, 1-15, 1-16, 1-28, 20-29, 1-30, and 1-33, are non-toxic or unable to form aggregation (Liu*, et al.*, 2004, Pike*, et al.*, 1993). For our study, the β-amyloid peptide we used required: (i) to be able to aggregate quickly for us to carry out the experiment; (ii) to be cytotoxic to cells in order for us to test the neuroprotective effects of our candidates. It has been shown that β-amyloid peptide fragment 25-35 exhibits a balance of rapid aggragation and produce strong toxic effect (Pike*, et al.*, 1993, Yankner*, et al.*, 1990). This fragment is widely used in other studies for mechanism elucidation of Aβ toxicity, Aβ-induced taupathies and therapeutic effect of chemical agents against Aβ toxicity on cell and animal models (Jayaprakasam*, et al.*, 2010, Lee*, et al.*, 2005, Martin*, et al.*, 2001, Zeng*, et al.*, 2010).

The *in vitro* platform of PC-12 cell line treated with $A\beta_{25–35}$ was producing a stable cytotoxic effect. The platform was rapid and highly reproducible. Due to the speed and the amount of samples that can be handled at once, this platform was used to screen our candidate TCMs - Tianma (TM), Gouteng (GT),

Chuanxiong (CX) and Yuanzhi (YZ). By 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, the viability of the cells could be rapidly determined. That reflected the cytotoxic effect of $A\beta_{25-35}$ and the protective effects of the candidate herbs. The fast throughput of MTT assay also enabled us to determine the dose range of the extracts, which is non-toxic but effective.

After that, we would use the flow cytometric assays to determine the anti-apoptotic and anti-oxidative effects of the more potent TCM candidates. Our target was to select the most potent one for the further studies. After the selection of the most potent TCM extract, its underlying action mechanisms were studied in 3 aspects of Aβ pathology – apoptosis caused by Aβ, oxidative stress caused by Aβ and Aβ-induced hyperphosphorylation of tau protein. Aβ-dimer, oligomer and plaque are all toxic to cells (Schnabel, 2011). They cause apoptosis, which is characterized by mitochondrial dysfunction, activation of caspase cascade, loss of phospholipid membrane asymmetry, DNA condensation and fragmentation and blebbing of plasma membrane (Harada and Sugimoto, 1999, Kaminsky*, et al.*, 2010, Mattson, 1997, Pike*, et al.*, 1993). Therefore, detection methods were also designed to target these changes.

In our study, we would detect the exposure of phosphatidylserine from cytosolic side to extracellular side of plasma membrane using Fluorescein isothiocyanate (FITC)-annexin V, which annexin V binds specifically to

phosphatidylserine and FITC is responsible for the florescence signal. A DNA stain propidium iodide (PI) would be added to detect the necrotic cells. PI is membrane impermeant, and it would be excluded by the viable cells. But it can enter the necrotic cells, which have impaired plasma membrane, to intercalate the DNA in nucleus and produce florescence signal (Vermes, *et al.*, 1995). The signals would be detected by flow cytometry, which would produce a graph like Figure 3.1. The lower left quadrant (PI: -ve; Annexin V: -ve) is the viable cells. The lower right quadrant (PI: -ve; Annexin V: +ve) is the early apoptotic cells. The upper right quadrant (PI: +ve; Annexin V: +ve) is the late apoptotic or the necrotic cells.

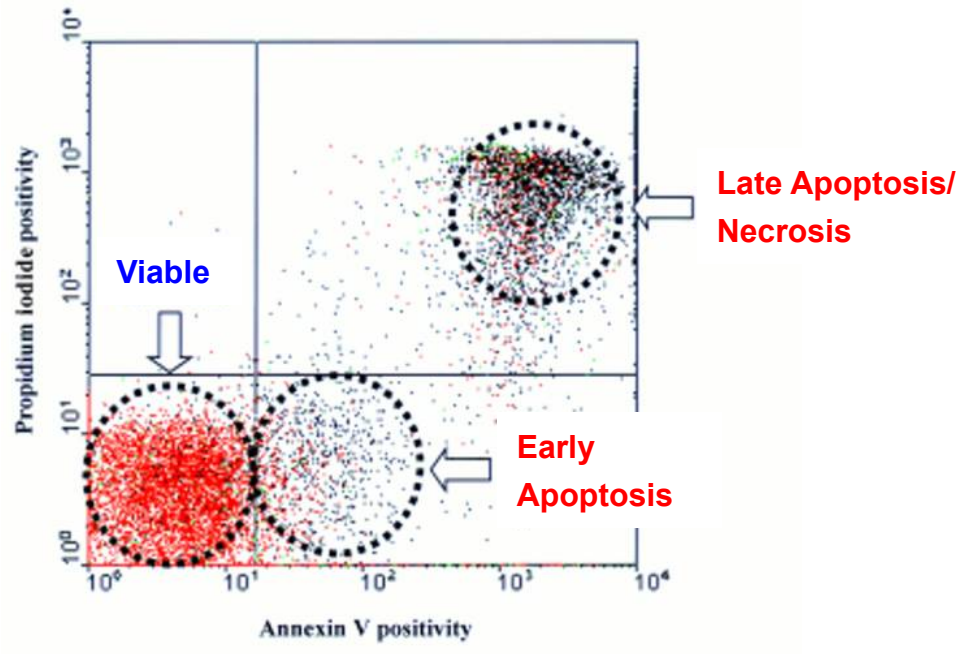


Figure 3.1 The sample plot for the PI-annexin V staining detected by flow cytometry.

Caspase cascade is well-known to play an important role in apoptosis. Among them, caspase-3 and its active form, cleaved caspase-3, is closely related and widely investigated on apoptosis during AD progression (Harada and Sugimoto, 1999, Uetsuki*, et al.*, 1999). In our study, the expression and activity of cleaved caspase-3 would be investigated, which in hope of suppressing the expression and activity of caspase-3. The inhibition of caspase-3 would be beneficial to the suppression of disease progession.

DNA condensation and degradation is another hallmark of apoptosis (Danial and Korsmeyer, 2004). DNA fragmentation can be detected using enzyme-linked immunosorbent assay (ELISA) for Bromodeoxyuridine(BrdU)-prelabeled DNA in the cell culture (Virag*, et al.*, 1998). BrdU is the analogue of the nucleotide thymidine, which would incorporate into the newly synthesized DNA in proliferating cells. Cells undergoing apoptosis would have genomic DNA cleaved by endogenous endonucleases to form fragments (Walker*, et al.*, 1994). Antibodies against BrdU are adopted in the ELISA, which the concentration of these fragments is detected by spectrophotometry

Oxidative stress always plays a key role in apoptosis (Buttke and Sandstrom, 1994, Finkel and Holbrook, 2000, Kannan and Jain, 2000). Reactive oxygen species (ROS) can oxidize and damage the DNA, which in turn activate poly-ADP-ribose transferase and cause the p53 accumulation. Damaged DNA and the subsequence conditions would induce apoptosis (Buttke and Sandstrom, 1994,

Kannan and Jain, 2000). ROS can also oxidize cellular membrane lipid and cause dysfunction of cell membrane (Buttke and Sandstrom, 1994). Moreover, ROS can directly induce the expression of genes responsible for apoptosis (Buttke and Sandstrom, 1994, Kannan and Jain, 2000). Adding up these factors, oxidative stress would be an important factor to induce apoptosis. Thus, reducing oxidative stress is a widely used approach of fighting against apoptosis (Gao*, et al.*, 2009, Liang*, et al.*, 2009, Shih*, et al.*, 2002, Sun*, et al.*, 2011).

ROS formation can be monitored by 2′,7′-dichlorofluorescin diacetate ($DCFH_2$-DA). $DCFH_2$-DA would be deacetylated by esterases to form 2′,7′-dichlorodihydrofluorescin (DCFH). Then DCFH would be oxidized by ROS to form 2',7'-dichlorodihydrofluorescein (DCF), which is highly fluorescent (Boldyrev*, et al.*, 2004, LeBel*, et al.*, 1992, Wang and Joseph, 1999). The fluorescence can be detected by flow cytrometry or fluorescent microplate reader.

ROS generated can be metabolized by anti-oxidative enzyme to non-oxidizing agents (Kannan and Jain, 2000). Well-studied anti-oxidative enzymes include superoxide dismutase, catalase and glutathione peroxidase (Coyle and Puttfarcken, 1993, Michiels*, et al.*, 1994, Reiter, 1998). There are commercial assay kits for the activities of these enzymes. In this study, these kits would be used to determine the activities of these enzymes. Increasing activities of these enzymes by TM would mean an increasing ability of the cells to combat Aβ-induced oxidative stress.

Amyloid beta peptide can induce tau phosphorylation in cell models (Busciglio*, et al.*, 1995, Takashima*, et al.*, 1993). Tau is the main microtubule-associated proteins (Cleveland*, et al.*, 1977). Hyperphosphorylated tau loses the ability to bind microtubules and causes aggregation to form neurofibrillary tangles (NFT) (Avila, 2000). The disruption of microtubules would damage the cytoskeleton of the cell (Buee*, et al.*, 2000). In this study, Western blotting would be used to determine the content of tau and phosphorylated tau in cell lysate. Reduced phosphorylation of tau can reduce the formation of NFT and stabilize the cytoskeleton of the cells.

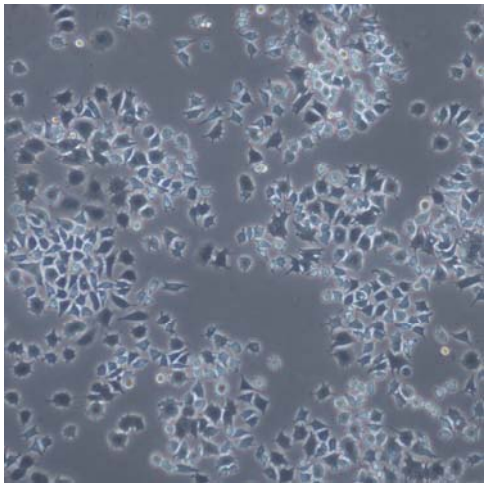
In the present study, the objectives are to screen the effect of our candidate TCM extract on Aβ-induced cytotoxicity. The most potent candidate will be subjected to further mechanistic study on anti-apoptosis, anti-oxidation and inhibition of tau phosphorylation. The study can give a brief picture of how the most potent candidate protects the cell against the toxic effect of Aβ.

3.2 Methods

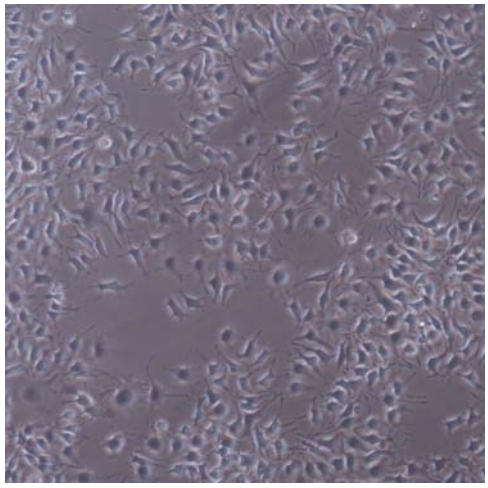
3.2.1 Cell Cultures

PC12 rat pheochromocytoma cells were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI medium 1640 supplemented with 10% (v/v) heat-inactivated HS, 5% (v/v) FBS, 50 U/ml of penicillin and 0.05 mg/ml streptomycin at 37 ℃ under 95% air/5% CO_2. Differentiation of PC12 cells was

achieved by treatment of 100μM nerve growth factor 2.5S for 2 days in 1% HS, 0.5% FBS (Figure 3.2). Cells were utilized for experiments during exponential growth.



Day 0



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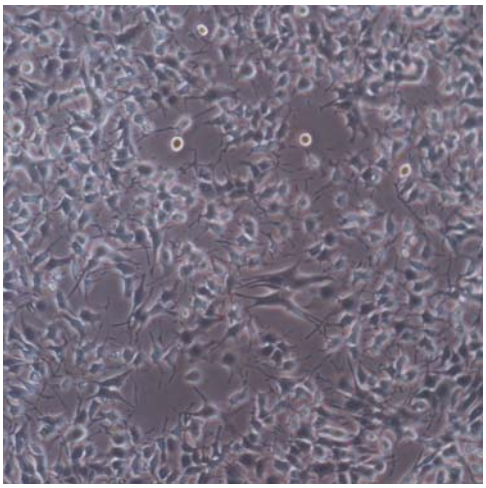

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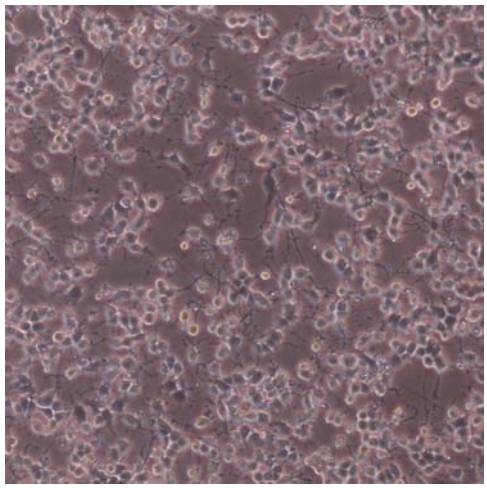

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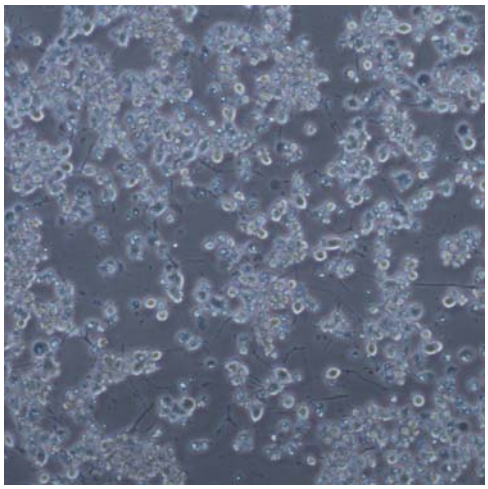


Day 7

Figure 3.2 Morphology of the PC12 cells during differentiation

3.2.2 Sample Treatment

$A\beta_{25-35}$ was dissolved in sterile distilled water at a concentration of 1.0 mM as a stock solution, and pre-aggregated at 37 ℃ for 7 days prior to use. Confluent cells were trypsinized, counted, and seeded into 6-well culture plates at a density of 3×10^5 cells/well and incubated for 24 h. After that, cells were treated with various concentrations of our herbal extracts and 20 μM of aggregated $A\beta_{25-35}$ for 48h.

3.2.3 Cell Viability Assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Briefly, the cells were plated in 96-well culture plates at the density of 1×10^4 cells/well and incubated for 24 h. After that, the medium was replaced with fresh medium, and the cells were incubated with $A\beta_{25-35}$ (20 μM) in the presence or absence of aqueous extract of TM, GT, CX and YZ (250-1000μg/ml) for 48 h. Thereafter, treated cells were tested according to section 2.4.1.

3.2.4 Acetylcholinesterase Activity Assay

The untreated cells were collected, and washed twice with ice cold PBS. PC-12 cells were lysed in cell lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM MgCl2, 0.1% Triton X-100) with 2 freeze/thaw cycles. The assay for acetylcholinesterase

(AChE) inhibition was performed according to the methods developed by Ellman *et al*.(Ellman*, et al.*, 1961). Briefly, the assay measure the conversion of acetylthiocholine to thiocholine by AChE, which thiocholine formed would react with dithiobisnitrobenzoate to form yellow coloured compound and measured spectrophotometrically (Figure 3.3). The concentration of protein was adjusted to 0.5mg/ml. The herbal extract was directly added to the sample during the experiment.

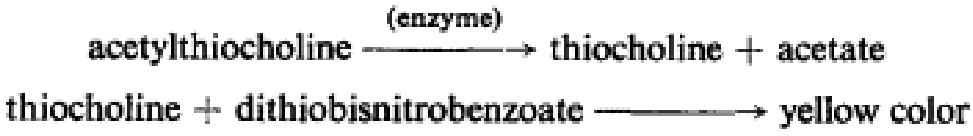


Figure 3.3 The Reaction Pathway in Ellman's Assay (Adopted from Ellman *et al.* (Ellman*, et al.*, 1961))

3.2.5 Flow Cytometric Detection of Apoptosis

Apoptotic cells among the treated cells were quantified by Annexin V-FITC and propidium iodide (PI) staining by flow cytometry (Vermes*, et al.*, 1995). Briefly, the treated cells were trypsinized and centrifuged at 450 xg at 25 ℃ for 5 min. The pellet was washed twice with ice cold PBS, and resuspended with Annexin V Binding Buffer binding buffer. Annexin V-FITC and PI were added according to manufacturer's instruction and incubated in dark at room temperature for 15 min. 300 µl of binding buffer were added to each sample. The stained cells were analyzed by fluorescence-activated cell sorter (FACS). Ten thousands events were

analyzed per sample.

3.2.6 Measurement of Apoptosis

The treated cells were collected, and washed twice with ice cold PBS. PC-12 cells were lysed in cell lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM MgCl2, 0.1% Triton X-100) with 2 freeze/thaw cycles. The supernatant was collected after centrifugation at 15000 xg for 3min, after that the total protein concentration was determined by the bicinchoninic acid (BCA) assay, using bovine serum albumin (BSA) as a standard. The samples were then applied to caspase-3 activity assays, according to manufacturer's instructions. The activities were normalized using the total protein concentrations.

3.2.7 Measurement of ROS Production

The 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) method was used to measure intracellular ROS production. The treated cells were collected, washed twice with ice cold PBS, and incubated with H_2DCF-DA (20 μM) in dark at 37 °C for 15 min. Then cells were washed once with PBS and harvested for fluorescence-activated cell sorter (FACS) analysis. Ten thousands events were analyzed per sample.

3.2.8 Measurement of the Anti-oxidative Enzyme Activities

The treated cells were collected, and washed twice with ice cold PBS. The cells were lysed in cell lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM MgCl2, 0.1%

Triton X-100) with 2 freeze/thaw cycles. The supernatant was collected after centrifugation at 15000 xg for 3min, after that the total protein concentration was determined by the bicinchoninic acid (BCA) assay, using bovine serum albumin (BSA) as a standard. The samples were applied to anti-oxidative enzyme activity assays, including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), according to manufacturer's instructions. The activities were normalized using the total protein concentrations.

3.2.9 Western Blot Analysis

Immunoblot analysis was performed on PC12 cells treated as described above. The treated cells were collected, washed twice with ice cold PBS, and lysed with 30 μl of RIPA buffer (62.5 mM Tris HCl [pH 6.8], 1% SDS, 10% glycerol and 5% 2-mercaptoethanol). The samples were boiled with the gel-loading dye (250 mM Tris HCl [pH 6.8], 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.04% bromphenol blue) at 95 °C for 5 min before loading onto the poly-acrylamide gel (12%) for SDS-PAGE. Protein kaleidoscope standard was used as a size marker. After the proteins were separated by SDS-PAGE, they were transferred onto a nitrocellulose membrane at 90 V for 90 min. The membrane was blocked with 5% skim milk for 1 h after the blotting procedure, followed by washing twice with 1× Tris-buffered saline (TBS; 20 mM Tris-HCl, 136 mM NaCl, pH 7.6) with 1% Tween-20 (called TTBS below) for 10 min. The primary antibody against Cleaved caspase-3, caspase-3, Bcl-2, Bax, Tau, pTau (Thr205/Ser396/Ser404) and β-actin were diluted at 1:200 to 1:5000 in

5% skim milk and incubated with the membrane overnight at room temperature. On the next day, the membrane was washed twice with TTBS for 10 min, followed by incubation with the secondary antibody (bovine anti-rabbit-horseradish peroxidase; Santa Cruz Biotechnology, CA) at 1:1000 dilution for 2 h at room temperature. The membrane was washed twice with TTBS for 10 min before detection using the Immun-Star™ WesternC™ Chemiluminescence Kit. The chemiluminescent signal was visualized and analyzed with the ChemiDoc™ XRS+ System and the Image Lab™ Software (Bio-Rads Laboratories, Hercules, CA, USA).

3.3 Results

3.3.1 In vitro Screening Assay and Neuroprotective Studies

3.3.1.1 Cell Viability Assay

The effects of Aβ treatment on cell viability of undifferentiated and differentiated PC12 cells were assessed for the establishment of the platform. The 48 h treatment of 10, 20 and 50 μM of Aβ on undifferentiated cells produced a 25.34±4.71%, 24.47±9.17% and 27.40±9.74%, respectively, while 48 h treatment of 10 and 20 μM of Aβ on differentiated cells produced a 28.74±15.34% and 26.72±11.95%, respectively (Figure 3.4). Due to the stability of the undifferentiated on Aβ-induced cytotoxicity, undifferentiated cells was treated with 20 μM of Aβ in the whole study. After that, we identify the potential toxicity of our selected herbs on PC12 cells. GT demonstrated a significant cytotoxicity that reduce the cell viability by 55.50±18.33% at 1000 μg/ml. On the other hand,

CX demonstrated a slight proliferative effect by increasing the cell viability by 14.90±10.01% and 22.30±8.42% at 250 and 1000 μg/ml, respectively (Figure 3.5). Therefore, the dose of TM, CX and YZ were chosen to be 125 to 1000 μg/ml, while the dose of GT was chosen to be 125 to 500 μg/ml as the non-toxic dose range used in the future studies.

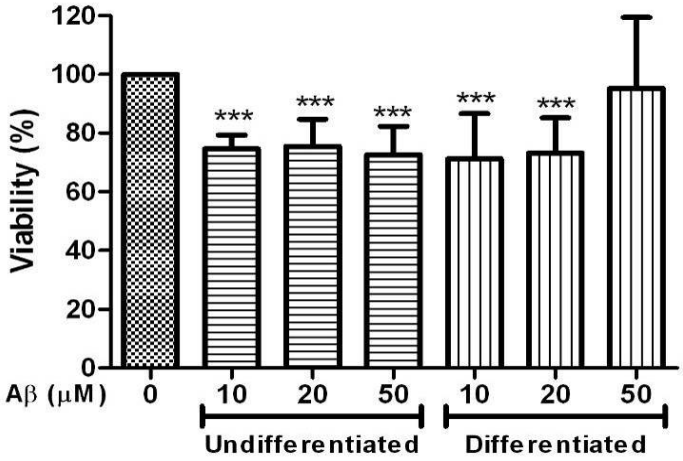


Figure 3.4 Neurotoxic effects of $A\beta_{25-35}$ on undifferentiated and differentiated PC12 cells. Data are expressed as percent of maximal cell viability in untreated control. Results are the means ± SD from five separate experiments. *** $p<0.001$ relative to control by one-way ANOVA.

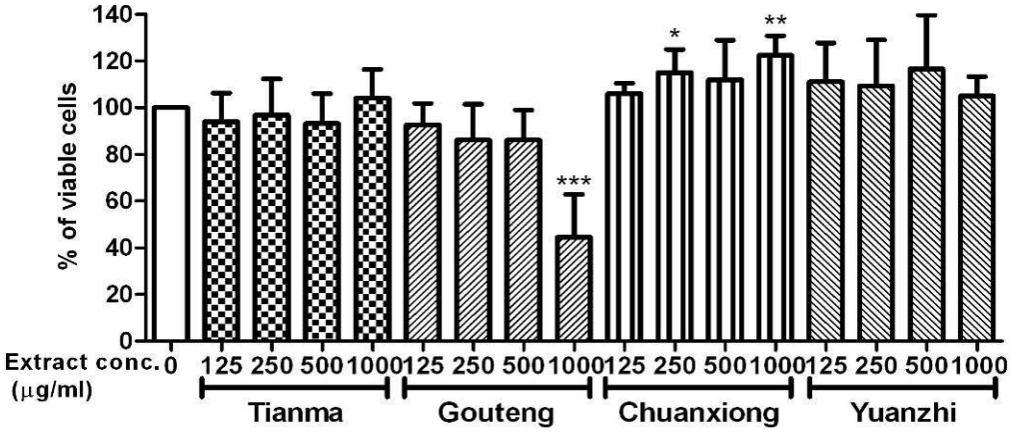


Figure 3.5 Safety dose elucidation of selected herbal extracts in PC12 cells. Results are the means ± SD from five separate experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, relative to control by one-way ANOVA.

After the establishment of the Aβ-induced cytotoxicity platform and the detection of the non-toxic dose range of our herbal extracts. The effects of our herbal extracts on the Aβ-induced cytotoxicity were investigated. The viability Aβ-treated control was decreased by 33.12±4.66%. The treatment of TM significantly increased the viability to 78.90±6.59% and 94.41±7.55% at 500 and 1000 μg/ml, respectively. The treatment of GT significantly increased the viability to 86.29±7.11% and 89.34±9.13% at 250 and 500 μg/ml, respectively. The treatment of CX significantly increased the viability to 75.39±6.86% and80.19±10.75% at 500 and 1000 μg/ml, respectively. Lastly the treatment of YZ significantly increased the viability to 79.47±21.42% at 500 μg/ml (Figure 3.6). From the results, TM was showing the best protective effect against Aβ-induced cytotoxicity. The effect of the well-known active ingredient of TM,

gastrodin, was investigated. The result showed that gastrodin could not rescue PC12 cells from Aβ-induced cytotoxicity (Figure 3.7). We also investigated a compound formula that was mentioned in Chinese Pharmacopeia 2010 that consist of two of our selected Chinese Medicine, CX and TM (Chinese Pharmacopoeia Commission, 2010). The ratio of TM and CX was 2:1 and 4:1, which is based on our previous result that TM had higher efficacy than CX. The results showed that the compound formula in ratio 2:1 significantly increased the viability of PC12 cells from 57.55±4.94% in Aβ control to 67.13±6.72%, 68.86±7.09% and 76.69±8.43% at 250;125, 500;250 and 1000;500μg/ml (dose of TM; CX), respectively. Similarly, the results showed that the compound formula in ratio 4:1 significantly increased the viability of PC12 cells from 57.55±4.94% in Aβ control to 66.94±7.21%, 69.26±7.43% and 76.80±7.43% at 250;67.5, 500;125 and 1000;250μg/ml (dose of TM; CX), respectively (Figure 3.8). The results demonstrated that the compound formula also had a significant protection against Aβ-induced cytotoxicity. However, the efficacy is similar to that of TM alone. Therefore, further studies on the neuroprotective effect were focused on TM, with CX as the spare candidates.

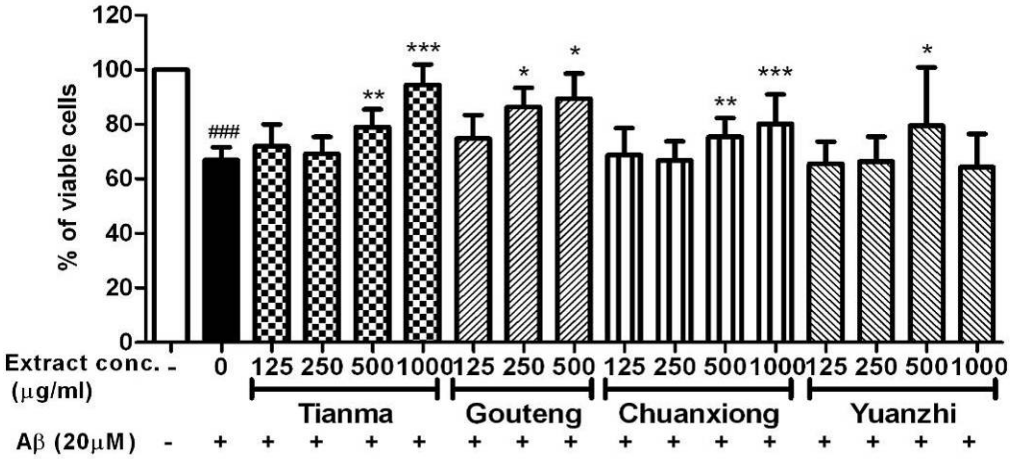


Figure 3.6 Protective effect of selected herbal extracts on Aβ-induced cytotoxicity in PC12 cells. Results are the means ± SD from three separate experiments. ### $p<0.001$, relative to control; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, relative to Aβ treatment only by one-way ANOVA.

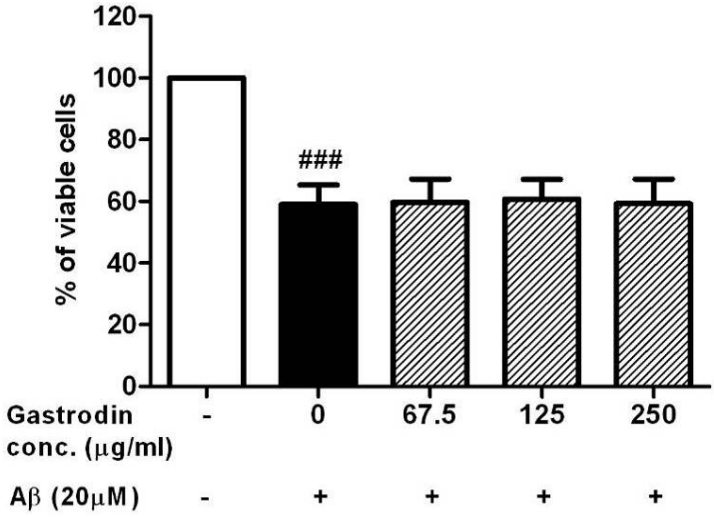


Figure 3.7 Protective effect of gastrodin on Aβ-induced cytotoxicity in PC12 cells. Results are the means ± SD from three separate experiments. ### $p<0.001$, relative to control by one-way ANOVA.

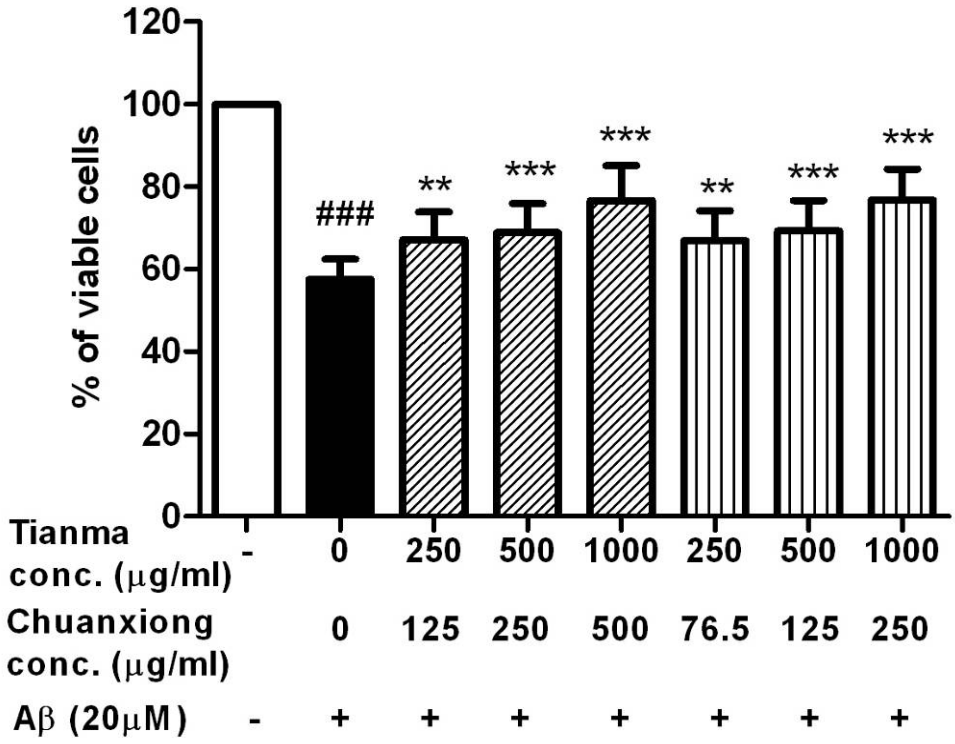


Figure 3.8 Protective effect of TM-CX compound formula on Aβ-induced cytotoxicity in PC12 cells. Results are the means ± SD from three separate experiments. ### $p<0.001$, relative to control; ** $p<0.01$ and *** $p<0.001$, relative to Aβ treatment only by one-way ANOVA.

3.3.1.2 Acetylcholinesterase Activity Assay

TM produced a marked dose-dependent inhibition of AChE activity, which inhibited AChE by 28.15%±9.99% at 1000 μg/ml. GT and CX produced a significant inhibition of AChE activity by 14.75%±6.71% and 7.89%±4.32% at 500μg/ml and 1000μg/ml, respectively. Meanwhile, YZ significantly and

dose-dependently promoted the AChE activity. However, the results from AChE inhibition by TM, GT and CX were not comparable to those of donepezil, which inhibit AChE by 63.88%±4.54% at 10 nM and 92.32%±3.01% at 1000nM (Table 3.1).

Table 3.1 Inhibitory effect of selected herbal extracts on AChE extracted from PC12 cells. Results are the means ± SD from five separate experiments. ** $p<0.01$ and *** $p<0.001$, relative to control by one-way ANOVA.

Treatment	AChE activity (% control)
Control	100.00%
TM (250μg/ml)	96.07%±2.58%
(500μg/ml)	91.46%±3.97%**
(1000μg/ml)	71.85%±9.99%***
GT (250μg/ml)	89.27%±4.05%
(500μg/ml)	85.25%±6.71%***
CX (250μg/ml)	98.31%±4.60%
(500μg/ml)	99.48%±6.56%
(1000μg/ml)	92.11%±4.32%**
YZ (250μg/ml)	109.24%±3.43%
(500μg/ml)	114.91%±10.60%***
(1000μg/ml)	136.54%±10.18%***
Donepezil (10nM)	36.12%±4.54%***
(1000nM)	7.68%±3.01%***

3.3.2 Detailed Underlying Mechanistic Studies

CX, GT and TM demonstrated a high efficiency on rescuing the PC12 cells from Aβ-induced cytotoxicity. Therefore, the anti-apoptotic and anti-oxidative effects were studied in order to further understand their underlying mechanism and further compared their efficiency, which the more potent one would be chosen for the *in vivo* studies.

3.3.2.1 Effect of Chuanxiong on Aβ-induced Apoptosis and Oxidative Stress

Apoptosis is one of the main causes for the neurodegeneration in Alzheimer's disease (Shimohama, 2000). Studies shown that apoptosis was also involved in $A\beta_{25-35}$-induced cell death in PC12 cells (Jang and Surh, 2005, Martin, *et al.*, 2001). Therefore, we investigated the effect of CX on $A\beta_{25-35}$-induced apoptosis by Annexin V-FITC and PI detected by flow cytometry. The normalized percentages of cells undergoing $A\beta_{25-35}$-induced early and late apoptosis were 14.1±3.5% and 2.6±0.3% respectively. The treatment of CX reduced the percentage of cells undergoing early and late apoptosis to 5.4±0.8% and 0.7±0.5% for 125 μg/ml, 4.3±2.3% and 0.06±0.4% for 250 μg/ml, and 7.3±3.0% and 0.1±0.5% for 500 μg/ml (Figure 3.9). The results suggested that TM could reduce $A\beta_{25-35}$-induced apoptosis dose dependently. But the dose of 500 μg/ml CX may already exert some cytotoxic effect to the PC12 cells.

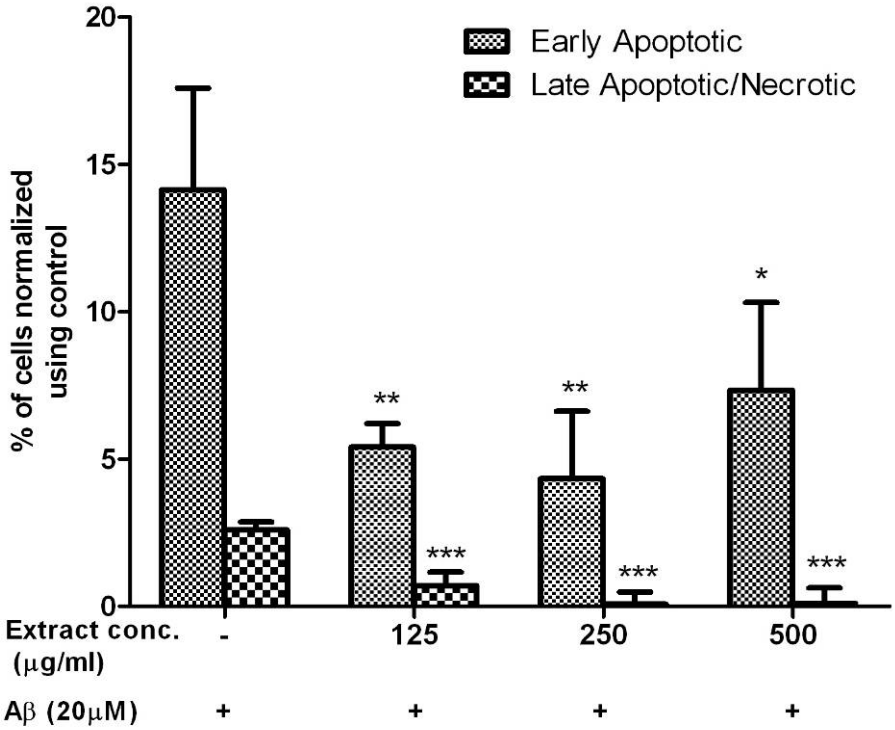


Figure 3.9 Anti-apoptotic effect of CX on Aβ-induced cytotoxicity in PC12 cells. CX reduced Aβ-induced apoptosis in flow cytometric analysis. Results are the means ± SD from three separate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to Aβ treatment only by one-way ANOVA.

Oxidative stress, which is due to the accumulation of ROS, is one of the major causes of apoptosis. In this study, the ROS content in cells was detected by H_2DCF-DA by flow cytometry. H_2DCF-DA can pass through the cell membrane and oxidized by ROS to form the fluorochrome 2′,7′dichlorofluorescein (DCF). Therefore, the florescence level of DCF reflects the intracellular ROS level (Boldyrev*, et al.*, 2004). Figure 3.10 shows that 20μM Aβ25-35 elevated the production of ROS from 100% to 145.2±16.3%, whereas the relative fluorescence

intensity in CX-treated groups decreased significantly to 94.4±9.3% and 92.8±11.0% for 250 and 500 μg/ml, respectively. The decrease of fluorescence reflected the efficacy of CX in reducing the ROS content.

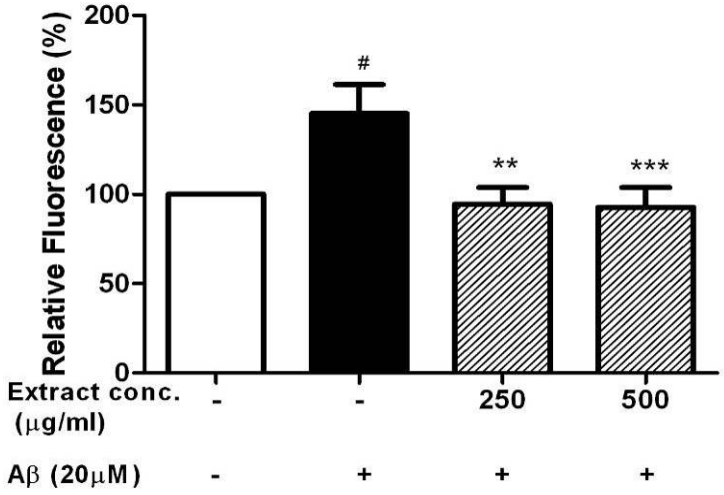


Figure 3.10 Anti-oxidative effect of CX on Aβ-induced cytotoxicity in PC12 cells. CX reduced Aβ-induced oxidative stress in flow cytometric analysis of H_2DCF-DA positive cells. Results are the means ± SD from three separate experiments. $^{\#}p<0.05$, relative to control; $^{**}p<0.01$ and $^{***}p<0.001$, relative to Aβ treatment only by one-way ANOVA.

3.3.2.2 Effect of Gouteng on Aβ-induced Apoptosis

GT was examined with the same platform as CX on its anti-apoptotic effect. The normalized percentages of cells undergoing $A\beta_{25-35}$-induced early and late apoptosis were 14.8±1.0% and 1.7±0.3% respectively. The treatment of GT did not rescue the cells from apoptosis. Instead, there is a trend to increase the

percentage of cells undergoing early apoptosis to 21.2±6.2% at 500 μg/ml (Figure 3.11). Due to the possible pro-apoptotic activity of GT, the investigation on GT was terminated.

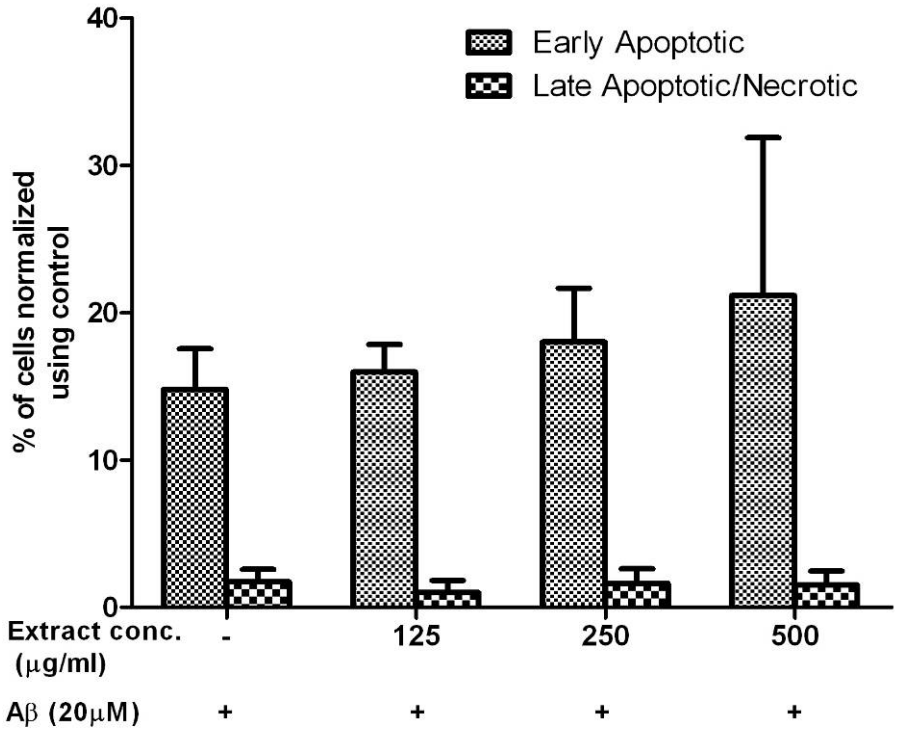


Figure 3.11 Anti-apoptotic effect of GT on Aβ-induced cytotoxicity in PC12 cells. GT demonstrated a pro-apoptotic trend in flow cytometric analysis. Results are the means ± SD from three separate experiments.

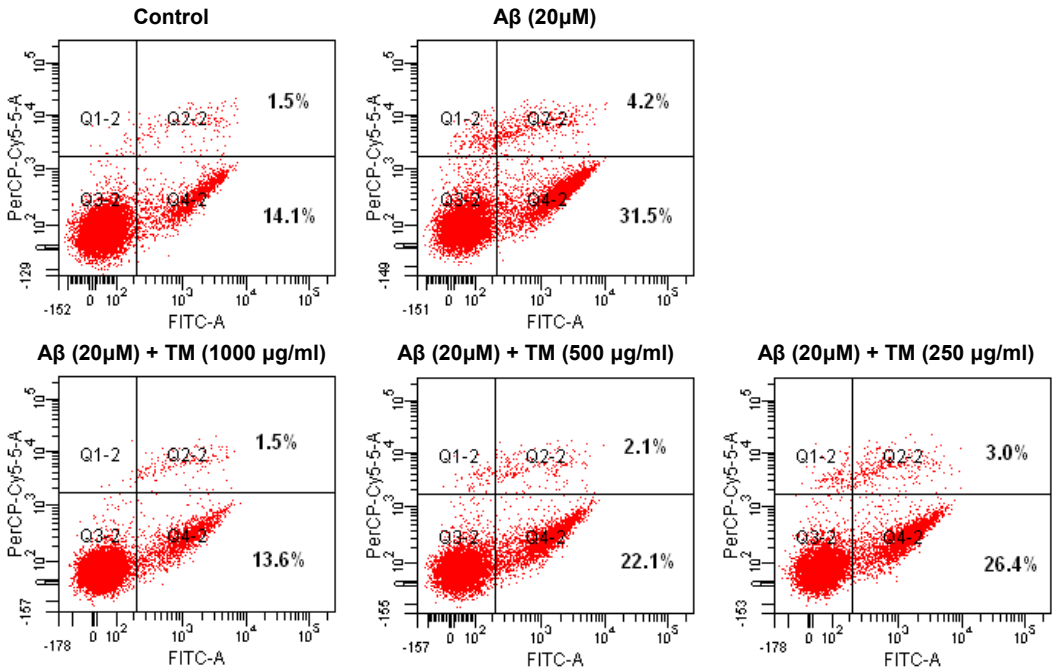
3.3.2.3 Effect of Tianma on Aβ-induced Apoptosis

TM was study with the same platform as CX on its anti-apoptotic effect. From figure 3.12A, the intensity of the red dots of the lower and upper left quadrant of the plot was lowered with TM treatment when comparing with Aβ

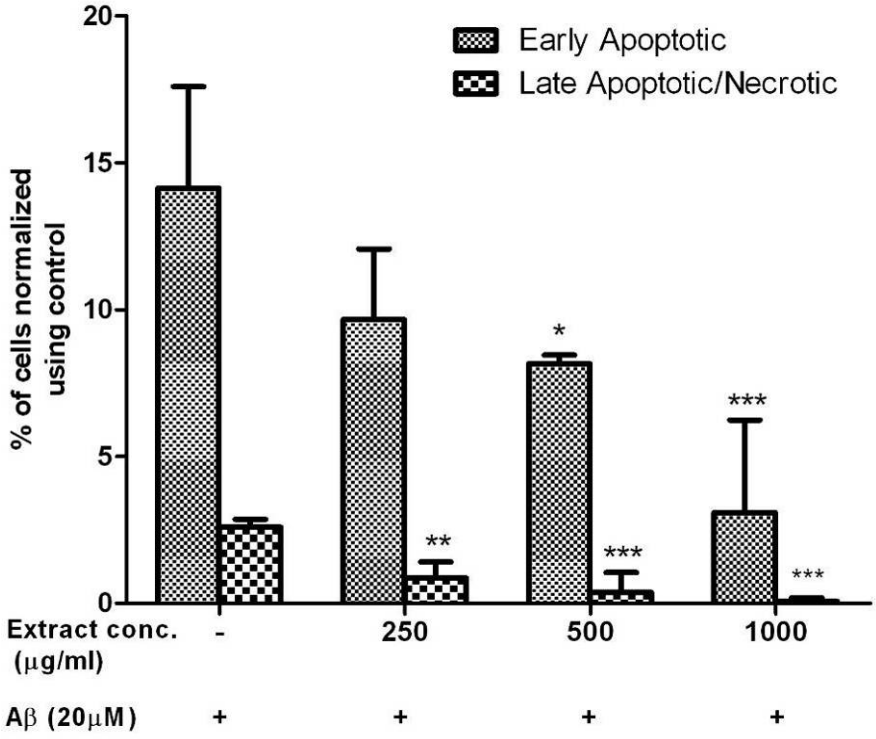
treatment only, which indicates the decreasing population of cells that undergoing early and late apoptosis, respectively. The normalized percentages of cells undergoing $A\beta_{25–35}$-induced early and late apoptosis were 14.1±3.5% and 2.6±0.3% respectively. The percentage of early and late apoptosis induced by Aβ25–35 with treatment of TM were 9.7±2.4% and 0.9±0.6% for 250 μg/ml, 8.2±0.3% and 0.4±0.7% for 500 μg/ml, and 3.1±3.1% and 0.1±0.1% for 1000 μg/ml (Figure 3.12B). The results suggested a dose dependent reduction $A\beta_{25–35}$-induced apoptosis by the TM treatment. Other studies demonstrated that caspases are crucial mediators of apoptosis, which caspase-3 activation is early event and initiates apoptotic damage. Therefore, the expression was assessed by western blotting. Aβ increased the cleavage of caspase-3 into active cleaved caspase-3 (Figure 3.13A) and increased Cleaved Caspase-3/Caspase-3 ratio by 105.3±19.09%. The 250 and 500 μg/ml treatment of TM reduced the Cleaved Caspase-3/Caspase-3 ratio to 24±13.11%, 13.3±15.14% more than the normal level, respectively. The 1000 μg/ml treatment of TM reduced the ratio back to the normal level (Figure 3.13B). The activity of caspase-3 was determined. Caspase-3 activity was increased 31.8±13.4% by $A\beta_{25–35}$ treatment, and the increase in activity was attenuated dose dependently by treatment of TM. One thousand μg/ml of TM totally suppressed the caspase-3 activation and reduced the activity level back to the state without $A\beta_{25–35}$ treatment (Figure 3.12C). Also, treatment of PC12 cells with 20 μM of $A\beta_{25–35}$ for 48 h caused a decrease in the ratio of Bcl-2/Bax as compared to the untreated control group (p <0.01) and the ratio of Bcl-2/Bax was 71.3±5.3% of the control, suggesting that $A\beta_{25–35}$ treatment was

capable of inducing apoptosis of PC12 cells. When PC12 cells were treated with TM at the concentrations of 1000 μg/ml and exposed to 20 μM of $A\beta_{25-35}$ for 48 h significantly enhanced the Bcl-2/Bax expression ratio (p <0.05) as compared with the $A\beta_{25-35}$ group, and the Bcl-2/Bax expression ratio was 98.0 ±14.0% of the control. These experimental results indicate the ability of TM on protecting PC12 cells against $A\beta_{25-35}$-mediated apoptosis.

A


Control
Aβ (20μM)
Aβ (20μM) + TM (1000 μg/ml)
Aβ (20μM) + TM (500 μg/ml)
Aβ (20μM) + TM (250 μg/ml)
PerCP-Cy5-5-A
FITC-A
Q1-2
Q2-2
Q3-2
Q4-2
1.5%
14.1%
4.2%
31.5%
1.5%
13.6%
2.1%
22.1%
3.0%
26.4%

B


Early Apoptotic
Late Apoptotic/Necrotic
% of cells normalized using control
20
15
10
5
0
*
**
***
***
***
Extract conc. (μg/ml)
-
250
500
1000
Aβ (20μM)
+
+
+
+

C

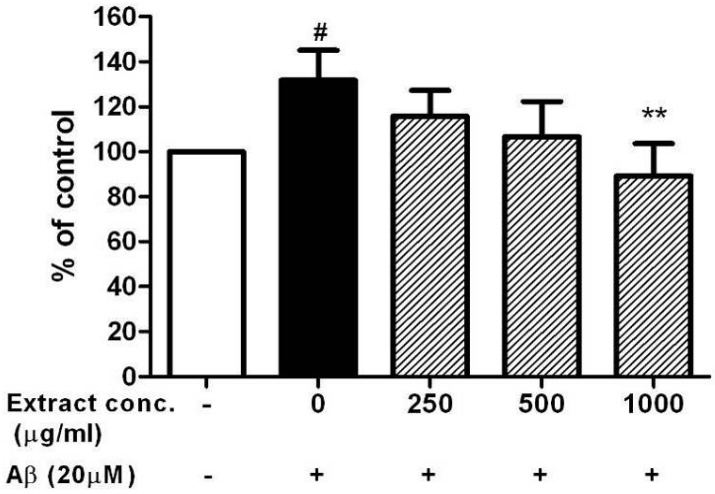
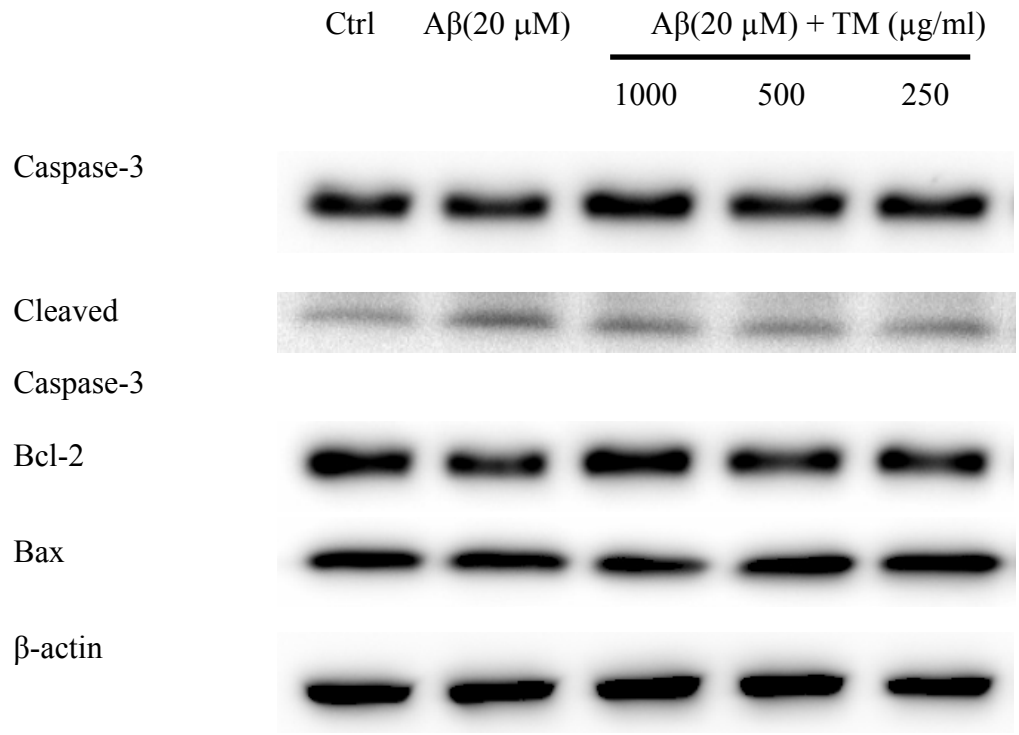
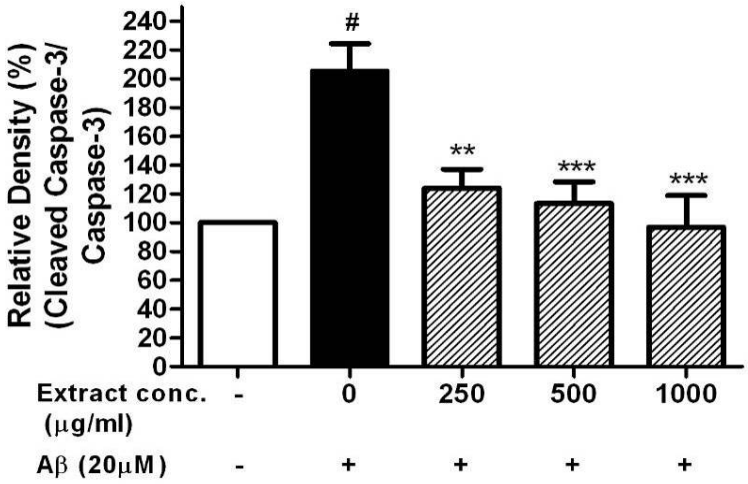


Figure 3.12 Anti-apoptotic effect of TM on Aβ-induced cytotoxicity in PC12 cells. (A) Representative plots for the flow cytometric analysis. (B) TM extract reduced Aβ-induced apoptosis in flow cytometric analysis. (C) TM extract attenuated Aβ-induced increase in caspase-3 activity. Results are the means ± SD from three separate experiments. $^{\#}p<0.05$, relative to control; $*p<0.05$, $**p<0.01$, $***$ $p<0.001$ relative to Aβ treatment only by one-way ANOVA.

A


Ctrl
Aβ(20 μM)
Aβ(20 μM) + TM (μg/ml)
1000
500
250
Caspase-3
Cleaved Caspase-3
Bcl-2
Bax
β-actin

B


Relative Density (%) (Cleaved Caspase-3/ Caspase-3)
240
220
200
180
160
140
120
100
80
60
40
20
0
#
**
***
***
Extract conc. (μg/ml)
-
0
250
500
1000
Aβ (20μM)
-
+
+
+
+

C

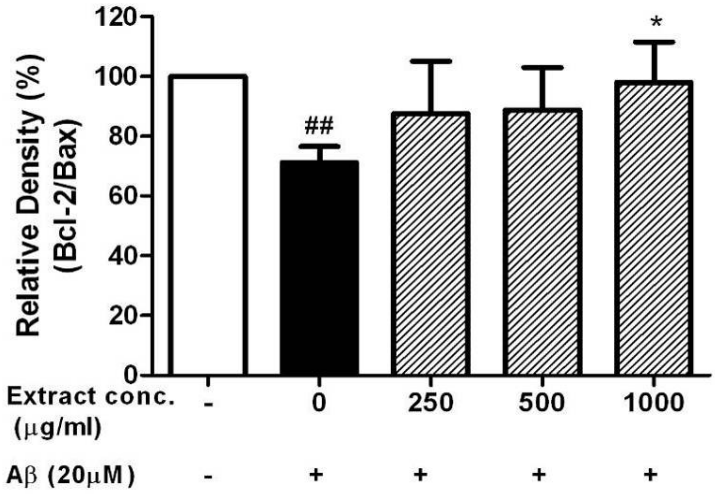


Figure 3.13 Western blotting results of the effect of TM on Aβ-induced caspase-3 cleavage and Bcl-2/Bax expression in PC12 cells. (A) Representative photos for the Western blots. (B) Ratio of cleaved caspase-3/caspase-3 on TM-treated cells. (C) Ratio of Bcl-2/Bax on TM-treated cells. Results are the means ± SD from three separate experiments. $^{\#}p<0.05$, relative to control; $*p<0.05$, $**p<0.01$, $***$ $p<0.001$ relative to Aβ treatment only by one-way ANOVA.

3.3.2.4 Anti-oxidative Effect of Tianma

The anti-oxidative effect of TM was studied, which could possibly by one of the underlying mechanisms of the neuroprotective effect of TM on PC12 cells against $A\beta_{25-35}$-induced apoptosis. Figure 3.14 shows that 20μM $A\beta_{25-35}$ elevated the production of ROS from 100% to 145.2±16.3%, whereas the relative fluorescence intensity in TM-treated groups decreased significantly to 110.9±7.5%, 103.7±23.1% and 99.0±15.1% for 250, 500 and 1000 μg/ml TM,

respectively. The decrease of relative fluorescence by TM reflected the reduction of oxidative stress, which possibly contributed by the activation of anti-oxidative enzymes.

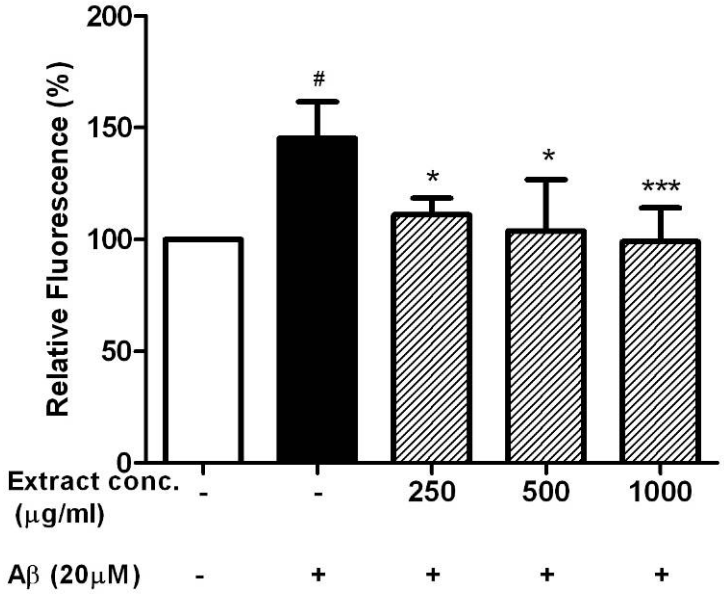
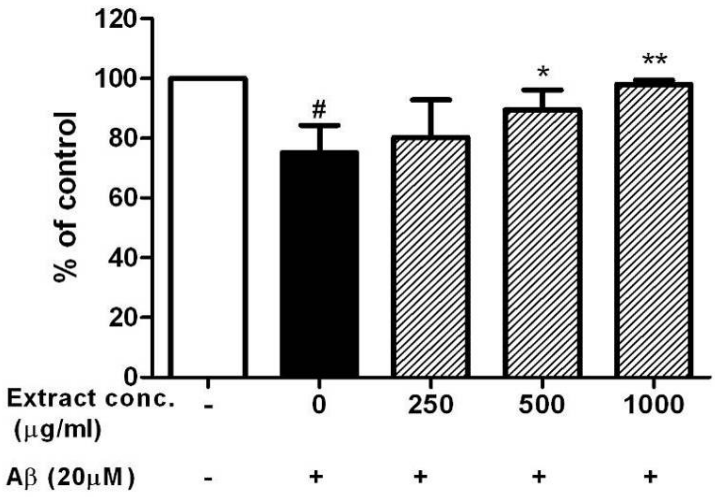


Figure 3.14 Anti-oxidative effect of TM on Aβ-induced cytotoxicity in PC12 cells. TM reduced Aβ-induced oxidative stress in flow cytometric analysis of H_2DCF-DA positive cells. Results are the means ± SD from three separate experiments. $^{\#}p<0.05$, relative to control; *$p<0.05$ and *** $p<0.001$, relative to Aβ treatment only by one-way ANOVA.

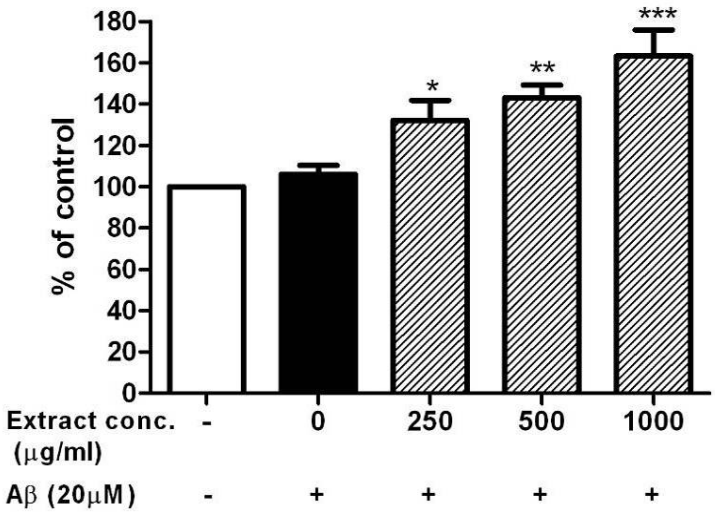
The activities of anti-oxidative enzymes (SOD, CAT and GPx) in untreated PC12 cells and those treated with 20μM $A\beta_{25-35}$ alone or with TM together are presented in figure 3.15. The exposure of PC12 cells to 20μM $A\beta_{25-35}$ decreased the activity of SOD by 24.8±9.1% (Figure 3.15A), but the activity of CAT was not

significantly affected (Figure 3.15B). The exposure induced a 19.7±4.9% increase in activity of GPx (Figure 3.15C), which was possibly due to the activation of p53 by Aβ which in turn activate GPx (Tan*, et al.*, 1999). TM treatment dose dependently reverted the activity of SOD to the normal activity level of the PC12 cells without $A\beta_{25-35}$ treatment, while the activity of CAT was enhanced dose-dependently and increased by 63.3±12.6% compared with the normal control when treated with 1000 μg/ml of TM. Moreover, 1000 μg/ml of TM further increased the activity of GPx to 45.0±7.7% higher than the normal control. Overall, treatment with different dose of TM significantly and dose-dependently enhanced the activities of SOD, CAT and GPx (Figure 3.15).

A



B



C

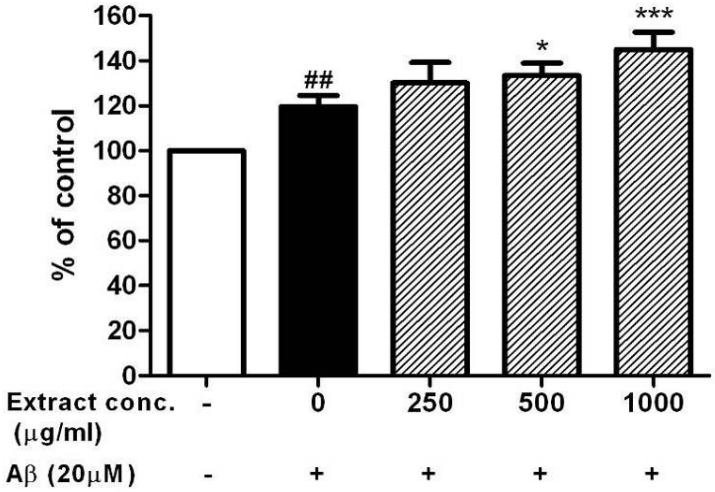
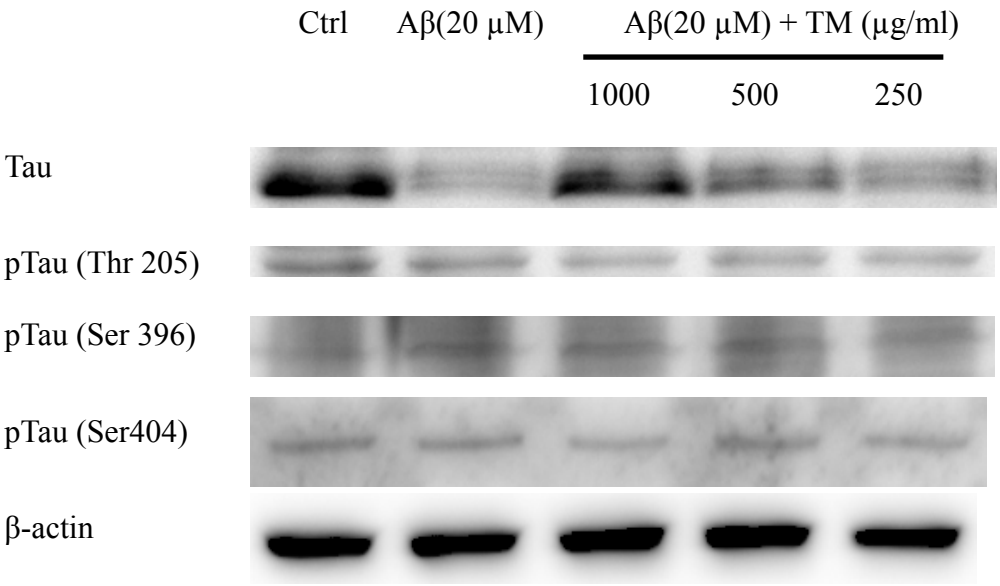


Figure 3.15 Effect of TM on anti-oxidative enzymes in Aβ-treated PC12 cells. TM extract increased the activities of anti-oxidative enzymes (A) superoxide dismutase (B) catalase and (C) glutathione peroxidase in Aβ-treated cells. Results are the means ± SD from three separate experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$, relative to control; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ relative to Aβ treatment only by one-way ANOVA.

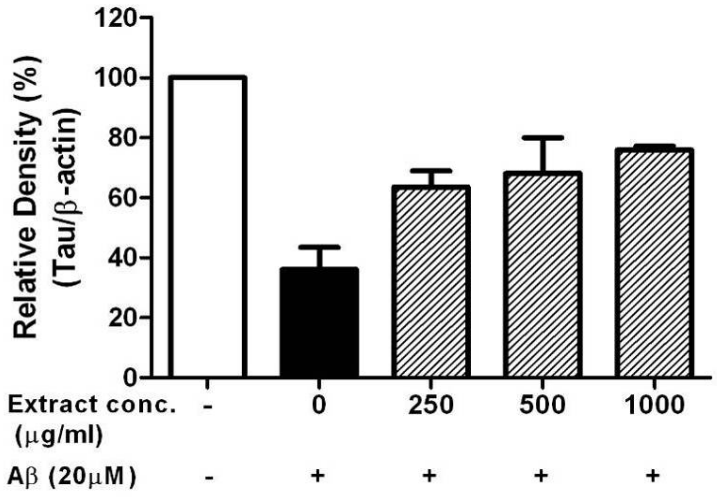
3.3.2.5 Inhibition of Tau Hyperphosphorylation by Tianma

The degree of tau hyperphosphorylation was determined preliminarily by Western blotting. In figure 3.16, relative tau phosphorylation at Ser 396 was increased to 150.0% of the control, when treated with 20 μM of $A\beta_{25-35}$ for 48 h. Relative tau phosphorylation at Thr 205 and Ser 404 did not change significantly by $A\beta_{25-35}$ treatment. Meanwhile, the total tau protein was reduced to 36.1±7.4% of the control by $A\beta_{25-35}$ treatment. On the other hand, phosphorylation of tau protein was inhibited by treating the cells with 250, 500 and 1000 μg/ml of TM together with 20 μM of $A\beta_{25-35}$ for 48 h. The relative tau phosphorylation at Thr 205, Ser 396 and Ser 404 was inhibited to 61.1%, 57.4% and 69.0±2.0% of the control, respectively, by 1000 μg/ml TM treatment together with 20 μM of $A\beta_{25-35}$ for 48 h (Figure 3.16B-D). Also, the total tau protein was increased to 63.6±5.4%, 68.2±11.9% and 76.0±1.3% of the control by 250, 500 and 1000 μg/ml TM treatment together with 20 μM of $A\beta_{25-35}$ for 48 h, respectively (Figure 3.16E). The preliminary results suggested that $A\beta_{25-35}$ could reduce the expression of normal tau protein, and 1000 μg/ml of TM could increase the expression of normal tau protein, and reduce the phosphorylation of tau at site Thr 205, Ser 396 and Ser 404.

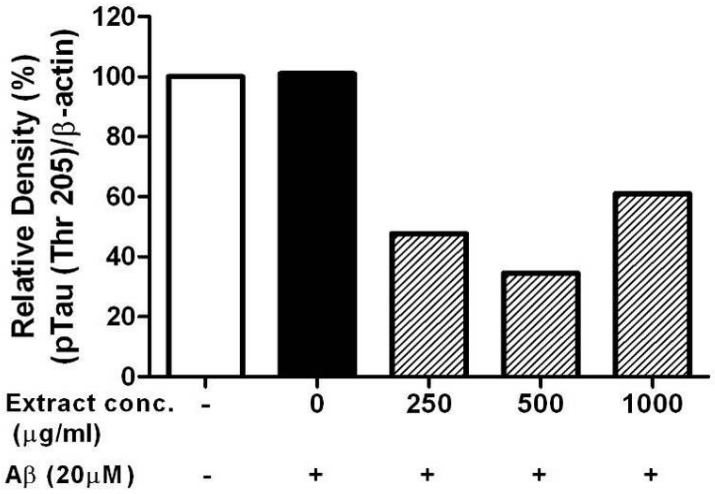
A


Ctrl
Aβ(20 μM)
Aβ(20 μM) + TM (μg/ml)
1000
500
250
Tau
pTau (Thr 205)
pTau (Ser 396)
pTau (Ser404)
β-actin

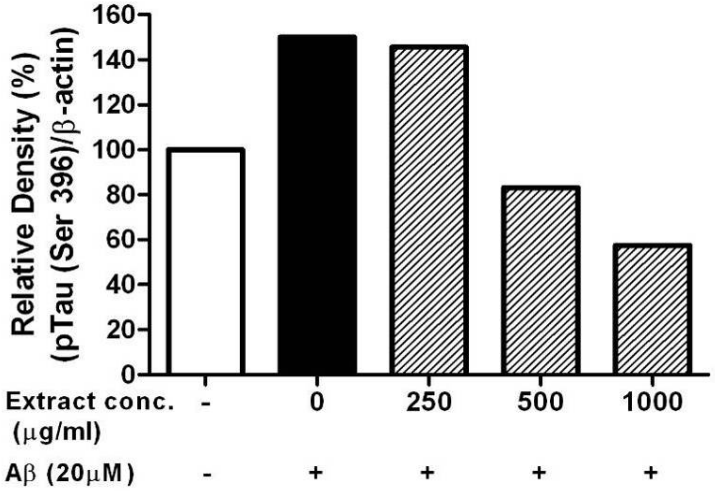
B


Relative Density (%)
(Tau/β-actin)
120
100
80
60
40
20
0
Extract conc. (μg/ml)
-
0
250
500
1000
Aβ (20μM)
-
+
+
+
+

C


Relative Density (%)
(pTau (Thr 205)/β-actin)
120
100
80
60
40
20
0
Extract conc. (μg/ml)
-
0
250
500
1000
Aβ (20μM)
-
+
+
+
+

D


Relative Density (%)
(pTau (Ser 396)/β-actin)
160
140
120
100
80
60
40
20
0
Extract conc. (μg/ml)
-
0
250
500
1000
Aβ (20μM)
-
+
+
+
+

E

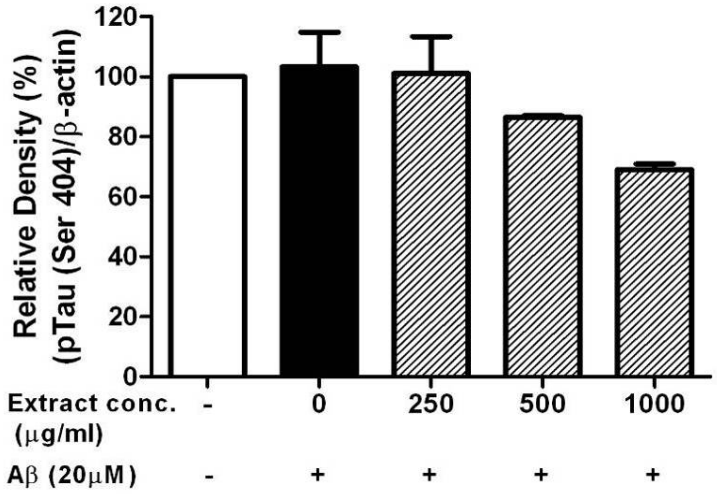


Figure 3.16 Western blotting results of the effect of TM on Aβ-induced tau phosphorylation. (A) Representative photos for the Western blots. (B-E) Expression levels of Tau, pTau (Thr 205), pTau (Ser 396) and pTau (Ser 404), respectively. The data was normalized by β-actin. Results are the means ± SD from one to two separate experiments. #$p<0.05$, relative to control; *$p<0.05$, **$p<0.01$, *** $p<0.001$ relative to Aβ treatment only by one-way ANOVA.

3.4 Discussion

Our *in vitro* experiments showed that Aβ-treated cultures exhibited characteristic features of ROS production, apoptosis and cell death in PC12 cells. All four selected Chinese Medicine was able to reduce the Aβ-induced cytotoxicity. TM and CX were able to reduce the apoptosis and oxidative stress mediated by Aβ. We have also presented the first evidence that the aqueous extract of TM could attenuate Aβ-induced cytotoxicity effectively, probably through increasing the activities of anti-oxidative enzymes so as to reduce overall oxidative stress and subsequently inhibiting Aβ-induced apoptosis.

The establishment of platform was done with both undifferentiated and differentiated PC12 cell lines. Our result showed that different doses of Aβ induced a more constant cytotoxicity in undifferentiated than in differentiated PC12 cells, which stability and repeatability was the most important consideration for a screening platform. Moreover, the differentiation of PC12 cell line could not be maintained in a healthy condition for long term. The neurite outgrowth started to degenerate and the cell morphology started to change at day 7. In addition, the detection of differentiate PC12 cells in flow cytometry was tested to be unstable with high basal level of apoptotic cells (data not shown). Therefore, although differentiated PC12 cells demonstrated a higher similarity to the neuron (Greene, 1978), undifferentiated PC12 cells was chosen in this study. However, the dose response of Aβ was not established in our assay, and a lower dose of Aβ can be used to find out the dose responsiveness of PC12 to Aβ. The main aim of the *in*

vitro screening platform was to select the most potential herbal extract for the underlying mechanistic studies and *in vivo* studies. Among of the 4 tested herbal extracts, all of them significantly protected PC12 cells from Aβ-induced cell death in a considerable manner, with the order of TM>GT>CX>YZ. However, GT was shown to have possible pro-apoptotic effect in flow cytometric study and was dropped out from the study. Although other studies demonstrated that GT and its active ingredient have neuroprotective effect, the discrepancy may due to fact that most of these studies either use organic extract of GT or its pure compounds (Lee*, et al.*, 2003, Xian*, et al.*, 2012), while our study used water extract. The chemical profiles of these two extract are different. Due to the higher extraction temperature and longer boiling time of the water extraction protocol, the degradation of neuro-active ingredient, rhynchophylline, would be more serious than the organic extraction process. The reduced content of rhynchophylline would certainly affect the efficacy of our extract. The prediction was based on a previous study that water extract of *Uncaria rhynchophylla*, which was extracted by 15 minutes boiling, was able to attenuate 6-hydroxydopamine-induced neurotoxicity in the PC12 cell model (Shim*, et al.*, 2009). In addition, there may be water-soluble cytotoxic content in GT that only appear in our water extract but not the organic extract. This may lead to the observed apoptotic effects. In the same study, CX demonstrated its potent anti-apoptotic effect, however, with no dose-dependence. The flow cytometric study also revealed the anti-oxidative effect of CX may be even more potent than TM, which its anti-oxidative effect was also mentioned in other studies (Jiang*, et al.*, 2011, Ramalingam and Yong-Ki,

2010). However, due to the higher potency of TM against Aβ-induced cell death and its consistency in dose-dependence, CX was chosen to be the spare candidate.

With the aim to find out the ingredient responsible for the neuroprotective effect, we also examined the anti-cytotoxic effects of the well-known active ingredient of TM, gastrodin (Xu*, et al.*, 2007), which turned out to be ineffective. Therefore, the effect of TM against Aβ-induced cell death should be contributed by compounds other than gastrodin, which may be the well-known bioactive ingredients, such as hydroxybenzyl alcohol, hydroxybenzaldehyde, vanillin and vanillyl alcohol (Huang*, et al.*, 2006, Jung*, et al.*, 2007), or the less-known polysaccharides (Lee*, et al.*, 2012). On the other hand, the possibility of increasing the potency of TM by using a compound formula was also investigated. The compound formula of TM and CX significantly protected neurons from Aβ-induced cell death. However, the result was similar to that of TM alone. To avoid increasing the complexity of the ingredient inside our extract, TM was investigated alone in the further studies.

Current management of Alzheimer's disease mainly include the drugs that inhibits the AChE, and maintain the concentration of neurotransmitter acetylcholine (Francis*, et al.*, 1999), which play a role in encoding of new memories (Hasselmo, 2006). It would be more promising to extrapolate our result to *in vivo* models or human if our herbal extract was able to inhibit AChE. Based on the study of Kim et al.(Kim*, et al.*, 2009), AChE was extracted from PC12

cells. Our herbal extract or the first-line drug for Alzheimer's disease, donepezil, was directly added to the extracted AChE and the potential inhibiting effect was determined. GT and CX produced a slight but significant inhibition of AChE, while the inhibition of AChE by TM was far more significant. However, donepezil, as a specific AChE inhibitor, nearly abolished the activity of AChE completely. Although the efficacy of TM was not comparable to donepezil, the AChE inhibiting effect could certainly contribute to the neuroprotective effect of TM.

For the *in vitro* mechanistic studies, PC12 cell line, which is originated from transplantable rat adrenal pheochromocytoma, was used. In the present study, we adopted this cell line and found that TM possessed protective effect against Aβ-induced cell death in MTT assay. Extensive evidence shows that neuron cell death in AD is mediated by apoptosis (Shimohama, 2000, Yuan and Yankner, 2000). For instance, DNA fragmentation was detected in neurons and glia of hippocampus and cortex by TdT-mediated dUTP nick end labeling in postmortem analysis of AD brain (Ankarcrona and Winblad, 2005). Studies also reveal that the extracellular accumulation of Aβ triggers the intracellular formation of neurofibrillary tangles (LaFerla*, et al.*, 2007), and leads to the loss of cholinergic neurons (Coyle*, et al.*, 1983). Hence, a common theory believed that the pathological neuronal loss in AD is through apoptosis, which may cause by Aβ accumulation and cytotoxicity (Mattson, 1997, Mattson, 2004). In order to elucidate the possible mechanisms of the neuroprotective effect, the anti-apoptotic

effects of TM were determined by flow cytometry using PI/Annexin V staining method, caspase-3 expression and activity assay. Our PI/Annexin V data demonstrated that TM could strongly attenuate not only the early stage, but also the late stage of apoptosis/necrosis induced by Aβ. The increased cleavage of caspase-3 into its active form is an important process in apoptosis (Stennicke*, et al.*, 1998). Active form of caspase-3 specifically cleaves 42 of the 58 known caspase substrates (Porter and Janicke, 1999), such as anti-apoptotic Bcl-2, DNA repair enzyme PARP, inhibitor of the caspase-activated DNAse, cytoskeletal protein gelsolin and other pro-caspases (Cryns and Yuan, 1998, Nicholson and Thornberry, 1997). These events lead to DNA fragmentation, activation of caspase cascade and dismantling of the cell, which are essential for apoptosis (Porter and Janicke, 1999). Our Western blotting data show that Aβ promoted the cleavage of caspase-3 into its active form, while TM suppressed that cleavage, leading to a decrease in pro-apoptotic cleaved caspase-3. Besides, we also found that TM also could suppress Aβ-induced caspase-3 activity, which provided further evidence in anti-apoptosis.

Although the exact underlying mechanism leading to Aβ-induced apoptosis was not well understood, oxidative stress caused by the Aβ plaque was widely believed to seriously impair various cellular function and play an important role apoptosis (Annunziato*, et al.*, 2003, Mattson*, et al.*, 1999). Therefore, reducing reactive oxygen species (ROS) production was a promising approach to inhibit Aβ-induced apoptosis. It has been previously reported that the non-polar fractions

of TM and its active constituents could inhibit ROS generation (Jung*, et al.*, 2007, Kim*, et al.*, 2007). In the present study, we found that the aqueous extract of TM also possessed strong anti-oxidative action, which inhibited ROS accumulation and reduced the H_2DCF-DA-labeling of PC12 cells. Anti-oxidative action could be mediated by 2 mechanisms: activation of anti-oxidative enzymes and direct free radical scavenging (Butterfield and Lauderback, 2002). Anti-oxidative enzymes, including superoxide dismutase and catalase, catalyze the reduction superoxides, a strong ROS, to hydrogen peroxide and then to water. Meanwhile, glutathione peroxidase catalyzes glutathione, which is a crucial endogenous anti-oxidative mechanism, to reduce hydrogen peroxide to water (Finkel and Holbrook, 2000). Our results demonstrated that the up-regulation of superoxide dismutase, catalase and glutathione peroxidase activity by TM during Aβ-insult. The up-regulation of these enzymes can promote the clearance of ROS, and that partially explained the anti-oxidative action for TM. Other studies also demonstrated that both non-polar and polar fractions of TM have hydroxyl radical scavenging activity and reduce lipid peroxidation(Jung*, et al.*, 2007, Lee*, et al.*, 2006, Shin*, et al.*, 2011). Its active constituents, including vanillyl alcohol, vanillin, hydroxybenzyl alcohol and hydroxybenzaldehyde, were found to be potent anti-oxidants (Kim*, et al.*, 2007, Kim*, et al.*, 2011). The presence of these compounds in aqueous extract of TM was shown in some studies (Teo*, et al.*, 2008, Wang*, et al.*, 2012). From the results of other studies and the present study, TM was able to activate the anti-oxidative enzymes and possess direct free radical scavenging. By the duo anti-oxidative effect of TM, the oxidative stress induced

by Aβ was inhibited.

Our results suggested that Aβ can induce tau phosphorylation at Serine 396, which also reduce the amount of tau protein. The results were similar to the previous study, which Aβ enhances phosphorylation of tau and reduce the content normal tau protein in hippocampal neurons (Takashima*, et al.*, 1998). The treatment of TM can attenuate the phosphorylation of tau protein at Threonine 205 and Serine 396/404 and restore the content of the normal tau back to the normal level. The inhibition of tau phosphorylation can reduce the content of pTau. The reduced availability of pTau can lead to a decreased formation of NFT, which in turn reduce the cytotoxic effect contributed by NFT. The maintenance of the normal tau level can retain its function of stabilizing the microtubules, and help preventing the disruption of cytoskeleton. Hence, the preliminary anti-phosphorylation effect of TM can stabilize the tau protein, leading to a stable cytoskeleton and reduction of NFT. These effects may partially contribute to the anti-apoptotic effect of TM on PC12 cell line. However, due to the results is only preliminary, more trials may be needed to confirm the results. Moreover, the effect of TM on Glycogen synthase kinase 3 beta (GSK3β), which is responsible for the phosphorylation of tau, can also be determined in the future (Lovestone*, et al.*, 1994, Takashima*, et al.*, 1998).

The present study demonstrated novel use of aqueous extract of TM against Aβ-induced neurodegeneration in PC12 cell line. Its effect is mediated through

the increasing activity of anti-oxidative enzymes and reducing oxidative stress in cells, which work together with the reduced cleavage of caspase-3 into its active form, the inhibition caspase-3 activity, the restoration of Bcl-2 and the possible inhibition of tau phosphorylation, leading to the attenuation of apoptosis.

Chapter 4
In vivo Protective Effects of *Gastrodia elata* Aqueous Extract on *Drosophila* model of Alzheimer's Disease

4.1 Introduction

The ultimate goal of our Chinese medicine study is to develop a complementary or alternative medicine for improvement of the dementia conditions for human, which include Alzheimer's disease. In order to estimate the effect of the herbal extract in human, neuroprotective efficacy should be determined step by step from the *in vitro* platform to *in vivo* animal studies, and finally to the human clinical trials. Since our TM extract demonstrated a significant neuroprotective effect against beta-amyloid protein (Aβ)-induced cytotoxicity in the *in vitro* conditions, determination of the efficacy of our TM extract on *in vivo* Alzheimer's disease model, which is mediated by Aβ, was our next objective. There are a number of model organisms for the studies of Aβ-mediated Alzheimer's disease, including transgenic mice, stereotaxic injection of Aβ in rats/mice and transgenic *Drosophila*. The most commonly used model is transgenic mice (Liang*, et al.*, 2010, Paris*, et al.*, 2010, Spuch*, et al.*, 2010). The model overexpresses γ-secretase (Presenilin 1) and wild type or mutants of APP leads to the cleavage of APP by the endogenous β-secretase and the overexpressed γ-secretase into Aβ. The mice slowly accumulate Aβ protein and form plaque. The disease phenotypes would slowly appear at 8 to 12 months, such as accumulation of aggregated amyloid plaque and impaired spatial learning and memory (Paris*, et al.*, 2010, Reiserer*, et al.*, 2007). However, the behavioral variation among the animals with same genotype is large (Reiserer*, et al.*, 2007) and therefore, the

previous studies were mainly focused on gene expression, protein expression and histological assessments (Yan*, et al.*, 2009). However, due to the instability of the behavioral changes, the long breeding time and the complexity for the transgenic mice, another commonly animal model, *Drosophila melanogaster* was used in the present study.

The using of fruitfly *Drosophila* was a long history of genetic analysis. In 1900, *Drosophila* was first bred in quantity at Harvard University (Stephenson and Metcalfe, 2013). In 1909, Nobel laureate Thomas Hunt Morgan started his work on *Drosophila*, which demonstrated the sex link inheritance, and spontaneous mutation of genes (Morgan, 1913). His great success on *Drosophila* led to the wide spread use of it in genetic analysis. *Drosophila* enjoys many advantages over the other model organisms (Greenspan, 2004, Marsh and Thompson, 2006, Moloney*, et al.*, 2010).

1. The life cycle of flies is short, which means a generation of flies can be generated in 1 to 2 weeks.
2. Flies have high reproductiveness, which means a female fly can lay hundreds of eggs and produce a large number of offspring.
3. Genome of flies was fully sequenced. The genetic similarity of flies with human is the highest among the model genomes which have been sequenced, with the similarity percentage of about 60% for human disease genes.
4. Method for genetic transformation of flies with transposable element vectors was well-established, which enable insertion of interested gene into flies for

expression.

5. The chromosomes of flies only consist of 1 pair of sex chromosome and 3 pairs of autosomal chromosomes.
6. Male and female can easily be distinguished and method for virgin female collection is available, which enable a simple and reliable genetic cross.
7. Existence of balancer chromosomes prevents the chromosomal crossover and introduces easily identified phenotypes, which facilitate screening of the flies with desirable genes.

The high throughput, reliable, highly editable but simple characteristic of *Drosophila* make it to be one of the most widely used model organism in biological research field. Several neurodegenerative diseases are developed in *Drosophila*, such as Alzheimer's disease, Parkinson's disease and polyglutamine disease (Bonini and Fortini, 2003, Crowther*, et al.*, 2006, Feany and Bender, 2000, Mugat*, et al.*, 2008). The characteristic pathologies of these diseases can be recreated by expression of the corresponding mutant human disease protein, such as Aβ, tau, alpha-synuclein and truncated Ataxin-3 with expanded polyglutamine, in *Drosophila*. The overexpressions of these proteins can mimic the human pathological conditions and cause neurodegeneration similar to the disease condition. These models provide a fruitful platform for the study of the neurodegenerative disease mechanisms and potential therapeutic interventions.

For Alzheimer's Disease, the neurodegeneration can be induced in the

Drosophila model by direct expression Aβ or cleavage of amyloid precursor protein (APP) into Aβ. Normally, *Drosophila* expresses different homolog for various genes involved in human AD pathogenesis, including APP, γ-secretase and tau (Finelli*, et al.*, 2004). However, due to the absence of β-secretase, APP cannot be cleaved into Aβ42, and there is no endogenous production of Aβ42 in flies (Moloney*, et al.*, 2010). Because of the intrinsic limitation of this model, therefore, there are 3 main types of Alzheimer's disease models for *Drosophila*, which involve direct overexpression of Aβ42 (Finelli*, et al.*, 2004), or overexpression of both human APP, human β-secretase (BACE) and *Drosophila* presenilin (Ye and Fortini, 1999), or overexpression of tau (Wittmann*, et al.*, 2001). In order to establish the platform for assessment of the herbs' effects against β-amyloid peptide-induced toxicity *in vivo*, Aβ-expressing *Drosophila* was selected as the model animal for the study. In the present study, the *Drosophila* model that overexpresses Aβ42 would be used, because Aβ, but not APP, was introduced to the cells to induce cytotoxicity in our *in vitro* model.

In the present study, 2 *Drosophila* models with same vector system but different gene expression systems were used. One used the *Glass Multiple Reporter* (*GMR*) enhancer-promoter and the other used the *GAL4/upstream activating sequence* (*UAS*) system. The models use the same vector with a secretion signal peptide to direct the secretion of Aβ42 produced in the nervous system of the fly. Pre-proenkephalin, the secretion signal peptide of rat encephalin, is used (Cescato*, et al.*, 2000). The cDNA encoding signaling peptide of rat

pre-proenkephalin was amplified from rat brain cDNA (Cescato*, et al.*, 2000). The cDNA encoding signaling peptide of pre-proenkephalin and Aβ42 was obtained by polymerase chain reaction (PCR) and was cloned into P element transformation vectors pGMR and pUAST. The transformation vectors were microinjected to the embryo of the flies and expressed (Finelli*, et al.*, 2004).

GMR promoter element would direct the expression of the protein at the eye imaginal disc, causing the degeneration of the ommatidia (eyes of flies). The advantage of expressing only in the eye is that flies producing a highly toxic protein may still be viable. Rapid and severe degeneration of the ommatidia (eyes of *Drosophila*) was achieved due to the presence of two copies of genes encoding for Aβ in our *Drosophila* with *GMR* promoter (Finelli*, et al.*, 2004). In this study, the effect of our TM extract on Aβ-induced degeneration of the ommatidia will be studied.

The *UAS* system is more complex. Tissue - specific expression of the *UAS-Aβ42* is achieved by crossing the transgenic flies with driver lines that control tissue-specific expression *GAL4*, which would bind with *UAS* to activate gene transcription (Duffy, 2002). *Embryonic lethal abnormal vision (elav)-GAL4* is a commonly used pan-neuronal driver that directs the expression of transgene throughout the brain, neuronal system and retina of the fly (Feany and Bender, 2000, Rogina and Helfand, 2004, Warrick*, et al.*, 1999). The advantage of this model is that the lethal gene can be carried in the parents without expressing.

Hence, the viability and the fecundity of the parents are not affected. Moreover, the Aβ-mediated behavioral defects and phenotypes, such as impaired locomotor activity, olfactory memory and reduced lifespan, was demonstrated in this model (Iijima*, et al.*, 2004). On the other hand, Aβ deposition and progressive neurodegeneration can be observed in the brain of the transgenic *Drosophila* (Iijima-Ando and Iijima, 2010), which correlation between behavioral defects/phenotypes and neurodegeneration can also be monitored. In this study, *UAS-Aβ42* would be crossed with $Elav^{C155}$*-GAL4* to express Aβ42 and gradually accumulate in the whole neuronal system (Iijima*, et al.*, 2004). The effect of our TM extract on Aβ-induced reduction in lifespan and locomotor activity will be studied (Iijima*, et al.*, 2008).

In the present study, the use of two *Drosophila* models enables the control of the expression location and expression level of Aβ, which leads to different disease phenotypes. A high expression level of Aβ can be tolerated for the *GMR* expression system, as the expression is restricted at the ommatidia. On the other hand, the lower expression level of Aβ in the *elav-GAL4/UAS* system can cause to a progressive degeneration of neurons in whole body of the *Drosophila*, which leads to the behavioral defects and the disease phenotypes. In addition, the use of two models with different gene expression system can eliminate the effect of the herbal extract on gene expression, which may lead to false positive results in the study.

Drosophila have 4 pairs of chromosomes. In the nomenclature of fly genetics, the name is presented in the order of *X/Y ;2 ;3 ;4* (Greenspan, 2004). In the present study, we would be using female virgins with *elav-GAL4* driver and male curly wing flies with *UAS-Aβ42*. They are *elav-GAL4/elav-GAL4* and *w/Y;UAS-Aβ42/CyO* respectively. That means *elav-GAL4* is at X-chromosome and *UAS-Aβ42 and CyO* is at 2nd chromosome, which *CyO* is the curly wing balancer to prevent the loss of *UAS-Aβ42* insert. So, the crossing scheme is listed in table 4.1. From the scheme, we can see that the male with straight wing would be collected as our experimental subjects for the longevity and climbing assays. The photos of the offspring were listed in Figure 4.1.

Table 4.1 The crossing scheme for virgin *elav-GAL4/elav-GAL4 Drosophila* and male *w*/Y;*UAS-Aβ42/CyO Drosophila*

		Female gametes	Phenotype
		elav-GAL4; +; +	
Male gametes	*w; CyO; +*	*elav-GAL4/w; CyO/+; +/+*	Female; Curly wing
	w; UAS-Aβ42; +	*elav-GAL4/w; UAS-Aβ42/+; +/+*	Female; Straight wing
	Y; CyO; +	*elav-GAL4/Y; CyO/+; +/+*	Male; Curly wing
	Y; UAS-Aβ42; +	*elav-GAL4/Y; UAS-Aβ42/+; +/+*	Male; Straight wing

On the other hand, for the ommatidia degernation study we would be using female virgin w^{1118} *Drosophila* to cross with male *GMR-Aβ42*^{K52}, *GMR-Aβ42*^{K53} *Drosophila*. The crossing scheme is listed in table 4.2. From the scheme, we can see that the male would be collected as our experimental subjects for the pseudopupil assays. The photos of the offspring were listed in Figure 4.1.

Table 4.2 The crossing scheme for virgin w^{1118} *Drosophila* and male *GMR-Aβ42*^{K52}, *GMR-Aβ42*^{K53} *Drosophila*

		female gametes	Phenotype
		w^{1118};+ ;+	
male gametes	w;+ ;*GMR-Aβ42*^{K52}, *GMR-Aβ42*^{K53}	w^{1118}/w;+/+ ;*GMR-Aβ42*^{K52}, *GMR-Aβ42*^{K53}/+	Female
	Y;+ ;*GMR-Aβ42*^{K52}, *GMR-Aβ42*^{K53}	w^{1118}/Y;+/+ ;*GMR-Aβ42*^{K52}, *GMR-Aβ42*^{K53}/+	Male

The objectives of the present study is to determine the in vitro effect of our TCM extract – Tianma (TM) on neurodegeneration induced by Aβ. The assessment was performed through the possible improvements of the Aβ-induced AD phenotypes, such as shortened lifespan, impaired locomotor activity and the degeneration of ommatidia, by our TCM extract.

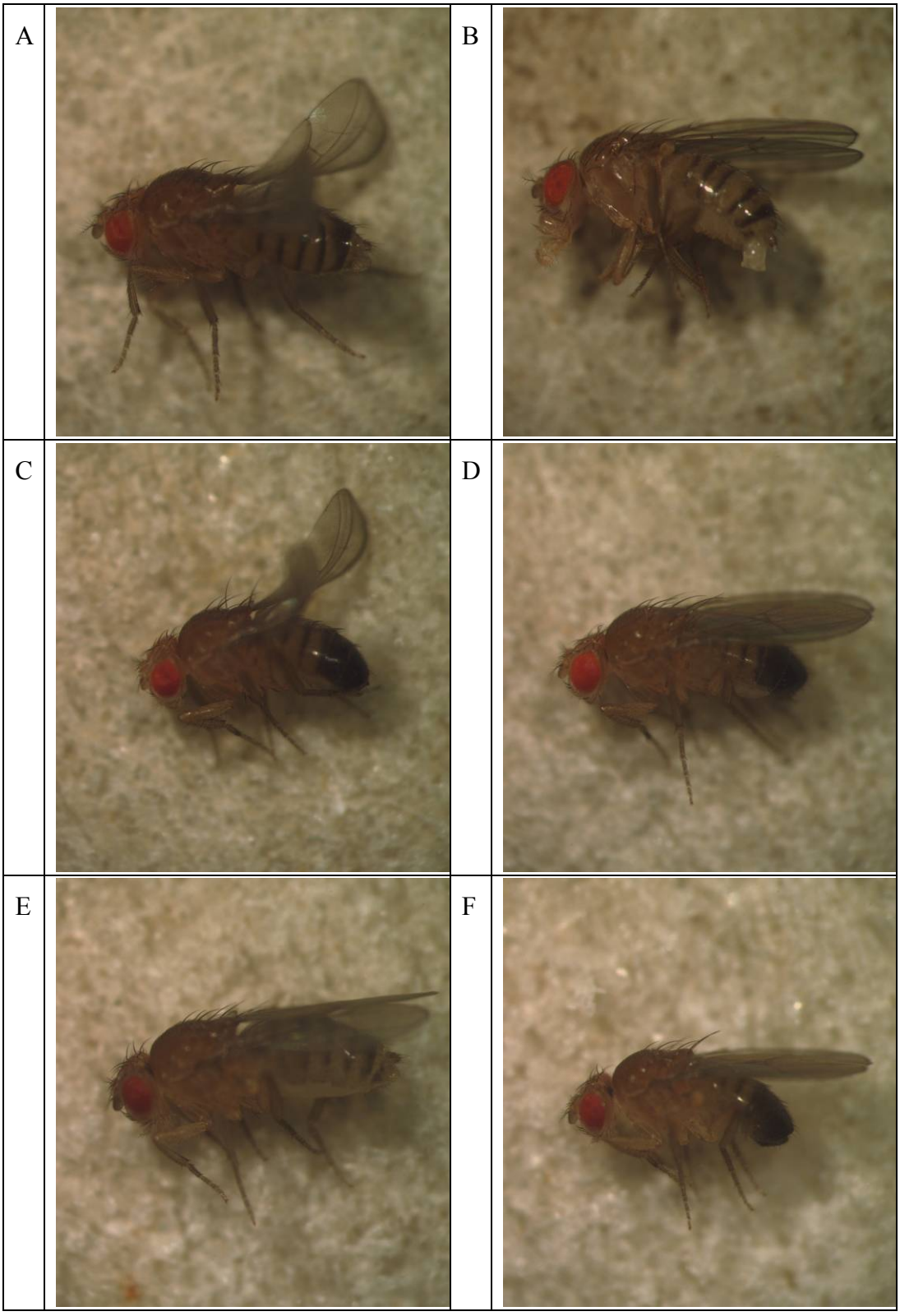


Figure 4.1 Photos of the phenotypes of *Drosophila* with respective genotype of

(A) *elav-GAL4/w; CyO/+; +/+,* (B) *elav-GAL4/w; UAS-Aβ42/+; +/+,* (C) *elav-GAL4/Y; CyO/+; +/+,* (D) *elav-GAL4/Y; UAS-Aβ42/+; +/+,* (E) w^{1118}*/w; +/+ ;GMR-Aβ42*^{K52}*, GMR-Aβ42*^{K53}*/+,* and (F) w^{1118}*/Y; +/+ ;GMR-Aβ42*^{K52}*, GMR-Aβ42*^{K53}*/+*

4.2 Methods

4.2.1 Preparation of Fly Media containing selected TCM extract or donepezil

Flies were fed with positive control donepezil or TM extract mixed with instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC, USA). Concentrations of donepezil used were presented in μmol/ml of fly media, while concentrations of TM extract used were presented in mg/ml of fly media.

4.2.2 Fly Strains

Drosophila strains used in this study were Oregon-R-C (OR) (#5), w^{1118} (#3605) and *elav-GAL4*^{C155} (#458). (Bloomington *Drosophila* Stock Center, Department of Biology, Indiana University, Bloomington, IN, USA). *UAS-Aβ42/CyO* and *GMR-Aβ42*^{K52} *; GMR-Aβ42*^{K53} heterozygous were gifts from Dr. M. Konsolaki (Rutgers University, USA). OR is a wild type *Drosophila*. w^{1118} is a white-eye mutant with a deletion in the sex-linked white gene. *Elav-GAL4*^{C155} is a mutant with an *elav-GAL4* insert on the X chromosome. *UAS-Aβ42*/*CyO* is a mutant with an *UAS-Aβ42* insert and a *Curly of Oster (CyO)* balancer on the 2nd chromosome. *GMR-Aβ42*^{K52}; *GMR-Aβ42*^{K53} heterozygous is a mutant with 2

copies of *Glass Multiple Reporter (GMR)-Aβ42* inserts on the 3rd chromosome.

For longevity and climbing assay, genotypes of *Drosophila* used in this study were as follows: Control: *elav-GAL4^{c155}/Y, Aβ42: elav-GAL4^{c155}/Y; UAS-Aβ42/+; +/+*. *Elav-GAL4^{C155}* line was crossed with w^{1118} line to produce control. *Elav-GAL4^{C155}* line was crossed with *UAS-Aβ42/CyO* to produce *elav-GAL4^{c155}/Y; UAS-Aβ42/+; +/+*. For the psuedopupil assay, *Drosophila* genotypes were as follows: Control: *OR*, Aβ42: *GMR- Aβ42^{K52}; GMR- Aβ42^{K53}* heterozygotes.

4.2.3 Validation of Aβ Expression in the *Drosophila* Model

Total Ribonucleic acid (RNA) was extracted from 30 *Drosophila* heads using TRIzol-chloroform method. Single-stranded cDNA was generated using oligo-dT primers from the Superscript First-Strand Synthesis System. PCR was carried out with Taq DNA Polymerase and a pair of gene-specific primers. The primer for amplifying the Aβ transgene was forward 5'-GAG ACT TTG CAT CTG GCT GCT A-3' and reverse 5'-TGC GTC TGC CTG CAC TGT A-3', according to Cao *et al.* (Cao*, et al.*, 2008). The sequence is encoding for rat preproenkephalin signaling peptide, which is the signaling sequence that linked with Aβ42 to direct its expression as a secretory protein (Ling*, et al.*, 2009). The *Drosophila* RpL32 RNA was amplified as an internal control. The primer for amplifying the RpL32 RNA was forward 5'-GCT AAG CTG TCG CAC AAA TG-3' and reverse 5'-TGT GCA CCA GGA ACT TCT TG-3', according to Laviolette *et al.* (Laviolette*, et al.*, 2005). Reactions were amplified through 35 cycles at the following parameters:

45 sec at 94°C, 30 sec at 55°C, and 90 sec at 72°C, followed by a final extension step at 72°C for 10 min. Then the reaction mixture was maintained at 4°C. The samples were stored at –20°C until use. Amplifications were confirmed by standard submarine gel electrophoresis, using 3% w/v low-melting agarose/TBE gels, stained with ethidium bromide.

4.2.4 Monitoring of Food Intake

The food intake was monitored using Blue No. 1 dye added to the fly media in concentration of 0.5% w/w. Food dye FD&C Blue No. 1 is not affected by gut pH and digestive enzymes, and remains in the digestive tract until it is passed in the fecal pellet (Figure 4.2) (Min and Tatar, 2006, Shimada*, et al.*, 1993). Twenty OregonR flies were allowed to feed for 24 h. The flies were then anesthetized by CO_2 and homogenized in PBS. The supernatant was collected after centrifugation at 15000 xg for 3min. Absorbance at 625nm was measured to estimate the quantity of dye intake, which reflected the amount of food intake. The data was normalized using the untreated group.

Figure 4.2 A 10-day-old *Drosophila* after consuming FD&C blue No. 1 dye. The dye is restricted to the proboscis and digestive tract (adopted from Rera *et al.* (Rera*, et al.*, 2011)).

4.2.5 Effect of TM on Longevity of Aβ Expressing *Drosophila*

Genetic crosses were performed in the vials containing the diet with treatments. The normal control, which did not express Aβ, was maintained on the normal diet. The Aβ expressing control and the positive control were maintained on the normal diet and diet containing 10 mmol donepezil/g of *Drosophila* media, respectively, whereas the two TM groups were fed with diets containing 1 or 5 mg TM/g of *Drosophila* media, respectively. Newly hatched male *Drosophila* were transferred to a new vial with same treatment (30 *Drosophila* per vial) and incubated at 25 °C. Dead *Drosophila* were counted on day 1 and 5 in a 7 day-cycle, and the remaining live *Drosophila* were transferred to a new vial containing the same diet. The feeding lasted for 65 days. One hundred and fifty *Drosophila* were tested for each group.

4.2.6 Climbing Assay

Locomotor function of *Drosophila* was measured according to climbing assay as previously reported by Lee *et al.* (Lee*, et al.*, 2009) with slight modifications. In brief, 30 male *Drosophila* were placed at the bottom of a 15 ml falcon tube, and given 10 s to climb up. At the end of each trial, the number of *Drosophila* that climbed up to a vertical distance of 8 cm or above was recorded. Each trial was performed three times. *Drosophila* were tested at selected time points during the survival assay.

4.2.7 Pseudopupil Assay

The control and Aβ42 *Drosophila* were treated with the same treatments as described above. *Drosophila* heads were examined under a light microscope (Olympus CX31; Olympus, Tokyo, Japan) as described previously (Wong*, et al.*, 2008). Briefly, the compound eye of 5 days old *Drosophila* was viewed under microscope in a dark-field. There were eight photoreceptors in each ommatidium, in which seven of them were visible. Each photoreceptor projected a darkly staining rod, the rhabdomere, into the center of the ommatidium. Under the microscope, the rhabdomeres appeared as bright spots and rhabdomeres in each ommatidium were counted. In the control group, 7 rhabdomeres could be observed in each ommatidium. One hundred ommatidia were observed from 5 to 10 eyes, and the average rhabdomeres count per ommatidium was calculated. Three trials were conducted for each group.

4.3 Results

4.3.1 Validation of Aβ Expression in the *Drosophila* Model

To verify that the amplified fragment is indeed the Aβ transgene, electrophoretic analyses were done on 3% agarose gel. A specific band for our Aβ *Drosophila* was observed showing the product size was slightly above 50bp. On the same gel, our internal control RpL32 was observed in normal *Drosophila* and our Aβ *Drosophila* as a band which the product size was about 100bp. Similar results was obtained for both lines of our Aβ *Drosophila* (Figure 4.3).

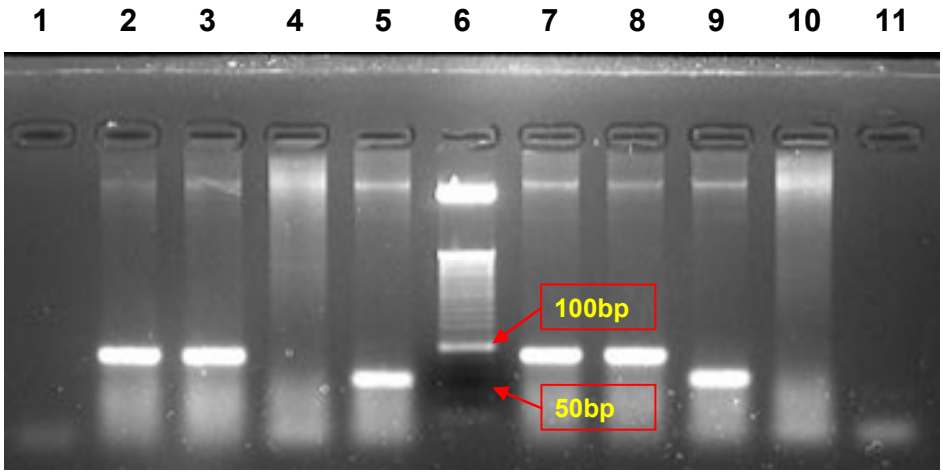


Figure 4.3 Agarose gel of PCR products from the head of both lines of our Aβ *Drosophila*. Lane 1 and 11: Blank; Lane 2 and 4: *elav-GAL4^{c155}/Y; +/+; +/+* Lane 3 and 5: *elav-GAL4^{c155}/Y; UAS-Aβ42/+; +/+* Lane 6: 25 bp ladder; Lane 7 and 9: OR; Lane 8 and 10 *GMR-Aβ42^{K52}; GMR-Aβ42^{K53}* heterozygous. Lane 2, 3, 7 and 8 were amplifying the RpL32 RNA. Lane 4, 5, 9 and 10 were amplifying the Aβ transgene.

4.3.2 Monitoring of Food Intake

There is no significant difference in volume ingested by *Drosophila* when fed with normal diet, with donepezil of 10 μmol/ml diet, or TM of 50 mg/ml diet. However, there is a trend that *Drosophila* with donepezil of 10 μmol/ml diet, or TM of 50 mg/ml diet ingest more than *Drosophila* fed with normal diet (Figure 4.4).

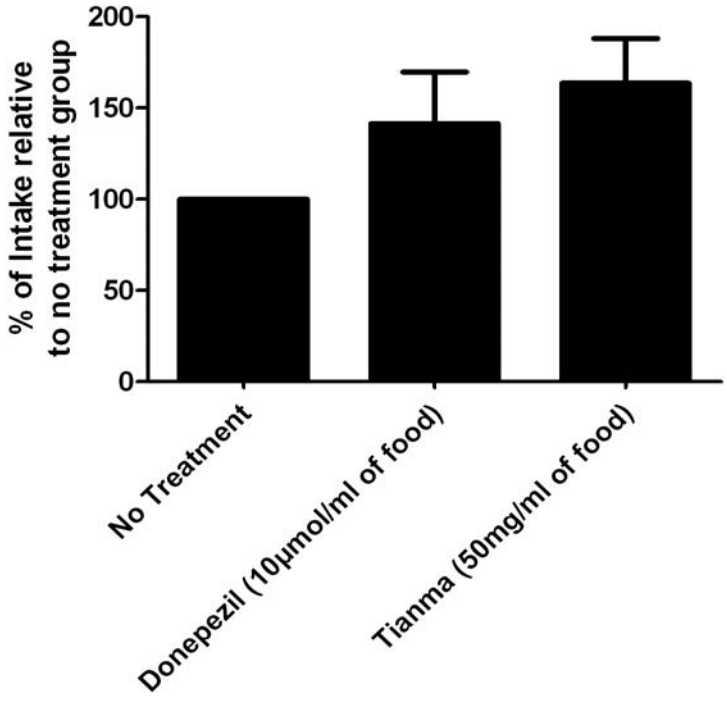


Figure 4.4 Effect of Tianma or donepezil in diet on volume of food intake of *Drosophila*. Results are the means ± SD from 4 independent crosses. Twenty *Drosophila* were collected from each group in each trial.

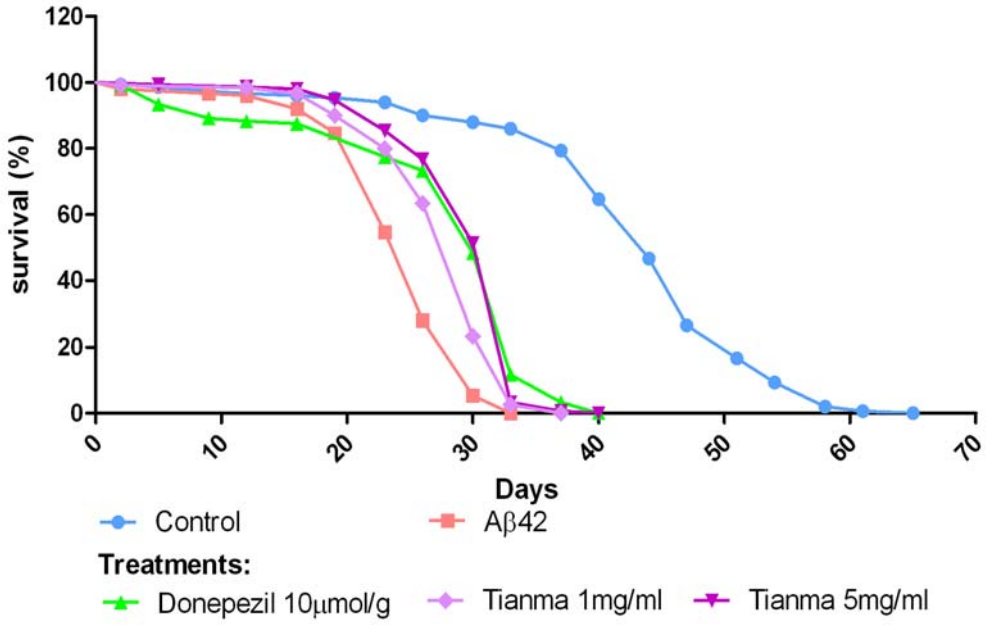
4.3.3 Tianma improves prolong the lifespan and locomotor abilities of

Aβ-expressing *Drosophila*

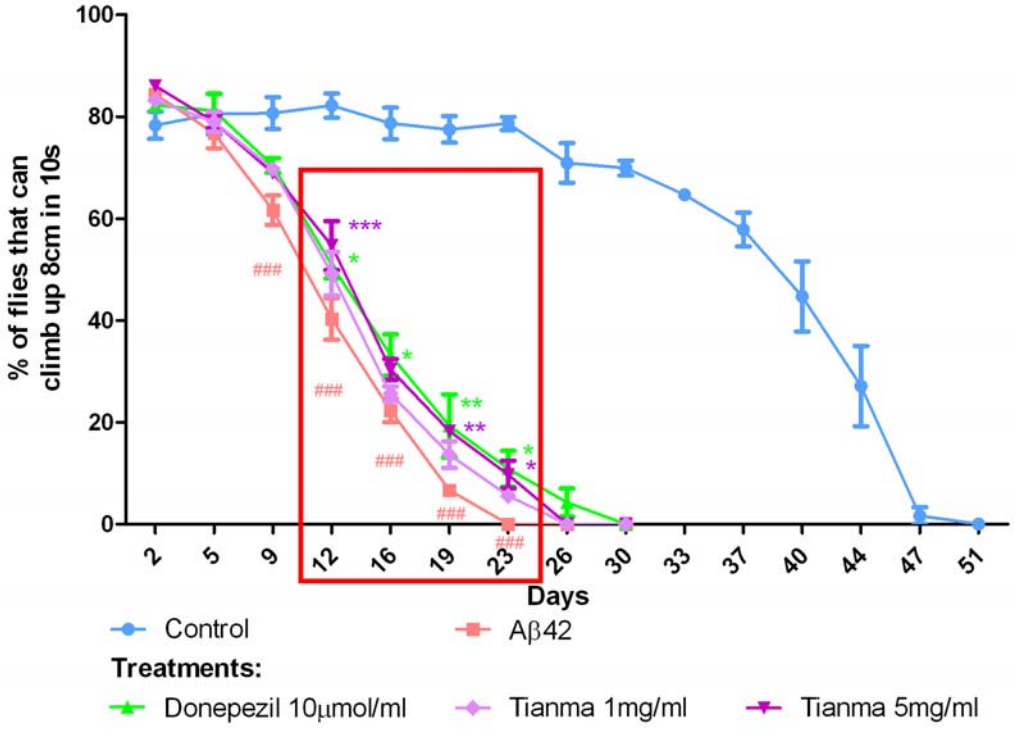
In the present study, we evaluated the neuroprotective effect of aqueous extract of TM, using *Drosophila* AD models. For lifespan experiment, Aß42 *Drosophila* showed a reduction of median and maximum lifespan by 17 days and 32 days when comparing with control, respectively. Both TM treatments significantly improved the survival of *Drosophila* (Figure 4.5A). At 1 mg TM extract/g of *Drosophila* media, median and maximum lifespan was increased by 4 days (12.0%) and 4 days, respectively (p<0.001 for mean increases). At 5 mg TM extract/g of *Drosophila* media, median and maximum lifespan were increased by 7 days (26.9%) and 7 days, respectively (p<0.001 for mean increases).

For locomotor abilities determination, Aß42 *Drosophila* showed significantly impaired locomotion from age of day 9 onwards (Figure 4.5B.i). TM treated flies showed an improvement in locomotor activity from age of day 12 to 23. At day 12, 19 and 23, 5 mg TM extract/g of *Drosophila* media resulted in a 14.4%, 11.6% and 9.74% improvement in locomotion, respectively (p<0.001, p<0.01, p<0.05) (Figure 4.5B.ii) when compared with the Aß42 *Drosophila* without TM treatment. Since TM extract exerted in the high mortality reduction and locomotor improvements in Aß42 flies, the pseudopupil assay was conducted so as to further confirm the neuroprotective effect of TM against Aß associated degeneration.

A


survival (%)
Days
Control
Aβ42
Treatments:
Donepezil 10μmol/g
Tianma 1mg/ml
Tianma 5mg/ml

B.i


% of flies that can climb up 8cm in 10s
Days
Control
Aβ42
Treatments:
Donepezil 10μmol/ml
Tianma 1mg/ml
Tianma 5mg/ml

B.ii

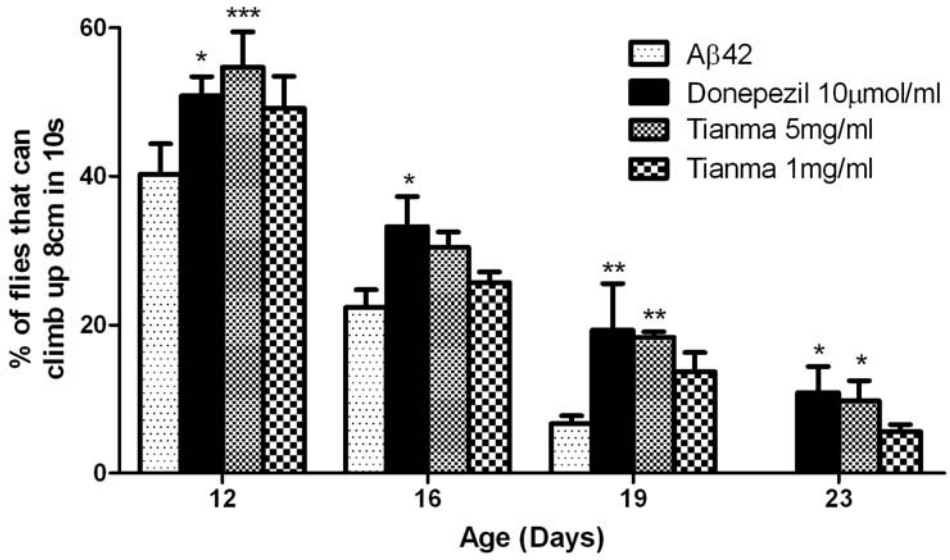


Figure 4.5 Intake of TM increases the **(A)** lifespan and **(B.i)** locomotor activity of Aβ-expressing *Drosophila*. **(B.ii)** an amplification of the region days 12 to 23 showing the differences between the Aß42 group and the treatment groups (The data of Aβ42 *Drosophila* on day 23 is zero). Results are the means ± SEM from five independent crosses. $^{###}p<0.001$, relative to control; *$p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to Aß42 *Drosophila* by two-way ANOVA for locomotor activity. Log-Rank analysis and chi-square comparison were applied to the survival data and $p<0.001$ was obtained when comparing Aß42 *Drosophila* and Donepezil 10μmol/ml or TM 1 mg/ml or 5 mg/ml treated ones (n=150).

4.3.4 Tianma rescues neurodegeneration in ommatidia of Aβ-expressing *Drosophila*

We analyzed the effect of Aß42 on degeneration of retinal tissue of *Drosophila*, which were mainly neurons. Aß42 *Drosophila* contained significantly

more degenerating rhabdomeres, comparing with OregonR. The number of degenerated rhabdomeres was 3.82±0.28. Aß42 *Drosophila* treated with TM (1 and 5 mg/ml of *Drosophila* media) had significanly rescued rhabdomere in each ommatidium, which increased 0.49 and 0.97 rhabdomere count per ommatidium, respectively (Figure 4.6), which reflected preventive effect of TM on neurodegeneration. The preventive effect was comparable to western medication donepezil (10 μmol/ml of *Drosophila* media), which there was 0.78 rhabdomere count per ommatidium than the Aß42 *Drosophila*. These results suggest that TM is effective at protecting neurons from Aß-induced neuron cell death and improves neuronal functions.

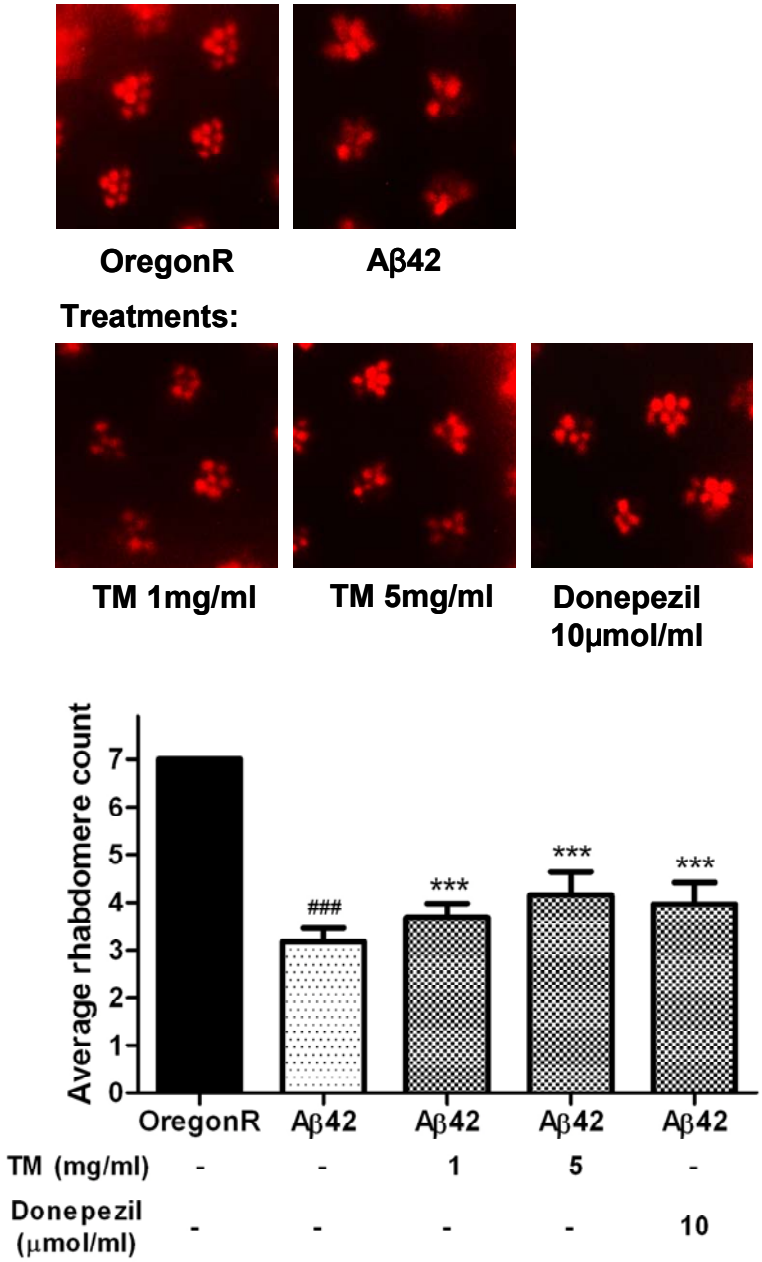


Figure 4.6 Rhabdomere count in the pseudopupil assay. $^{###}p<0.001$, relative to OregonR; *** $p<0.001$ relative to Aß42 *Drosophila* with no treatment by one-way ANOVA. Results are the means ± SEM from 3 independent crosses. One hundred ommatidia were observed from 10 eyes of 5 *Drosophila* from each group in each trial.

4.4 Discussion

The present study demonstrated that the aqueous extract of TM could significantly ameliorate the adverse behavioral or phenotypical changes from Aβ in *Drosophila*. On the *Drosophila* with GMR promoter to express Aβ, the Aβ-mediated degeneration of ommatidia was improved by TM treatment. On the *Drosophila* with *elav-GAL4/UAS* system to express Aβ, TM treatment improved the impaired locomotor function and prolonged the lifespan of the *Drosophila*.

The gel electrophoresis results revealed that verified the specific expression of preproenkephalin signal peptide in our models. The expression of preproenkephalin signal peptide also indicated the co-expression of Aβ42, which was encoded in the same vector with preproenkephalin signal peptide. The expression of pre-proenkephalin would direct the Aβ42 produced to be secretory protein (Ling*, et al.*, 2009), and secreted to the extracellular matrix. Aβ42 would aggregate in the extracellular matrix, exerting neurotoxicity and cause neurodegeneration.

Prior of the drug treatment, we have to make sure that the food intake of *Drosophila* was not interfered by TM extract or donepezil, therefore, we used Blue No. 1 dye mixed *Drosophila* media to feed the *Drosophila*. Food dye FD&C Blue No. 1 is not digested or denatured in the digestive tract and remains in the

gut until excretion. Hence, by quantifying the amount of Blue No. 1 dye in the *Drosophila* gut, the amount of food intake can be determined. We found that the amount of food intake was not affected by the addition of TM or donepezil into the food. If the herbal agents/drug affect the food intake, it implied that the tested substances might cause anorexia in *Drosophila*, which would cause harmful effects to *Drosophila* and reduce the lifespan (Walters*, et al.*, 2012). In addition, the dietary restriction in *Drosophila* would lead to extension of lifespan (Grandison*, et al.*, 2009), which may lead to the false positive or false negative results.

Using the *Drosophila* models, the *in vivo* effects of TM aqueous extract in Alzheimer's disease were studied. The doses of TM were 1X and 5X equivalent to the effective dose we used in the *in vitro* study. Firstly, we found that TM aqueous extract reduced the neurotoxic effect of Aβ to ommatidia. The degree of degeneration of ommatidia reflected the extent of neurodegeneration (Jackson*, et al.*, 1998), based on the fact that rhabdomeres were neurons in nature. Overexpression of Aβ causes plaque formation and neuronal degeneration, which was responsible for the eye morphological changes (Finelli*, et al.*, 2004, Iijima*, et al.*, 2008). The intake of TM extract reduced the adverse effect of Aβ associated plaque formation and rescued the eye phenotype. Similar findings were observed in the other *Drosophila* model with systemic pan-neuronal Aβ42 expression. *Drosophila* would respond to a knock-down stimulus by quickly climbing up the wall of a plastic tube. This simple assay was widely used to assess age-related

decline in locomotor activity in models of neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease (Feany and Bender, 2000, Hong*, et al.*, 2011, Lee*, et al.*, 2009). TM aqueous extract significantly prolonged the lifespan and improved locomotor dysfunction of the flies. We also found that the beneficial effects of TM were comparable with the western medicine, donepezil. Hong et. al. (Hong*, et al.*, 2011) recently reported that Chinese traditional medicinal prescription, SuHeXiang Wan improved the longevity and locomotor ability using the same *Drosophila* model system.

The present study established a stable and rapid *in vivo* platform for the assessment of the efficacy of herbal extract on Aβ-induced Alzheimer's disease phenotypes. The platform will be useful for the further screening of the other herbal extract, which shows positive effect in the *in vitro* studies. In summary, the results of the current study suggested that TM aqueous extracts confers an improvement to AD-like pathology of Aβ42 overexpressing in different *Drosophila* models. The *in vivo* neuroprotective effect of TM on *Drososphila* consolidates our findings in the *in vitro* study, which prove TM is not only effective on the cell line, but also on animal model. Further research on *Drosophila* can be done according to our *in vitro* results, which Aβ-mediated apoptosis, oxidative stress and tau hyperphosphorylation and the efficacy of TM on attenuating them can be investigated.

Chapter 5
In vitro Protective Effects of *Gastrodia elata* Aqueous Extract against Excitotoxcity and Acetylcholine Deficit in Primary Cortical Neurons

5.1 Introduction

In chapter 3, PC12 cell line was used to investigate the effect of the selected herbal extracts on the Aβ-induced cytotoxic effect. This platform is simple and robust, which can be easily adopted as a high-throughput screening system. However, the PC12 cell line is dopaminergic in nature (Greene and Tischler, 1976) and complete subunit of glutamate receptor is absent (Edwards*, et al.*, 2007), and therefore the cellular excitotoxicity is not inducible in the cell line (Froissard and Duval, 1994, Penugonda*, et al.*, 2005). Cytotoxicity is mediated by the oxidative glutamatergic toxicity pathway (Kawakami*, et al.*, 2011). In our preliminary study, glutamate was unable to induce cytotoxicity by treatment of 24 h and concentration of 20 mM (data not shown). At the concentration, the pH value of the culture medium was already acidic, which was not feasible for the maintenance of a health culture. Therefore, another *in vitro* platform is needed to test the glutamate-induced excitotoxicity. Various types of neurons, such as the hippocampal neurons, cortical neurons, oligodendrocytes and cerebellar granule cells, possess glutamate receptors. Amongst them, hippocampal and cortical neurons are closely related to cognitive ability and memory, and the population of cortical neuron is much higher than hippocampal neurons in brain. Therefore, primary cortical neuronal cells are used in the present study. Nevertheless, as mentioned in chapter 1, cholinergic deficit is another cause of neurodegernation during Alzheimer's disease. Inhibition of acetylcholinesterase can preserve

acetylcholine level and slow down the deterioration of cognitive function in Alzheimer's disease patients, which is the main protective mechanism against Alzheimer's disease of the current medications (Rogers and Friedhoff, 1996). In order to further investigate the effect of the selected herbal extract on acetylcholinesterase activity in cholinergic neuron, primary cortical neuronal cells, which are expressing high level of acetylcholinesterase, are required.

Excitotoxicity is the final common destructive metabolic pathway of many different neurodegenerative diseases as mentioned in chapter 1 (Dong*, et al.*, 2009). Briefly, it is caused by the excessive excitation of neurotransmitter receptor - glutamate. Glutamate is one of the main excitatory neurotransmitter which is responsible for memory, and is the main mediator of excitotoxicity (Mark*, et al.*, 2001). Excitation of NMDA receptor by glutamate causes the influx of calcium ions (Dodd*, et al.*, 1994). The disruption of calcium homeostasis leads to the activation of a series of enzymes. These enzymes in turn catalyze a series of membrane, cytoplasmic, and nuclear degradative events and damage the neurons (Nicotera*, et al.*, 1992). To mimic the toxicity, previous studies used glutamate to activate the NMDA receptors and induce excitotoxicity in primary cortical neurons or other types of neurons (Lemus-Molina*, et al.*, 2009, Yu*, et al.*, 2003). Apoptosis and oxidative stress is once again the downstream pathway of excitotoxicity (Dong*, et al.*, 2009). Therefore, monitoring the cell viability, apoptotic condition and level of ROS can reflect the efficacy of our herbal extract against excitotoxicity.

The isolation and culture of neurons from the adult mammalian brain generally fail (Brewer, 1997). There are two main difficulties faced. (1) Isolation of cells from an intertwined network of thousands of adhesive contacts without causing irreversible damage of sheared axons and dendrites (Brewer and Torricelli, 2007), because adult neurons are generally unable to regenerate. (2) Separating the different cell types, especially neurons from glia, to perform experiments on specific cell types, because the proportion of glia in adult brain is much higher than that in embryonic or neonatal brain (Ling, 1976). Based on these findings, the present study started with embryonic tissue with fewer connections and higher content of neurons.

The culture was maintained in neurobasal medium with B27 supplement. The B27 medium supplement consisted of essential fatty acids, antioxidants, vitamins and hormones, which maintained the viability of primary neurons culture from various regions of the rat brain for weeks (Brewer, 1997, Brewer and Cotman, 1989, Brewer*, et al.*, 1993). Two regions of rat brain were commonly used, the cerebral cortex and the hippocampus. Hippocampus is a part of cerebral cortex, which is vital for the encoding and recall of episodic memory (Lisman and Grace, 2005), while the whole cerebral cortex was divided into parts that control different functions, such as locomotor, sensory, visual, memory and olfactory functions. In the present study, cortical neurons were used due to the requirement of a large quantity of neuronal cells. On DIV 2, cytosine arabinoside was added to

the culture. Cytosine arabinoside is a mitotic inhibitor that inhibits the DNA synthesis and locks the cells in the S phase. However, cells that remain in G_1 phase were spared from the cytotoxic effects of cytosine arabinoside (Tobey, 1972). Therefore, cytosine arabinoside was able to kill the proliferating glial cells and spare the non-proliferating cortical neurons (Yu*, et al.*, 2009). Hence, a model with high proportion of primary cortical neurons was established in the present study.

In the present study, the objectives are to test or confirm the effect of TM extract on glutamate-induced excitotoxicity, Aβ-induced cytotoxicity and its subsequent acetylcholinesterase activation. The primary neuron model is actually the best *in vitro* model to mimic the brain environment, based on the fact that it was freshly extracted from the brain and is neuron in nature. However, the model is more time consuming, more susceptible to contamination, cost higher to maintain and is involving the sacrifice of animals. Therefore, this model was used after narrowing down the selected TCM into one.

5.2 Methods

5.2.1 Preparation of Primary Cortical Neurons and Treatment

Primary cultures of rat cortical neurons prepared from 1-2 day-old neonatal Sprague Dawley (SD) rats. Isolated rat cerebral cortex was treated with 0.25% trypsin at 37 ℃ for 20 min, and triturated with a pasteur pipette, dispersed cells were diluted to a concentration of 5×10^5 cells/ml on poly-L-lysine precoated

96-well plates or 6-well plate. Cultures were maintained in neurobasal medium with 2% B27 supplement, 2 mM GlutaMAX, 5% (v/v) FBS, 50 U/ml of penicillin and 0.05 mg/ml streptomycin at 37 ◦C with 95% air and 5% CO2. On day *in vitro* (DIV) 2, 5 μM of cytosine arabinoside were added to the medium to suppress the proliferation of the glia cells. The medium was renewed every 2 days during the culturing period. The glutamate-induced excitotoxicity was carried out on DIV 5. On DIV 5, cultured cortical neurons were exposed to various concentrations of Tianma and selected dose of glutamate for 24 h, or 10 μM of aggregated $A\beta_{25–35}$ for 48 h.

The neurite morphology after the glutamate treatments of 50, 100 and 200μM were assessed by immunoflorescence staining of the neurites. The culture media were removed and rinsed twice with PBS. The cells were fixed with acetone-methanol (1:1) at room temperature and rinsed with PBS. The fixed cells were permeabilized with 0.3% Triton-X 100 in PBS and rinsed with PBS. Blocking was done by incubating cells in 1% BSA in PBS at room temperature for 1 h. Then, cells were incubated with rabbit anti-MAP2 antibody in 1% BSA in PBS at 4 degree overnight. After incubation, the cells were rinsed with PBS and incubated with Alexa Fluor® 488 Conjugated anti-rabbit secondary antibody in 1% BSA in D-PBS at room temperature for 90 minutes. The cells were rinsed with D-PBS and counter stained with Hoechst 33342 for 20 seconds. After washing by PBS, the cells were observed under florescence microscope with suitable excitation (Hoechst: 350nm, Alexa Fluor® 488: 495nm). From the results,

100 μM of glutamate resulted in degeneration of neurites and was selected to induce the excitotoxicity.

5.2.2 Cell Viability Assay

The treated cells in 96-well plates were tested according to section 2.4.1.

5.2.3 Acetylcholinesterase Activity Assay

The primary cortical neurons were treated with 10 μM of aggregated $A\beta_{25-35}$ for 48 h in 6-well plate. The treated cells were collected, and washed twice with ice cold PBS. Primary cortical neurons were lysed in cell lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM MgCl2, 0.1% Triton X-100) with 2 freeze/thaw cycles. The assay for AChE inhibition was performed according to the methods developed by Ellman *et al*. (Ellman*, et al.*, 1961). Briefly, the assay measured the conversion of acetylthiocholine to thiocholine by AChE, which thiocholine formed would react with dithiobisnitrobenzoate to form yellow coloured compound and measured spectrophotometrically.

5.3 Results

5.3.1 The effect of glutamate in induction of excitotoxicity

Exposure of cultured cortical neurons to various concentrations of glutamate for 24 h resulted in the degeneration of the neurite, which indicated in green colour in Figure 5.1. We can see the amount of neurite decreased as the dose of glutamate increase. At 200 μM of glutamate, the neurite completely disappeared

and the neucleus of neurons appeared as bright blue spots, which could be an indicator of apoptosis.

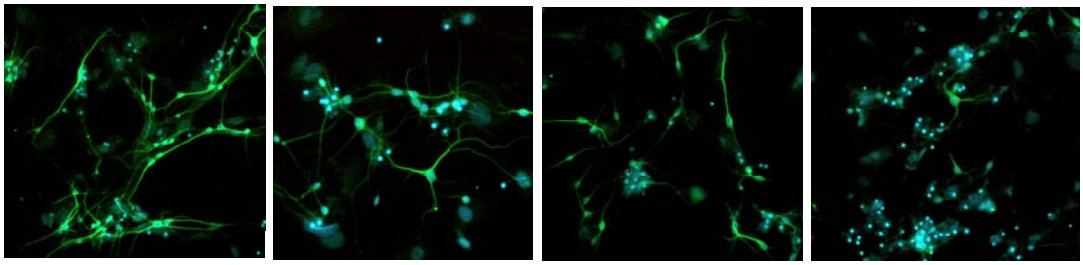


Figure 5.1 The effect of different concentrations of glutamate on primary cortical neurons. The excitotoxicity effect on primary cortical neurons was analyzed by immunofluorescent staining using rabbit anti-MAP2 primary antibody and Alexa Fluor® 488 Conjugated anti-rabbit secondary antibody (green); hoechst 33342 (Blue) (Magnification = 20X)

5.3.2 The effect of TM on excitotoxicity

From different doses of glutamate, 100 μM of glutamate was chosen to induce excitotoxicity. Exposure of cultured cortical neurons to 100 μM of glutamate for 24 h resulted in 20.28±6.97% decrease in viability, while the treatment of 125 to 1000 μg/ml of TM did not improve the viability. Meanwhile, the treatment of 100 μM of memantine, a medication on Alzheimer's disease (AD) and a specific NMDA receptor antagonist (Kutzing*, et al.*, 2012), improved the viability of the cortical neurons back to 103.00±2.43% of control (Figure 5.2).

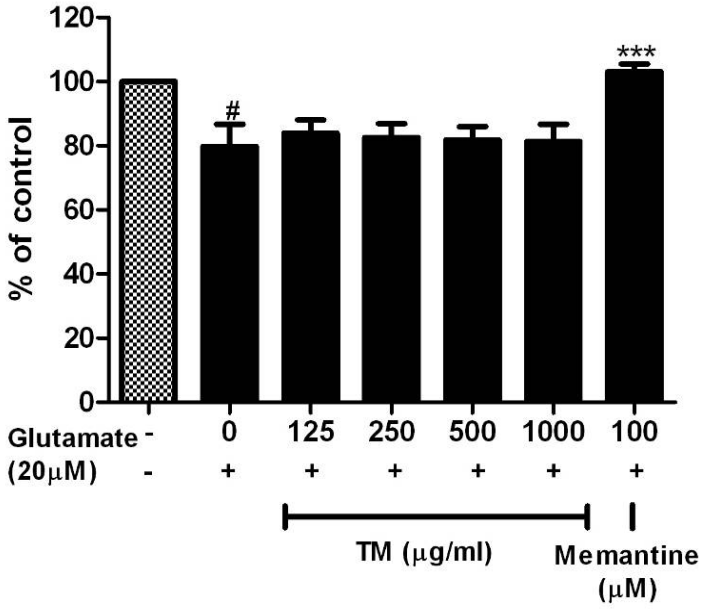


Figure 5.2 Effect of TM and memantine on glutamate-induced cytotoxicity on primary cortical neurons. Results are the means ± SD from three separate experiments. # $p<0.01$, relative to control; *** $p<0.001$, relative to glutamate treatment only by one-way ANOVA.

5.3.3 The effect of TM on Aβ-induced cytotoxicity

The effects of TM on the Aβ-induced cytotoxicity were verified in primary cortical neurons, which is neuron in nature instead of a tumour cell. The viability of Aβ-treated control was decreased by 51.84±3.25%. The treatment of TM significantly increased the viability to 59.03±3.28%, 59.73±1.23%, 61.39±1.62% and 68.41±2.07% at 125, 250, 500 and 1000 μg/ml, respectively (Figure 5.3).

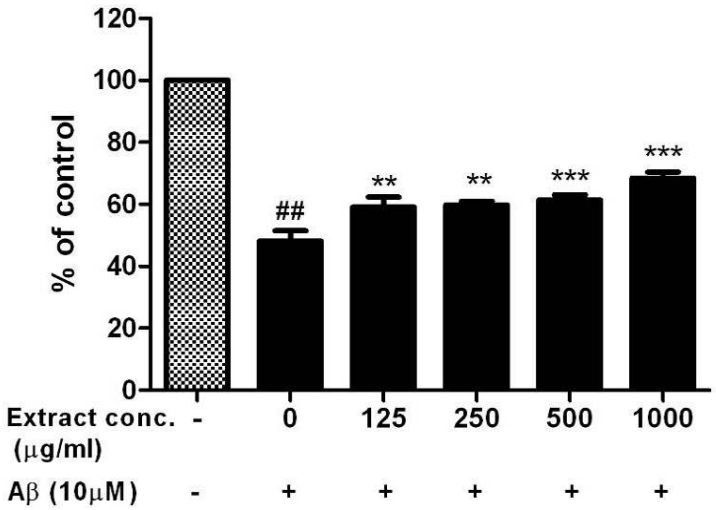


Figure 5.3 Protective effect of TM on Aβ-induced cytotoxicity on primary cortical neurons. Results are the means ± SD from three separate experiments. ## p<0.05, relative to control; * p<0.01, ** p<0.05 and *** p<0.001, relative to Aβ treatment only by one-way ANOVA.

5.3.4 The effect of TM on acetylcholinesterase in Aβ-induced primary cortical neurons

Due to the low expression of AChE in PC12 cell line, primary cortical neurons is a better platform to investigate the activity of AChE, which was found to be increased in AD pathology and contribute to the cholinergic deficit and memory impairment (Micheau and Marighetto, 2011). The effects of TM on the AChE activity was determined by Ellman's assay (Ellman*, et al.*, 1961). The AChE activity of Aβ-treated control was increased by 181.00±17.52%. The treatment of TM significantly inhibited the activity back to 148.30±32.19%, 132.00±33.42% and 112.30±11.15% at 250, 500 and 1000 μg/ml, respectively

(Figure 5.4).

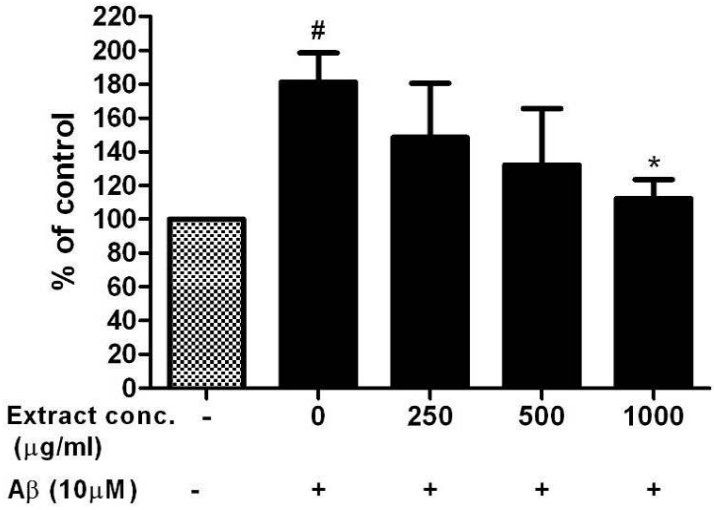


Figure 5.4 Anti-acetylcholinesterase effect of TM on Aβ-induced cytotoxicity in primary cortical neurons. TM extract inhibited the activities of acetylcholinesterase in Aβ-treated neurons. Results are the means ± SD from three separate experiments. $^{\#}p<0.05$, relative to control; $^{*}p<0.05$ relative to Aβ treatment only by one-way ANOVA.

5.4 Discussion

Studies were widely done in exploration of new drugs to inhibit glutamate-induced excitotoxicty on primary neurons (Lee*, et al.*, 2003, Lemus-Molina*, et al.*, 2009, Weller*, et al.*, 1993, Yu*, et al.*, 2003). Our positive control, mematine, was able to protect cerebellar and cortical neurons against glutamate toxicity (Weller*, et al.*, 1993). The mechanism was by the direct blockage of the NMDA receptors. Some herbal extracts, such as the fruit of

Alpinia oxyphylla Miq., also demonstrated protective effect against glutamate-induced excitotoxicty on the primary cortical neurons (Yu*, et al.*, 2003). In the present study, exposure of cultures to a glutamate (100 to 200 μM) for 24 h resulted in degeneration of neuritis and some extent of apoptosis. The results showed that TM was ineffective against excitotoxicity, which reveal that the neuroprotective effect of TM may not through inhibition of excitotoxicity. However, the decrease in cell viability induced by glutamate is not very significant, which possibly due to the insufficient concentration of glutamate, or the presence of anti-oxidants in the culture medium, serum and supplements (Brewer, 1997). These factors may interfere with the action of TM and result in the loss of effectiveness on excitotoxicity. Therefore, improvements can be made by increasing the concentration of glutamate, and using medium or buffer without anti-oxidants (Lemus-Molina*, et al.*, 2009). However, the short comings is that the medium or buffer may not have sufficient nutrients for the survival of the neurons in long term, which may result in the apoptosis due to starvation instead of glutamate-induced excitotoxicity. Further study is required to optimize the conditions to confirm our results.

The study on PC12 cell line revealed the protective effect of TM against Aβ-induced cytotoxicity. To consolidate the result, the effect of TM against Aβ-induced cytotoxicity was tested on primary cortical neuron. Based of the fact that primary cortical neuron is more delicate (Hansen*, et al.*, 1995), the concentration of $A\beta_{25\text{-}35}$ was decreased from 20 μM to 10 μM to avoid high

proportion of apoptotic cell, which may be too severe and the damage was irreversible. In the study, TM increased the viability of $A\beta_{25\text{-}35}$-treated primary cortical neurons in a dose dependent manner. The results matched with our findings on PC12 cells. As the mechanism elucidation was already done on PC12 cells, the same experiments were not repeated in this platform. However, the AChE activity in undifferentiated PC12 cells is not as high as primary cortical neurons. Although our previous results revealed that the direct addition of TM can inhibit the AChE, the *in vitro* effect of TM on AChE was unknown. Moreover, study had observation of deficiency in ACh and excessive amount of AChE around amyloid plaques in autopsy from AD patients (Davies and Maloney, 1976). *In vitro* study on cultured retinal cells revealed that Aβ-induced oxidative stress was probably the cause of the enhancement of AChE activity (Melo*, et al.*, 2003). Additionly, AChE and Aβ would aggregate to form complexes and increase the neurotoxic effect (Munoz and Inestrosa, 1999). On the other, AChE, in turn, would modulate the fibrillogenesis of Aβ fragment 12-28 and 25-35, which promote the amyloid plaque formation (Alvarez*, et al.*, 1997). *In vivo* study also demonstrated the over-expression of AChE would result in an increase in plaque formation using doubly transgenic mice, which express human amyloid precursor protein (APP) and human AChE (Rees*, et al.*, 2003). Therefore, AChE and Aβ would form a vicious cycle, which promote the activity and cytotoxic effect of each other and leading to neurodegeneration. To consolidate and further our previous result with the Aβ-induced cytotoxicity, the present study tested the effect of TM on AChE of primary cortical neuron with Aβ-induction. The results

from our AChE assay suggested that Aβ enhance AChE, which is the same as the other studies (Melo*, et al.*, 2003). TM significantly inhibited AChE activity in a dose-dependent manner. Based on our previous results, TM was able to reduce Aβ-induced oxidative stress, which may be the explanation of the reduced AChE activity. However, there is also possibility that TM directly inhibited AChE activity, as shown by our previous results. The inhibition of AChE can explain the effect of TM to increase the survival of primary cortical neurons after Aβ-induced cytotoxicity.

The present study demonstrated that TM was unable to inhibit the excitotoxicity. However, it was able to attenuate the AChE activation and cell death induced by Aβ. The inhibition of AChE activity by TM can attenuate the promotion of fibrillogenesis of Aβ, and slow down the breakdown and depletion of acetylcholine, and subsequently slow down plaque formation and cholinergic deficit, which are two important pathogenic processes in AD. Therefore, the present study further consolidates TM's function in slowing down the progression of AD.

Chapter 6
In vivo Protective Effects of *Gastrodia elata* Aqueous Extract against Trumatic Brain Injury in Rat

6.1 Introduction

As TM was shown to be neuroprotective on *in vitro* cell line and primary culture and *in vivo Drosophila* models, while TM is also traditionally used for treatment of various types of dementia, we speculated that TM can be used to treat other types of neurological disorder, such as brain trauma. In the present study, the right parietal cortex and the underneath hippocampus of the rats was damaged traumatically instead of using Aβ, which aim to test the efficiency of TM on traumatic brain injury.

Traumatic brain injury (TBI) is a one of the common causes of morbidity and mortality (Leon-Carrion*, et al.*, 2005). The incidence rate of TBI is between 150–250 cases per 100,000 population per year worldwide. A study reported the annual average death rate was 23.6 per 100000 population in West Virginia, USA. Normally, the physical traumatic head injury often associates with numerous neurological disorders in cognitive function, motor function, sensation and emotion. Therefore, dementia and physical disability are often the consequences of brain injury (Bloom*, et al.*, 2001).

TBI refers to a bump, blow, or jolt to the head that damage the brain. The damage can be a closed one, which caused by the movement of the brain within the skull. TBI are often due to physical crashes and falls during sports activity, slipping or car accidents. On the other hand, the damage can also be a penetrating

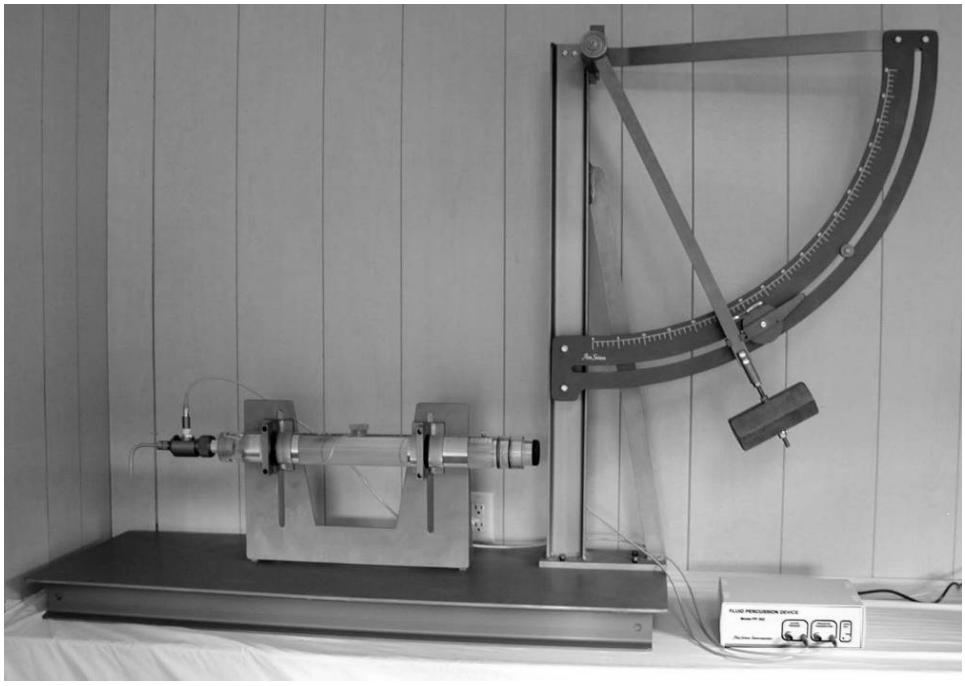
one, which caused by the entrance of foreign objects to the brain. The damages are often done by firearm, sharp object or heavy blunt objects, which have many different causes (Cernak, 2005).

The initial stage of traumatic brain injury is characterized by direct brain damage, accumulation of cerebrospinal fluid (CSF) in the ventricles of the brain and impaired brain energy metabolism. The physical injury leads to physical injury of the neurons, increased membrane permeability and causes edema (Werner and Engelhard, 2007). Accumulation of CSF causes a malnourishment of brain tissue and an "ischemia-like" condition. Insufficiency of oxygen leads to anaerobic glycolysis, and cause the accumulation of lactic acid. Some of the neuron may undergo apoptosis due to the breakdown of energy supply. The remaining cells need to face the failure of energy supply by ATP, which causes the failure of energy-dependent membrane ion pumps and the later stage of traumatic brain injury. The ion imbalance causes the membrane depolarization and subsequent release of neurotransmitters. The excessive release of neurotransmitters, such as L-glutamate and aspartate, will cause the activation of voltage-dependent calcium and sodium-channels (Yi and Hazell, 2006). Excessive calcium activates lipid peroxidases, proteases, and phospholipases, leading to the increase in intracellular free fatty acids, free radicals and cellular catabolic processes. The free radicals will cause oxidative stress and the catabolic processes would direct the cell towards apoptotic pathway. The whole TBI process leads the release of various inflammatory mediators including proinflammatory cytokines, prostaglandins, free radicals, and complement, which attracted the activated

macrophage and microglia. Moreover, the apoptotic neurons will also attract even more activated macrophage and microglia (Fitch and Silver, 1997), leading to inflammation. The injured neurons and normal adjacent neuronal tissue will be eliminated. Reactive astrocytes will produce glial fibrillary acidic protein and modify the extracellular matrix, ultimately lead to scar formation (Stichel and Muller, 1998). Simply, neuronal tissue would face physical injury, impairment of energy metabolism, oxidative stress, inflammation and apoptosis, throughout the process of TBI.

To mimic the TBI, mice and rat models were often used. The most commonly used induction methods are fluid percussion injury (FPI) (Figure 6.1A) and controlled cortical impact (CCI) (Figure 6.1B). Both of them would produce an impact (penetrating) type injury. The major difference is that FPI use the pressure pulse of saline to deform the brain, while CCI use the air-driven metallic piston to impact the brain (Cernak, 2005) (Figure 6.1C). CCI was used in this study due to its advantages of more control on the mechanical parameters, such as impact velocity, impact time, impact depth and probe diameter. CCI causes highly reproducible and controllable acute neuronal injury (Brody*, et al.*, 2007). Different behavioural defects can be induced, such as motor coordination or cognitive defects, which the degree and types can be controlled by the impact velocity, time, depth and location. In the present study, the right parietal cortex, which controls the locomotor action, was damaged by CCI. Therefore, motor coordination defect was tested to determine the neuronal damage of CCI and the neuroprotective effect of TM.

A



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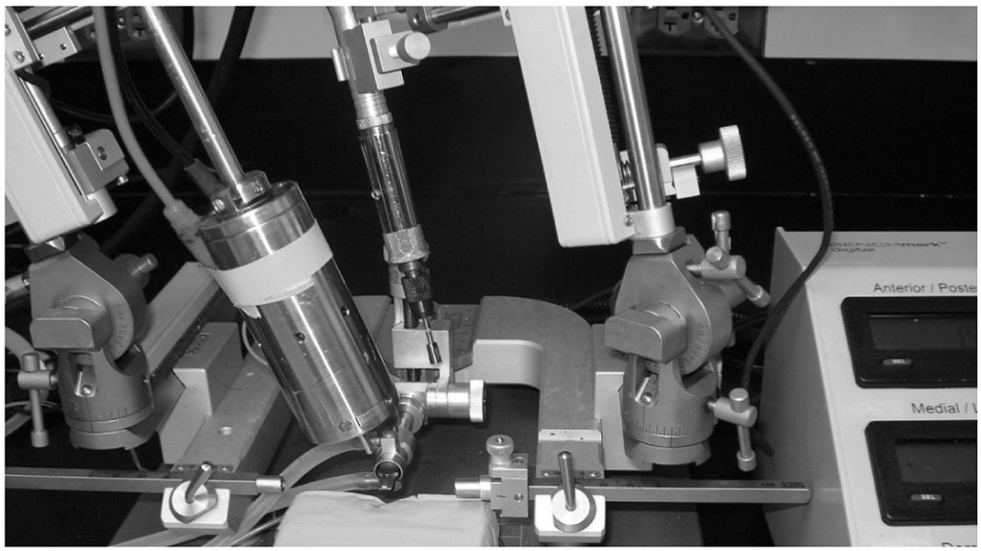


C

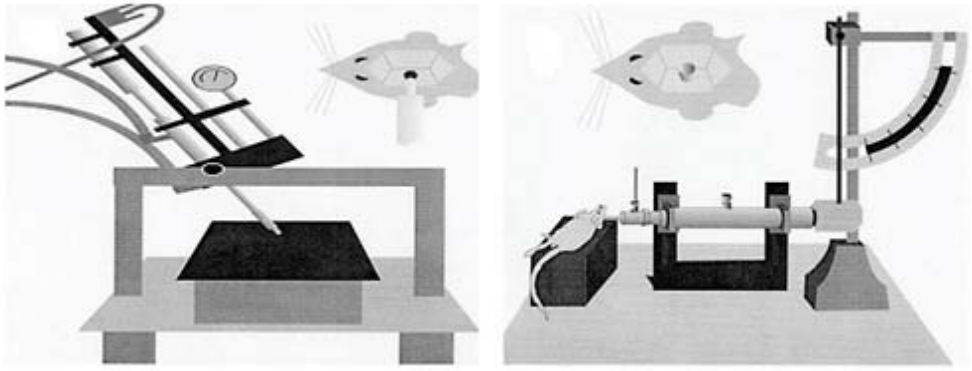


Figure 6.1 Common induction methods for TBI. (A) Photograph of the fluid percussion device. (B) Photograph of the impactor device mounted on the left arm of a stereotaxic device. (C) Rodent models of TBI (left: CCI; right: FPI) (adopted from (Brody*, et al.*, 2007, Erdman*, et al.*, 2011, Kabadi*, et al.*, 2010))

There is a wide varity of model to study the locomotor performance of the rats, such as Rotarod, open field test, beam-walking task, gait analysis (Basso*, et al.*, 1995, Matsuura*, et al.*, 1997). In the present study, rotarod would be used. Rotarod system is a automated system to measure the motor coordination of the rat or mice (Bogo*, et al.*, 1981). This system consists of a rod that rotated at accelerating rate, and high enough to induce avoidance of fall. Rats were placed on the rod, and they would try to stay on the rod to avoid the fall (Figure 6.2). The setting provides a model that started at a slow enough speed so that all rat could stay on, yet became difficult enough that all rat would eventually fall off (Jones and Roberts, 1968). The duration for the rat to be able to stay on the rotating rod is directly proportional to its locomotor performance. Rats with brain injury at locomotor controlling regions would fall off earlier than the normal rats. The rotarod system provides a sensitive and reproducible platform for quantitative

measurement of motor incoordination in rat and mice (Jones and Roberts, 1968). Previous study suggested that the pre-training could help to ensure the consistent group basal locomotor ability over repeated test sessions (Rustay*, et al.*, 2003). During the pre-training, the rotarod performance was mainly affected by the motor learning process, which is controlled by complex interaction between supplementary motor area, prefrontal parietal cortex, basal ganglia, and cerebellum, which is similar as in human (Jueptner*, et al.*, 1997). After pre-training, the running on rotarod became a conditioned reflex. The rotarod performance was mainly related to the locomotor function, which mainly controlled by motor cortex, motor divisions of the basal ganglia and cerebellum (Hikosaka*, et al.*, 2002). Without pre-training, repeated testing would result in improvement in the latency to fall, which was mainly due to the learning. Due to the sensitivity, reproducibility, its involvement in various brain area, its simpleness and the quantitative data it gives, rotarod is one of the most commonly used assays for trauma-related motor incoordination (Hamm*, et al.*, 1994).

Figure 6.2 Photograph of the RotaRod System.

In the present study, the objective is to evaluate the efficacy of TM on

protection against CCI-induced brain trauma on rats. Rotarod assessment was used to determine the extent of neuronal damage in rats' brains, which is directly related to the brain-controlled motor coordination. The target of the study was to demonstrate the possible neuroprotective effect of TM on the brain trauma, which reflected by the improved performance on the rotarod assessment in TM-treated TBI rats.

6.2 Methods

6.2.1 Traumatic Brain Injury

Rats were anesthetized with ketamine and xylazine (80 mg/kg body weight). After ensuring complete sedation of the animal by a firm pinch on the paw, the animal was positioned in a stereotactic frame. Then, the skin above the skull was prepped with Betadine, and a small incision was made in the skin above the skull along midline using a sterile scalpel-blade exposing the skull. A ~5 mm circular craniotomy was performed, lateral to the mid-sagittal suture on the right side, and below the bregma, with the use of dental microdrill. The dura was kept intact during craniotomy. The exposed cortex with intact dura was subjected to controlled cortical impact injury (CCI). The position of the impactor was carefully centered above the craniotomy area. Controlled cortical impact was produced, using an electromagnetically controlled cortical impact device with a 3.0 mm diameter impactor rod-tip, impacting the cortex at the velocity of 4.0 m/s with impact depth of 2.50 mm and impact time of 50 ms to produce injury within the right parietal cortex. At the end of CCI procedure, bone wax was applied to the exposed dura and craniotomy-surface. The skin-incision was closed with a

followed by the application of Betadine over the sutured area. Same surgical procedures were done to the sham-injured control animals except the controlled cortical impact procedures. The animal with traumatic brain injury (TBI) was kept warm during the experiment.

6.2.2 Drug Preparation and Administration

Tianma (505mg/kg and 1515mg/kg) dissolved in water and was administrated orally daily from day 1 to day 7 post-TBI. The dose was the 1X and 3X human equivalent dose. Accroding to Chinese Pharmacopoeia 2010, the maximum daily intake of TM was 10 g, in the form of raw herb. By assuming the weight of an adult is 60 kg and our extraction yield of TM is 48.90%, the maximum dose of TM in human is 81.5 mg/kg. The dose was converted to animal doses based on body surface area. The dose would be increased by 6.2 times for rats. Therefore, the 1X human equivalent dose of TM was 505 mg/kg, and 3X human equivalent dose was 1515 mg/kg.

6.2.3 Assessment of Motor Performance

Locomotor performances were tested using an accelerating rotarod motor test. The animal was placed on the accelerating (10–30 rpm within 100 seconds, 30 rpm until 150 seconds) rotarod cylinder, and the rat's task was to run and to maintain its equilibrium on the rotating rod, which rotated at a gradually increasing speed. When the rat falls off the rod, its endurance time was recorded in seconds. All rat were pretrained by undergoing five trials performed daily for 2 days before TBI was induced to ensure stable baselines. Before TBI, the rats were

tested to establish the baseline rotarod score. After TBI, the rats were tested on days 3 and 6 post-TBI. The rotarod score is calculated as the time duration of the rat staying on the rotating rod.

6.3 Result

6.3.1 Effect of TM on the weight of the rats

From the results, the weight of the rat was not significantly affected by the surgical procedures and the TBI (Figure 6.3). There was no significant drop in the weight of the rats, indicating the rats were in good health condition. Moreover, the treatments of TM did not cause significant weight changes, which indicated that the dose of TM given to the rats was not causing health problems to them.

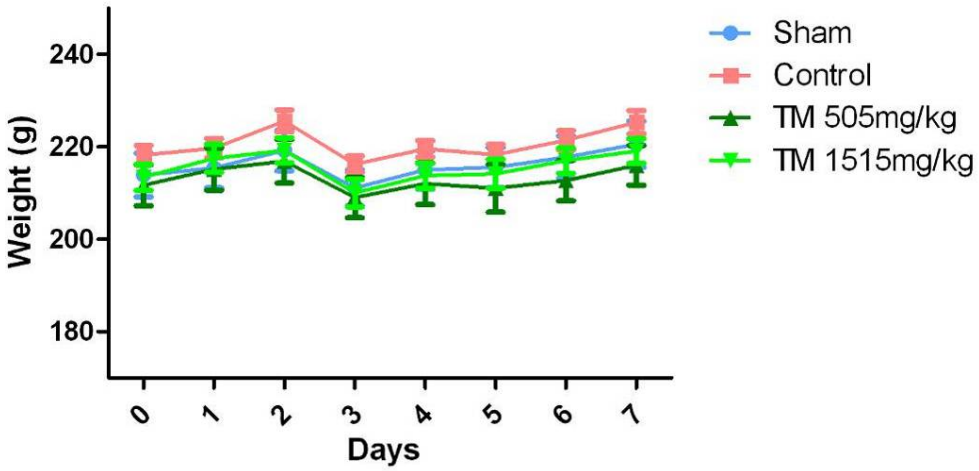
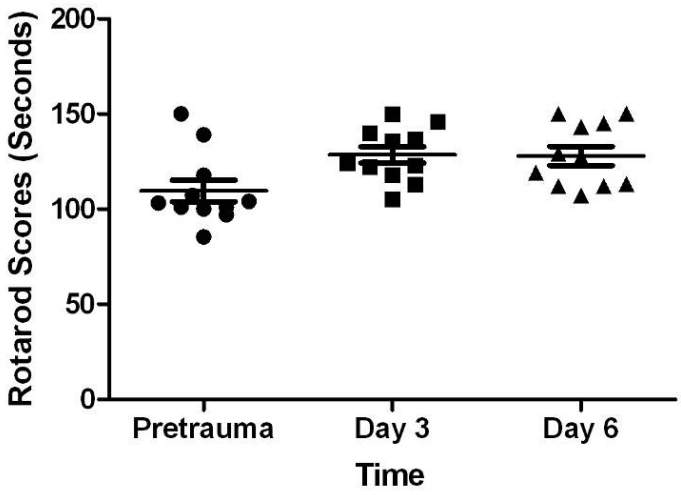


Figure 6.3 The weights of the rats during the whole experimental period. Day 0 was the day when the TBI was induced by CCI, while day 7 was the day when the rats were sacrificed.

6.3.2 Assessment of Motor Performance

Figure 6.4 shows the raw data of the individual rotarod score of the rats in each group, which included that data in our preliminary study and the 2 experimental trials. From figure 6.4A-C, we can observe that there is some rats' rotarod scores remain at 150 sec, despite of any operation or interventions. These rats are described as "over-trained" (which would be discussed later) in our experiment and their scores are excluded from the final calculation of the rotarod scores in figure 6.5.

A



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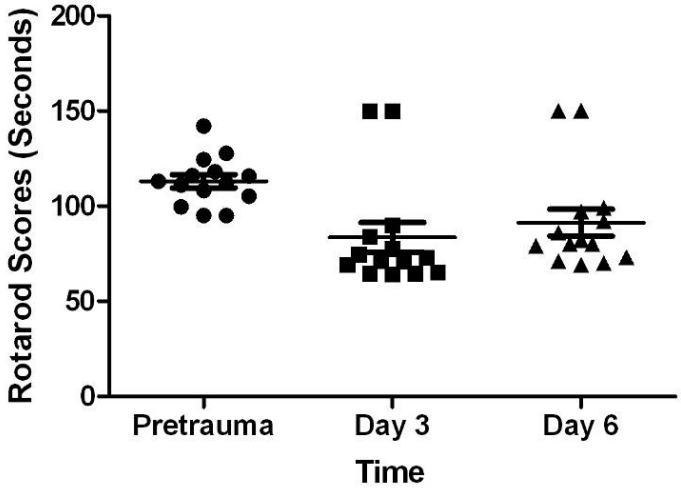
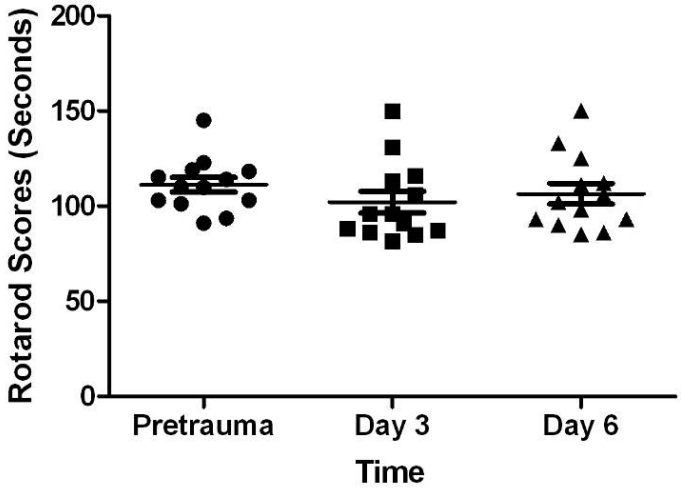

Rotarod Scores (Seconds)
200
150
100
50
0
Pretrauma
Day 3
Day 6
Time

C


Rotarod Scores (Seconds)
200
150
100
50
0
Pretrauma
Day 3
Day 6
Time

D

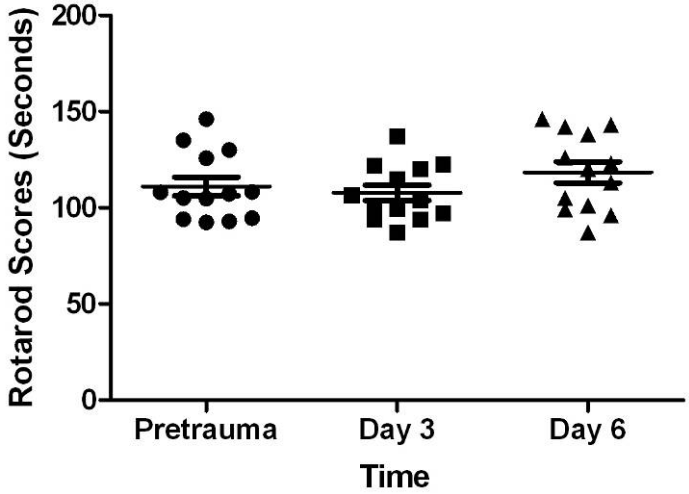

Rotarod Scores (Seconds)
200
150
100
50
0
Pretrauma
Day 3
Day 6
Time

Figure 6.4 The raw data of TBI experiment before the exclusion of over-trained rats for (A) Sham-operation group (B) Control group (C) TM 505 mg/kg treatment group (D) TM 1515 mg/kg treatment group

From figure 6.5, the rotarod scores of the rats before the operation have no significant difference. On day 3 and 6 post-TBI, the rotarod scores of control rats were significantly lower than those of the sham-operation group by 59.5±2.3 sec (p<0.001) and 47.2±2.7 sec (p<0.001). The results demonstrated that the surgical procedure of TBI was able to induce the motor incoordination. Moreover, the rotarod system was a useful tool to evaluate neurological motor function in rats after TBI. We compared the rotarod scores of post-TBI rats treated with TM 505 mg/kg and 1515 mg/kg with those that had received no treatment (Figure 6.5). Rotarod scores on day 3 and 6 post-TBI rats were significantly improved by daily TM treatment when compared with those received TBI only, and the improvements were 26.9±4.8 sec (p<0.01) and 34.4±4.5 sec (p<0.001) on day 3 and 20.9±4.3 sec (p<0.01) and 34.1±5.6 sec (p<0.001) on day 6 for TM treatment of 505 mg/kg and 1515 mg/kg, respectively.

The trend of the rotarod performance of each group was also investigated. For the sham group, the rotarod performance was improved on day 3 and 6 by 19.4±4.8 sec (p<0.05) and 15.6±5.3 sec, when compared with the pretrauma level,

which possibly due to the learning. For the TM treatment group of 505 mg/kg and the control group, their rotarod performances were lowest on day 3, which decreased by by 37.5±2.3 sec (p<0.001) and 10.5±4.8 sec, when compared with the pretrauma level. Their rotarod performance on day 6 was improved, which was 29.1±2.7 sec (p<0.001) and 8.1±4.3 sec lower than the pretrauma level. However, their rotarod performances never return to the pretrauma level. For the TM treatment group of 1515 mg/kg, the rotarod performance did not significant impaired by the TBI surgery on day 3 and 6 when compared with the pretrauma level.

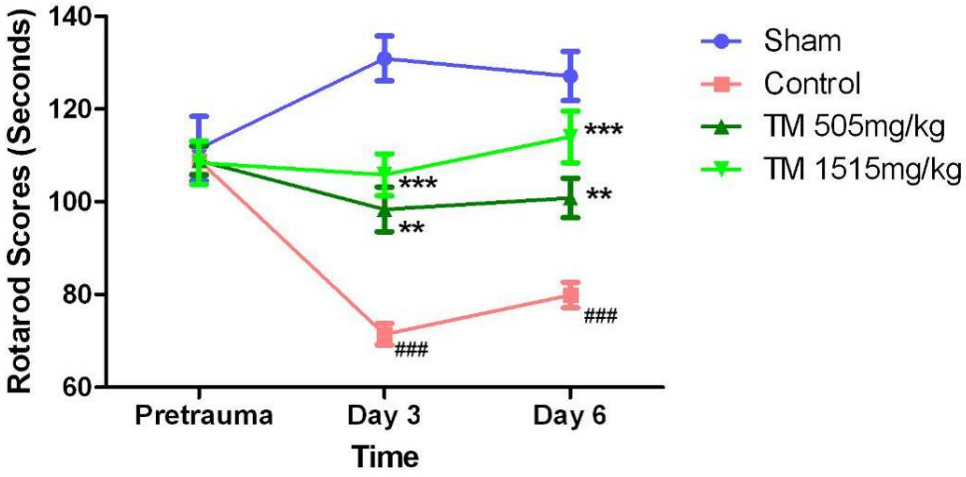


Figure 6.5 Rotarod performances of the TBI rats after the treatments of 2 doses of TM. The assessment was done on day 3 and day 6 post-TBI. Results are the means ± SD from 9 to 11 rats in 2 trials. $^{###}p$<0.001, relative to sham operation group; ** p<0.01, *** p<0.001 relative to control TBI group by two-way ANOVA.

6.4 Discussion

Although the mechanism of the neuroprotective action of TM is not fully understood, multiple potentially beneficial effects were shown. In the present study, the CCI would damage the neurons in the right parietal cortex and the underneath hippocampus, resulting in the deficit in balance, locomotion and memory. The reduced rotarod scores after TBI demonstrated that the successfully induction of motor function deficit by CCI. More importantly, TM treatment ameliorated the motor function deficits caused by CCI. The TM (1515 mg/kg)-treated animals retained their pre-injury locomotor activity by day 3 post-injury and have improved activity for the remainder of the assessment period, whereas the untreated animals never recovered to their pre-injury levels throughout the 6-day assessment period. The study indicate the neuroprotective effect of TM on TBI rats, which demonstrated by the improvements in motor function by TM.

Pre-training, a process to eliminate the dominance effect of learning, was done by undergoing five rotarod trials performed daily for 2 days before TBI. The pre-training ensured the consistant baseline rotarod performance, which was maintained at about 100 sec. The rats with retarded rotarod performance, which mainly due to deficit in learning or insufficient fear response to the height, was excluded before the CCI procedure. The exclusion of the retarded rotarod performance rats before TBI also eliminate the factors of learning and fearness to height. Thus, the rotarod results after TBI have a low deviation, and the difference

between groups was mainly due to the difference in motor performance. However, among the trained rats, we can see some of the rats performing extremely well on the rotarod, even with the motor impairment. They cannot be eliminated by pre-training as their performance is similar to the other rats. During the expierment, although TBI caused the impairment in motor function, they managed to overcome the impairment by jumping or by special gait using the right side of the body, which was not damaged by TBI. They are named as "over-trained" rats and their performance was excluded from the data analysis. By the exclusion of rats with abnormal performance, the accuracy and the stability of the model was ensured. Moreover, to determine the reproducibility and the stability of the model, the study was divided into 2 trials. From the results, we can see there is not significant deviation between 2 trials, which indicates our surgerical procedure and our rotarod assessment platform was well-established, stable and reproducible.

The brain injury induced by CCI would involve "ischemia-like" condition, malnourishment of brain, excitotoxicity, calcium imbalance, oxidative stress, and inflammation. From the study of Yu et al. (Yu*, et al.*, 2005), water, methanol and ethyl ether extract of TM were able to reduce ischemia condition induced by middle cerebral artery occlusion (MCAO) on rat, which water extract of TM showing the highest efficiency. The ingredients of TM, gastrodin and 4-hydroxybenzyl alcohol, were also able to reduce the infarct volume caused by ischemia condition of MCAO (Yu*, et al.*, 2005, Zeng*, et al.*, 2006). Their protective effects against ischemia condition were confirmed and elucidated *in*

vitro by oxygen-glucose deprivation (OGD) model (Yu*, et al.*, 2013, Zeng*, et al.*, 2006). Among them, gastrodin was able to reduce the intracellular calcium level and nitric oxide (NO) level (Zeng*, et al.*, 2006), which was effective against calcium imbalance and inflammation. Methanol extract of TM was also able to prevent serum-deprivation-induced apoptosis on PC12 cells (Huang*, et al.*, 2004), which suggest the possible protective effect of TM on malnourishment of cells. Some studies suggest ingredients of TM, such as 4-hydroxybenzyl alcohol, gastrodin, 4-hydroxybenzaldehyde, 4-methylphenyl-β-D-glucopyranoside and 3, 5-dimethoxybenzoic acid-4-O-β-D-glucopyranoside, were able to reduce glutamate-induced cytotoxicity on PC12 cells (Huang*, et al.*, 2006), while vanillin and 4-hydroxybenzaldehyde were able to reduce glutamate-induced cytotoxicity on IMR-32 human neuroblastoma cells (Lee*, et al.*, 1999). These results suggest the possible anti-excitotoxic effect of TM. Moreover, there were also a wide range of studies showing the anti-oxidative effect different extract and ingredients of TM (Jung*, et al.*, 2007, Kim*, et al.*, 2007, Kim*, et al.*, 2011, Shin*, et al.*, 2011, Yu*, et al.*, 2013, Yu*, et al.*, 2005, Yu*, et al.*, 2011). Last but not the least, there were studies showing the effect of TM against inflammation. The ethanol extract of TM suppress the microglia activation by kainic acid in rats (Hsieh*, et al.*, 2005), which microglia activation plays an important role in inflammation-mediated neurodegeneration (Liu and Hong, 2003). TM ingredient, 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde, were able to carrageenan-induced paw edema and arachidonic acid-induced ear edema in mice, which acted by inhibition of cyclooxygenase (COX) I and II (Lee*, et al.*, 2006). Moreover, 4-hydroxybenzyl alcohol also suppressed the production of NO and expression of inducible nitric

oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated RAW264.7 macrophages, which iNOS-derived NO is involved in pathological conditions, such as inflammation, and result in cell damage (Lim*, et al.*, 2007). The previous study suggested that the action of TM may possibly through rescuing ischemia-reperfusion damage, nourishment of brain, anti-excitotoxic effect, lowering intracellular calcium, anti-oxidation, and anti-inflammation. However, the exact neuronal protective action of TM on TBI rats was yet to be studied.

Last but not the least, the presence of blood-brain barrier (BBB) in mammlian may block those beneficial active ingredients from the brain. The positive results from the present study indirectly indicate that some active ingredients of TM are able to pass through the BBB and exert the beneficial effects. Therefore, the present study demonstrated the neuroprotective effect of TM on the brain trauma, and its possibility in passing through the BBB.

Chapter 7
General Discussion and Conclusion

7.1 Significance of the Study

Neurodegenerative diseases and dementia are amongst the most prevalent diseases in the world. It is also one of the top mortality causes. Treatment and management of dementia patients pose a heavy burden to the economy. Hence, prevention or suppression of the diseases in an economical way becomes a target of research. Although progression of dementia is a complex and inter-related process, the pathology of Alzheimer's disease, which is the most prominent form of dementia, and its involvements of beta-amyloid (Aβ) plaque, hyperphosphorylated tau, acetylcholinesterase and excitotoxicity are widely studied and confirmed (Dodd*, et al.*, 1994, Hardy and Selkoe, 2002, Kihara and Shimohama, 2004, Mandelkow and Mandelkow, 1998).

In the present study, we investigated the effect of the aqueous extract of 4 Chinese herbal medicines – Tianma, Gouteng, Chuanxiong and Yuanzhi, on our *in vitro* PC12 cell platform. From the literature review, the active ingredients of Gouteng, such as rhynchophylline and isorhynchophylline, significantly decreased Aβ-induced cell death (Xian*, et al.*, 2012). Meanwhile, butanol extract of Chuanxiong was able to prevent serum deprivation-induced apoptosis in rat pheochromocytoma cells (Lin*, et al.*, 2007, Lin*, et al.*, 2009). Water and butanol extract of Chunaxiong can also inhibit the aggregation of Aβ (Shuai*, et al.*, 2013). The ethyl ether fraction of Tianma can protect IMR-32 neuroblastoma cells against Aβ-induced cell death (Kim*, et al.*, 2003), and the methanol extract of

Tianma can rescue the PC12 cells from serum-deprived apoptosis (Huang*, et al.*, 2004). Moreover, the ingredients of Tianma, such as 4-hydroxybenzyl alcohol, gastrodin and 4-hydroxybenzaldehyde, were able to exert protective effect on PC12 cells against glutamate-induced cell death (Huang*, et al.*, 2006). For Yuanzhi, researches have been done on primary cortical neurons, and water extract of Yuanzhi was able to attenuate the cell damage induced by Aβ (Naito and Tohda, 2006). The above findings suggest the potential *in vitro* neuroprotective effect of the four herbs in the present study. Therefore, these four herbs were subjected to our screening assay, which PC12 cells would be incubated with the water extract of the four herbs and Aβ, and the protective effect of the extracts against Aβ-induced cytotoxicity would be deteremined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results demonstrated that the protective effect of the extracts against Aβ-induced cytotoxicity was Tianma > Chuanxiong > Gouteng > Yuanzhi. Meanwhile, all extracts, except Yuanzhi, was also exert direct inhibition to the acetylcholinesterase activity. The efficacy was Tianma > Gouteng > Chuanxiong. Therefore, anti-apoptotic effect of Tianma, Gouteng and Chuanxiong were investigated. Among them, Tianma and Chuanxiong demonstrated anti-apoptotic effect against Aβ-toxicity, while Gouteng demonstrated slight pro-apoptotic effect. Based on the finding, Tianma and Chuanxiong were selected for further investigation on their anti-oxidative effect. The results showed that Tianma possessed higher anti-oxidative potency than Chuanxiong. Therefore, Tianma was selected as our candidate herbal extract in the later studies. The present study started from the investigation of the in vitro effect of TM against Aβ-induced

cytotoxicity, hyperphosphorylated tau and acetylcholinesterase activation and glutamate-induced excitotoxicity. After that, the study proceeded to the research of the neuroprotective effect of TM against Aβ and traumatic injury on *in vivo* models.

Previously, Kim *et al.* (2003) reported that the ethyl ether fraction of TM was able to protect Aβ-induced IMR-32 neuroblastoma cell death (Kim*, et al.*, 2003). However, the content of active ingredients in the ethyl ether extract was expected to be different from the aqueous extract. Although the dose of extract used in the study was 10 μg/ml, the extraction yield of the extract was only 1.12%. When comparing with the present study of extraction yield of 48.90%, the dose was equivalent to 420.61 μg/ml in the present study, which is similar to the present dose of 500 μg/ml. Moreover, the study only use MTT assay to demonstrate the protective effect without further elucidation of any protective mechanisms. A complete picture from the *in vivo* effect to the downstream neuroprotective mechanisms was yet to be revealed, and the present study was the novel one targeting these.

In the present study, the action mechanism of TM in protecting PC12 cells against Aβ-mediated cytotoxicity was investigated in 3 aspects – apoptosis, oxidative stress and tau phosphorylation. Aβ treatment causes early and late apoptosis, partially due to the activation of caspase-3 and the reduced level of Bcl-2. Aβ also induces oxidative stress on the PC12 cells, which reduction of superoxide dismutase activity is observed. In addition, the Aβ treatment reduces

the content of normal tau protein, and increased the phosphorylation of tau at site Serine 396. The phosphorylated tau would aggregate to form neurofibrillary tangles, which is cytotoxic and would contribute to the apoptosis.

TM can reduce the early and late apoptosis induced by Aβ, possibly through the inhibition of cleavage and activity of caspase-3, and the restoration of Bcl-2 level. TM can also reduce the oxidative stress in the cells treated with Aβ. The possible explanation for the effects are the increased activity of the anti-oxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The reduced oxidative stress can also contribute to the anti-apoptotic effect of TM. Preliminary results also demonstrated that the treatment of TM reverse the reduced amount of normal tau protein, and reduce the phosphorylation of tau at site Threonine 205, Serine 396 and Serine 404. The reduction of tau phosphorylation would reduce the content of phosphorylated tau, and maintain the stability of the cytoskeleton. Overall, TM demonstrated neuroprotective effect on PC12 cell line against Aβ-induced toxicity. On the other hand, studies revealed that amyloid precursor protein (APP) processing leads to the production of Aβ, which have strong correlation with neurodegeneration in AD pathology (Mattson, 1997). Mishra *et al.* (2011) demonstrated that that TM was able to inhibit β-site APP-cleaving enzyme 1 activity and promote α-secretase activity (Mishra*, et al.*, 2011). The inhibition of β-site APP-cleaving enzyme 1 (β-secretase) reduce the cleavage of APP into Aβ (Vassar*, et al.*, 1999) and the activation of α-secretase increase the cleavage of APP into soluble-APP-α (Colciaghi*, et al.*, 2004, Nistor*, et al.*, 2007). Therefore, TM is an herbal extract

that can exert duo beneficial effect towards Aβ-pathology by inhibiting the production and toxicity of Aβ. From the promising *in vitro* effect of TM, the *in vivo* efficacy was studied to verify the *in vitro* effect and estimate the effect on the Aβ-induced behavioral changes.

Transgenic *Drosophila* was selected as our in vivo model in the present study. Two Aβ-expressing *Drosophila* lines were used. The first *Drosophila* line expresses Aβ at the ommatidia, causing ommatidia degeneration. The second one expresses Aβ at the whole neuronal system, causing more systemic changes like the accumulation of Aβ in brain, the impaired locomotor function and the reduced lifespan. The novel use of Alzheimer's disease *Drosophila* on the treatment of TM revealed that the treatment was able to improve Aβ-induced neurodegenerative and neurobehavioral phenotypes – it can rescue the ommatidia from degeneration, improve the locomotor function and increase the lifespan. Studies indicated that the iron-binding protein ferritin and the H_2O_2 scavenger catalase effectively suppressed the toxicity of wild-type and Arctic mutant of $A\beta_{1-42}$, and increased the lifespan and locomotion function of the $A\beta_{1-42}$-expressing *Drosophila* (Rival*, et al.*, 2009). The research suggested the importance of oxidative stress in the neurodegenerative process of the $A\beta_{1-42}$-expressing *Drosophila*. As anti-apoptotic and anti-oxidative effects of TM was found in our *in vitro* results, TM may also inhibit apoptosis and reduce the oxidative stress in *Drosophila* with the same mechanism, leading to the observed improvements.

As TM has shown a promising effect against Aβ-induced neurodegeneration

in vitro and *in vivo*, the pathological pathways, which are common for various neurodegenerative diseases, were studied. The platform we used was the primary cortical neuron culture isolated from neonatal rats. This *in vitro* platform demonstrated a high similarity with human neurons and widely used in various research on neurology. The excitotoxicity and the Aβ-induced cytotoxicity/ acetylcholinesterase activation were studied. Our results demonstrated that TM has no protective effect on the primary cortical neuron against excitotoxicity. However, vanillin and p-hydroxybenzaldehyde were demonstrated to be able to inhibit glutamate-induced excitotoxic effect on neuroblastoma cells in another study (Lee*, et al.*, 1999). The difference in efficacy may due to the different dose of glutamate used to induce apoptosis, which 500 μM was used in that study but only 100μM was used in the present study. The dose of glutamate used in the present study only induced about 20% reduction in viability of cells, which may shed the possible effect of TM. In the same study, protective effect against Aβ-induced cytotoxicity/ acetylcholinesterase activation was observed, which once again validate our previous *in vivo* and *in vitro* results. Inhibition of acetylcholinesterase can reduce the degradation of acetylcholine, which is the important neurotransmitter for memory, and prevent the cholinergic deficit. Thus, the progression of the memory loss can be slow down. The novel discovery of inhibitory effect of TM on acetylcholinesterase activation means TM also possess the same action mechanism of the current medication used for treatment of Alzheimer's disease, on top of its anti-apoptotic effect against Aβ.

As there are similarities between the pathological process of different neuro-

degenerative diseases, anti-apoptotic, anti-oxidative and anti-acetylcholinesterase effect of TM may also be beneficial to other types of dementia. Therefore, we used the rat traumatic brain injury (TBI) model to investigate the effect of TM on another type of dementia. Although we did not assess the memory function of the rats, we used rotarod test, which a quantitative and stable assay, to determine the degree of brain injury. TM improved the rotarod performance of the traumatic brain injured rats, which the 3 times human equivalent dose of TM was able to maintain the locomotor function at the pre-injury level. The results reflected that TM is effective in protecting neurons from degeneration during the pathological process of brain trauma. In other studies, inflammation after TBI was often investigated (Goss*, et al.*, 1995, Nimmo*, et al.*, 2004). Inflammation is closely related to apoptosis in the pathological process after TBI (Morganti-Kossmann*, et al.*, 2001). The stimulation of Toll-like receptor 4 (TLR4) activates nuclear factor kappa-B (NF-κB), which promotes the activation of genes of inflammatory cytokine (Akira and Takeda, 2004), such as such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β) and interleukin-6 (IL-6), and leads to their expression in brain, which ultimately cause the neuronal apoptosis (Dong*, et al.*, 2011). In other studies, compounds from *Salvia miltiorrhiza* and *Tripterygium wilfordii* demonstrated anti-inflammatory effect, which inhibited the elevation of TNF-α, IL-1β, and IL-6 in TBI rats' brain, and possessed neuroprotective effect (Chen*, et al.*, 2011, Lee*, et al.*, 2012). For our herbal extract of TM, other studies revealed that active ingredients of TM, such as 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, vanillyl alcohol and vanillin, exert anti-inflammatory effect on rat macrophage cell lines and arachidonic acid-induced edema on rat

paws. The anti-inflammatory effect of these ingredients may lead to inhibition of neuronal inflammation and subsequent apoptosis during pathological process after TBI.

In our study, the best known active ingredient of TM, gastrodin, does not protect PC12 cells against cytotoxic effect of Aβ. The neuroprotective effect may be partially contributed by other active ingredients, such as 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, vanillyl alcohol and vanillin. However, these ingredients are mostly non-polar and should be present in our extract in a low concentration, which provide a limited effect. Therefore, we suggest the possibility of the involvement of polysaccharides, which is rich in the water extract in general (Lee*, et al.*, 2012), in the protective mechanisms of our TM water extract. However, further studies are needed to determine the neuroprotective effect of the polysaccharides.

Overall, the present study screened the water extract of Tianma, Gouteng, Chunaxiong and Yuanzhi, and determined that water extract of Tianma (TM) possess highest efficacy against Aβ-induced cytotoxicity. The present study is the first report of the use of TM to protect against the Aβ-induced cytotoxicity *in vitro* and *in vivo*, on PC12 cells, primary cortical neurons and *Drosophila*. The protective mechanism was determined to be involving the anti-apoptotic and anti-oxidative action of TM, through the inhibition of pro-apoptotic caspase-3 and activation of anti-oxidative superoxide dismutase, catalase and glutathione peroxidase, respectively, which was a novel discovery. Additionally, this is also

the first report of TM possessing anti-acetylcholinesterase activity, which can help maintaining the cholinergic transmission of neurons and delay the dementia progression. Last but not the least, the present study is a novel report of the anti-neurodegenerative of TM against the traumatic brain injury in rat, which indicates that the protective effect of TM may not be limited to the Aβ-pathology. The findings shed light on the development of TM as a therapeutic agent against Alzheimer's disease, and the expansion of the investigation as the use of TM to a neuroprotectant against different types of neurodegenerative disorders.

7.2 Limitations and Future work

There are several limitations in the present study. Firstly, PC12 cell line is derived from rat pheochromocytoma, which is a cancer cell, in nature. Some positive controls, like donepezil or tacrine, were failed to protect the PC12 cells from Aβ-induced cytotoxicity in our preliminary test (data not shown), which may due to the discrepancy between Aβ cytotoxic mechanism and their specific protective mechanisms. Moreover, PC12 cells are not cholinergic in nature, which explain the low responsiveness to the acetylcholinesterase inhibitors donepezil or tacrine. Based on the above reasons, there is a lack of positive control in our PC12 cell model. Although there are similarities between PC12 cells and neurons, the rescue effect of TM on PC12 cells may not exactly the same as on the neuronal cell. Translation of the results from PC12 cells to the primary neurons was primarily done by the MTT assays in the present study. Primary neurons in the study are from neonatal rats, while the maturation of the nervous system in rat is at least 21 days (Miller and Peters, 1981). This characteristic may lead to discrepancies in results when comparing with adult or aged neurons, which are the ones the Alzheimer's disease affecting in clinical settings. Therefore, there are many follow-up works that can be done to confirm that the protective mechanisms are consistent in the 2 cell types.

Secondly, Alzheimer's disease and dementia mainly cause deficit in cognitive ability in human. However, our assays mainly focus on neurodegeneration-related changes, such as reduced lifespan, impaired locomotor function or degeneration of ommatidia. Meanwhile, due to the limitation of

equipment, cognitive test was not done on Drosophila. Moreover, the underlying mechanisms of the neuroprotective effect of TM have not been elucidated. From our *in vitro* results, we speculate that the protective effect is through the inhibition of apoptosis, activation of anti-oxidative mechanisms and the inhibition of acetylcholinesterase. Further studies can be done on *Drosophila* to validate these.

Another limitation of the present study is that in our *in vitro* models, $A\beta_{25-35}$ fragment was used to induce toxicity, while Aβ42 was expressed in the *Drosophila* model. In human situation, Aβ40 and Aβ42 were responsible for the Alzheimer's disease pathology. The use of $A\beta_{25-35}$ in our PC12 cell model may be responsible for fluctuation of the viability from 50% to 70% in our viability assays, which may relate to its stability.

The ultimate goal of the study is to further develop TM as novel non-toxic preventive/treatment interventions for life-threatening neurodegenerative diseases, such as AD. In mammalians, the presence of blood-brain barrier (BBB) may block those beneficial active ingredients from the brain. Although BBB exists in *Drosophila* and serves the function of blocking the passage of ions and small molecules (Juang and Carlson, 1994, Stork*, et al.*, 2008), the *Drosophila* BBB is morphologically different from mammalian one (Daneman and Barres, 2005, Freeman and Doherty, 2006). Nevertheless, some previous studies demonstrated that intravenous administration of gastrodin and hydroxybenzyl alcohol were able to pass through BBB in rats (Wang*, et al.*, 2008, Wang*, et al.*, 2007). However, the pharmacokinetics of TM in human brain and the pharmacokinetics of other active

ingredients of TM in mammalian brain are yet to be investigated.

For the rat traumatic brain injury model, the observed pathological changes include elevated intracranial pressure (brain oedema), decreased blood and cerebral perfusion pressures, neurodegeneration, histological deformations and behavioural defects, which only the locomotor performance was assessed due to the limitation of assay platforms. Establishment of new assay in assessing the cognitive ability, which is more relevant to dementia, is necessary. Histological assessment on the brain is also important for the elucidation of underlying protective mechanisms of TM. Therefore, the limitation of the present study can be improved by the further investigation on these aspects.

Although the *Drosophila* is genetically similar to human, its morphology and the method to induce Aβ neurotoxicity is different from that of human. On the other hand, for the traumatic brain injured rat, although the impact is simulating the events happened during brain trauma in human, the opening of the skull before the impact may induce a difference.

For the future work, the project can be studied deeper, broader and more practical, which means 1) further elucidation of more underlying mechanism; 2) studying the other types of dementia and neurodegenerative diseases and using different models for the current study; 3) focusing on the practical issues, like the blood-brain barrier, the pharmacokinetics, and possibly the clinical trial as a supplement.

For further elucidation of underlying mechanism in our PC12 cell line, disruption of calcium homeostasis, which increases the intracellular calcium, was often a target for investigation related to Aβ-induced apoptosis (Hosoda*, et al.*, 2001, Iuvone*, et al.*, 2004, Park*, et al.*, 2008, Yallampalli*, et al.*, 1998). Measurement of intracellular calcium can be done using a fluorescence probe fura-2/AM. In the presence of Ca^{2+}, maximum fura-2 fluorescence at 510 nm is observed at an excitation wavelength of 340 nm, while the maximum florescence in Ca^{2+}-free condition is at 380 nm. Therefore, the concentration of intracellular Ca^{2+} is proportional to the ratio of fluorescence at 340/380 (Lambert, 2006). Additionally, fura-2 is impermeable across the cell membrane. Therefore, acetoxymethyl (AM) esters of fura-2 were used, which the AM esters would be cleaved by intracellular esterases to yield fura-2 (Di Virgilio*, et al.*, 1988). The assay can detect the effect of TM extract on restoring calcium homeostasis. Another aspect which is often investigated is the DNA fragmentation (Iuvone*, et al.*, 2006, Misiti*, et al.*, 2005, Xian*, et al.*, 2012). DNA fragmentation result from the chromatin DNA digested by endonucleases during apoptosis. Therefore, DNA fragmentation is a key feature of apoptosis and a clear evidence of apoptosis. Quantification of DNA fragmentation can be done by DNA laddering in DNA gel electrophoresis (Iuvone*, et al.*, 2006, Misiti*, et al.*, 2005) or DNA fragmentation Enzyme-linked immunosorbent assay (ELISA) (Xian*, et al.*, 2012). The amount of DNA fragmentation would reflect the extent of apoptosis. $A\beta_{25–35}$ could mediate DNA fragmentation (Iuvone*, et al.*, 2006, Misiti*, et al.*, 2005, Xian*, et al.*, 2012), and the capability of TM to reduce the fragmentation can be further studied.

In the present study, the $A\beta_{25-35}$-induced increase in phosphorylated tau and the inhibition of the process by TM can be further confirmed. Regarding this, glycogen synthase kinase 3 (GSK-3), which is responsible for the tau phosphorylation, can also be investigated by Western blotting (Iuvone*, et al.*, 2006, Zeng*, et al.*, 2010, Zhang*, et al.*, 2009). With further investigation in these aspects, the *in vitro* screening and mechanism elucidation platform on PC12 cell line would be a very robust one.

Regarding to the *in vivo Drosophila* platform, a behavioural assay can be added. The olfactory assay was used in the mechanistic study of Aβ-expressing flies (Chiang*, et al.*, 2010). During training session, the *Drosophila* was sequentially exposed to two odors, 3-octanol and 4-methylcyclohexanol. Flies were subjected to shock pulse during exposure to the first odor, but not during exposure to the second odor. To measure the learning ability, flies were transferred to the middle of a T-maze and were forced to choose between the two odors. Then, flies in each T-maze arms were counted. A learning index was calculated as the fraction of flies avoiding the shock associated odor minus the fraction of flies avoiding the unshocked control odor (Tully and Quinn, 1985). The higher learning index indicates a better learning ability. Further study can be focus on the novel use of this platform on the assessment of drug efficacy on Aβ-expressing flies. As TM may also act through the inhibition of aggregation of Aβ, the extent of Aβ aggregation can also an aim for the future studies. The aggregation of Aβ can be detected by the Thioflavin S staining, which labels beta-pleated sheets. Aβ aggregates in the brain of the *Drosophila* can be stained and the Thioflavin

S-labeled Aβ aggregate can be visualized under fluorescence microscope as green bright spots (Greeve*, et al.*, 2004, Iijima-Ando*, et al.*, 2009, Iijima*, et al.*, 2008). The amount of bright spots would reflect the extent of Aβ aggregation. With this method, the effect of TM on aggregation of Aβ can be investigated.

For the *in vitro* primary cortical neuron platform, future study can include all the assessment that was done on the PC12 cells, due to the fact that primary cortical neurons were more similar to the neurons in the brain. The determination of neurite outgrowth by immunofluorescence staining, which was used in our excitotoxicity assessment, can also be extended to the assessment of Aβ-induced cytotoxicity. The inhibition of neurite outgrowth can damage the function of synapse, and impair the neurotransmission (Hu*, et al.*, 2007).

There are lots of future studies that can be done on the TBI rat platform. The brain of the rat can be sampled for the histological assays to assess the show overall dorsal cortical and hippocampal tissue loss and distortion in architecture (Brody*, et al.*, 2007). Nissl-stain, such as cresyl violet, can stain the endoplasmic reticulum of neurons and glia in purple, which can demonstrate the loss of neurons caused by the TBI. Moreover, the locomotor coordination can be further assessed by CatWalk method, which consists of a runway to record the gait of the rat. The assay evaluates a large number of locomotor parameters, such as the time needed to cross the runway, regular step pattern, paws' contact area and interlimb coordination (Neumann*, et al.*, 2009, Vlamings*, et al.*, 2007). The assay gives more information on the motor performance. Therefore, the assay is able to give

more detail on 1) which part of the locomotor action was affected by TBI; and 2) how TM help improve the motor performance. On the other hand, to study the effect of TBI on memory and learning, the behavioural study of rat, such as Morris water maze, should also be done in the future. Morris water maze consist of a circular pool of water and a hidden platform under the water. The task of the rat is to locate and swim onto the platform within a period of time. Training was done with the platform visible on the water. After the training, the platform was lower under the water and the testing started. Time spend on the quadrant with the platform and time or distance spend to reach the platform are recorded (Brody and Holtzman, 2006, Morris*, et al.*, 1982). More time spend on the quadrant with the platform and faster in reaching the platform indicate a better learning and spatial memory (Brody and Holtzman, 2006). TBI rat would spend less time on the target quadrant and need more time to locate the platform (Brody*, et al.*, 2007). Whether TM exerts beneficial effect to TBI rat on their water maze performance would be a very important finding to reflect the effect of TM on TBI rats' cognitive ability.

In order to study the other types of dementia and neurodegenerative diseases, other types of inducer would be used. The effect of TM on stroke and ischemia damage was already investigated (Kim*, et al.*, 2007, Xu*, et al.*, 2007, Yu*, et al.*, 2005, Zeng*, et al.*, 2006). Based on our findings, TM have beneficial effects on locomotor activity, while Parkinson's disease is mainly related to the locomotor dysfunction at the initial stage. Therefore, Parkinson's disease may be another target for the further development of TM. Six-hydroxydapamine (6-OHDA)-induced neurotoxicity was widely used in *in vitro* and *in vivo* models

for Parkinson's disease research. For *in vitro* model, the protocol of treatment was similar, with Aβ substituted by 6-OHDA (Shim*, et al.*, 2009). For *in vivo* rat model, the induction was done by stereotactic injection of 6-OHDA into the striatum (Vercammen*, et al.*, 2006). The animals' motor performance was assessed by Rotarod, CatWalk or other behavioural tests (Monville*, et al.*, 2006, Rodriguez*, et al.*, 2001, Vlamings*, et al.*, 2007).

By using different models for the current study, the effect of TM of Alzheimer's disease can be investigated in different aspects. There are two commonly used models: the transgenic APP/PS1 mice and the Aβ stereotactic injection on rat. APP/PS1 mice overexpresses human amyloid precursor protein (APP) and human presenilin 1 (PS1), which leads to the cleavage of APP to Aβ by PS1 (Spuch*, et al.*, 2010, Yan*, et al.*, 2009). The accumulation of Aβ can be seen in the brain of the mice and can be histologically identified (Perez*, et al.*, 2010). However, behavioural changes were not often appearing. On the other hand, the stereotactic, intracerebroventricular injection of aggregated Aβ into the rat rapidly produces behavioural deficits that can be tested by different behavioural assessments (Choi*, et al.*, 2007, Stepanichev*, et al.*, 2003). By these, the effect of TM on mammalian model of AD can be determined in morphological and behavioural aspect.

If we want to develop TM into a supplement or a therapeutic intervention, practical issues, such as the permeability through the blood-brain barrier (BBB) and the pharmacokinetics of TM in the circulatory system and brain in

mammalian models, must be investigated. The commonly use model is rat. TM can be given by oral gavage. At different time point, the rats are sacrificed and cerebrospinal fluid (CSF) and blood was collected (Wang*, et al.*, 2007). The CSF and blood are then deproteinized and the active ingredients of TM in the samples are quantified by High-performance liquid chromatography (HPLC). If there is any active ingredient of TM found in CSF, the ingredient is permeable through the BBB. For the pharmacokinetics study, the concentration of certain ingredient and its metabolite (e.g. gastrodin and *p*-hydroxybenzyl alcohol) in the blood and CSF after TM administration would be determined (Lin*, et al.*, 2008). The concentration of the ingredients would indicate its absorption efficacy and the metabolism. This information will be very useful in determination of TM dose in the clinical trial and avoids its interference with the other medications.

In addition, neurodegenerative disease occurs in aged human in clinical situation, while the induction method and the treatment done in our experiments are relatively acute. These factors may affect the clinical relevance of our studies. To improve, some aged animal model may be used in the future studies. SAMP10 mouse, which is a model of brain aging characterized by cerebral atrophy, would be a good model to study the effect of TM on aged animal in the future (Shimada and Hasegawa-Ishii, 2011). Moreover, the safety of co-administration of TM and the commonly use medication against Alzheimer's disease should also be investigated.

After the validation of the permeability of active ingredients in TM across

blood-brain barrier and the neuroprotective results in aged animals, clinical trials may follow. TM can be treated according to the dose reported in the Chinese Pharmacopeia 2010 (i.e. 10g raw herb per day) on patients with Alzheimer's disease or other types of neurodegenerative diseases. The clinical effect would indicate the efficacy of TM on protecting neuronal system against different insults, which may shed light on the development of a new medication.

7.3 Conclusion

Four traditional Chinese medicines commonly prescribed for treating head-related disorders - Tianma, Gouteng, Chuanxiong and Yuanzhi, were selected for screening their protective activity against *in vitro* toxicity induced by aggregated beta-amyloid (Aβ). Aqueous extract of all candidates were found to increase the viability of PC12 cell line, with efficacy of Tianma > Chuanxiong > Gouteng > Yuanzhi. Amongst them, Tianma and Chuanxiong was selected for further anti-apoptotic and anti-oxidative stress assays. Both treatments resulted in significant anti-apoptotic and anti-oxidative stress efficacy, with Tianma to be more potent. The action mechanisms of the neuroprotective of Tianma (TM) were further elucidated. TM apparently promoted the survival of Aβ-treated PC12 cells by inhibiting apoptosis, which contributed by the maintenance of the expression of anti-apoptotic Bcl-2, reduced activation of caspase-3 and inhibition of its activity. Oxidative stress level in Aβ-treated cells was inhibited by TM via activation of superoxide dismutase, catalase and glutathione peroxide, which facilitate the breakdown of the reactive oxygen species. Preliminary results also suggested the inhibitory effect of TM on Aβ-mediated tau phosphorylation, resulting in decreased phosphorylation tau content. The anti-oxidative and anti-tau phosphorylation effect of TM can reduce the apoptotic effect of Aβ on PC12 cells, and increase the survival of the cells under Aβ-treatment. TM treatment also reduced Aβ-mediated cytotoxicity and acetylcholinesterase activation in rat primary cortical neuron culture. The results validate our finding in PC12 cells and discover another possible action mechanism of our TM extract – through the inhibition of acetylcholine breakdown to slow down the cholinergic deficit and

subsequent memory loss. For in vivo study, investigation of the effect of TM on neurodegenerative phenotypes and behavioral changes in *Drosophila* was carried out. Transgenic Aβ-expressing *Drosophila* model is a useful animal model for investigating Alzheimer's disease. Results showed that TM was effective in delaying the locomotor function impairment and delay the mortality by reducing neurodegeneration, which reflected by the reduction of ommatidia degeneration. Moreover, in this study, the effect of TM on anti-neurodegeneration was determined by another model of dementia – traumatic brain injury model in rats. TM was able to rescue the impaired locomotor function. The body weights of the brain injured rats were not affected by TM treatment. From the results, we can conclude that TM was able to rescue the neurons from Aβ-induced neurodegeneration *in vitro* and *in vivo*. In addition, TM has potential neuroprotective effect against other form of dementia. The findings shed light on the further research and development of TM to a neuroprotective health product.

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