Significance of a Cognition-Enhancing Chinese Herb Fructus Alpiniae oxyphyllae as a Source for Potential Neuroprotective Agents

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Abstract

Neuroprotection is the mechanisms and strategies used to protect against neuronal injury or degeneration in the central nervous system (CNS) following acute disorder or a result of chronic neurodegenerative disease, such as Glaucoma, Alzheimer's disease (AD) and Parkinson's disease (PD).

Natural herbs have long been used in Asian societies for treating neurodegenerative disorders. In an effort to find effective neuroprotective compounds from Chinese medicine, glutamate-induced neurotoxicity on cultured rat cortical neurons was employed as an *in vitro* model in our screening programme. Twelve Chinese herbs were selected for this project based on their ethnomedical use in the treatment of neurodegenerative disorders. Alcoholic extracts of these Chinese medicines were investigated for their neuroprotective action. Among all the herbs tested, Fructus Alpiniae oxyphyllae (FAO), or Yi Zhi Ren in Chinese, which is traditionally regarded as a cognition-enhancing herbal drug, was found to have the most potent activity in attenuating the glutamate-induced cell death. Pretreatment of FAO extract significantly reduced lactate dehydrogenase (LDH) release and the glutamate-triggered activation of caspase-3 in cultured cortical neurons. In addition, FAO was able to mitigate the neurotoxicity when co-cultured or post-treated with glutamate. Moreover, FAO could effectively rescue the H_2O_2-caused neurotoxicity. These experimental findings render FAO a promising neuroprotective crude drug.

Further isolation and purification were performed on the alcoholic extract of FAO, and one active compound, namely chrysin, was obtained. Pretreatment of this compound in cortical neurons could significantly reduced LDH release and the glutamate-triggered activation of caspase-3. In addition, chrysin could dramatically reduce the H_2O_2-induced neurotoxicity and oxidative stress induced by glutamate.

Glaucoma represents a group of neurodegerative diseases characterized by structural damage to the optic nerve and slow, progressive death of retinal ganglion cells (RGCs). To test the anti-glaucoma action of chrysin, we used the chronic ocular hypertension glaucoma model as an *in vivo* animal model. Our results showed that a single intravitreous injection of chrysin was effective in rescuing RGCs in the chronic ocular hypertension glaucoma rats and the effects could last for 4 weeks.

Taken together, the present PhD project has identified FAO and one of its compounds chrysin to have potent neuroprotective effects. The discovery may expand the clinical usages of FAO as a neuroprotective agent and paves the way for further development of chrysin into a pharmaceutical agent for neuogenerative diseases.

論文摘要

隨著社會人口老齡化的加劇，老年性疾病已成爲一個明顯影響人類健康的突出問題。在諸多的老年性疾病中，神經退行性疾病是一類高發病，其發病率正逐漸攀升。神經退行性疾病一般是指神經組織非正常退變引起的一類進行性功能缺陷與衰退疾病，如老年癡呆（Alzheimer's disease），帕金森氏病（Parkinson's disease），肌萎縮側索硬化症（Amyotrophic lateral sclerosis），亨廷頓氏舞蹈症（Huntington disease），青光眼（Glaucoma）等。由於這類疾病常伴有嚴重的認知障礙及精神異常症狀，甚至癱瘓和感覺障礙，嚴重危害著病人的健康，同時也給家庭、社會帶來了沉重的負擔。因此加強對神經元保護作用，避免或延緩神經元退行性病變的研究必將促進神經病醫學的發展，爲神經退行性疾病的治療和預防提供堅實的理論基礎和依據。

我國中醫藥有著悠久的歷史和良好的治療效果，在幾千年的實踐發展中，已形成了自己獨特的理論體系。特別是針對疑難病和慢性病，中醫藥有它的獨到之處。因此，進行中藥的基礎研究，特別是中藥防治疾病的物質基礎即有效成分的研究至關重要。本論文先通過谷氨酸誘導的大鼠大腦皮質神經元損傷模型，對十二味治療神經退行性疾病常用中藥的乙醇提取物進性了篩選。結果證明益智仁乙醇提取物具有最有效的神經保護作用。其次，我們利用活性追蹤手段對益智仁乙醇提取物進行進一步的分離，從中得到一個具有神經保護作用的黃酮類化合物白楊素。

在其後的研究中，我們使用谷氨酸誘導的大鼠大腦皮質神經元損傷模型對白

楊素的神經保護作用進行了進一步的評價。利用 LDH （lactate dehydrogenase）法和 Caspase-3 法分別檢測了白楊素對大鼠大腦皮質神經元的神經膜保護效應以及神經凋亡的保護作用，同時利用 H_2O_2 引起的神經氧化應激模型對白楊素的抗氧化能力進性了考察。結果表明，在對神經元預處理與後處理實驗中，白楊素有效的減少谷氨酸引起的 LDH 釋放以及 caspase-3 活化。白楊素並且能明顯拮抗 H_2O_2 誘導的氧化應激反應。另一方面，爲了進一步確定白楊素的神經保護作用，我們建立了體外慢性高眼壓大鼠模型。在實驗中，單次玻璃體內注射白楊素可以減少視網膜神經節細胞的死亡，並且這種保護作用可以有效地維持 4 個星期。

本課題的研究結果發現益智仁乙醇提取物以及其中一個黃酮類成分白楊素具有明顯的神經保護作用，爲進一步從益智仁中開發新的治療神經退行性疾病藥物打下堅實的基礎。

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List of Symbols and Abbreviations

Symbols

α	Alpha
β	Beta
δ	Delta
μ	Mu/Micro
κ	Kappa
γ	Gramma

Abbreviations

6-OHDA	6-hydroxydopamine
ACG	Angle-close glaucoma
ACh	Acetylcholine
AchE	Acetylcholinesterase
AD	Alzheimer disease
AIF	Apoptosis-induced factor
ANOVA	One-way analysis-of-variance
APCI	Atmospheric pressure chemical ionization
ApoE ε 4	Apolipoprotein E ε 4
APP	Amyloid precursor protein
ARVO	Association for Research in Vision and Ophthalmology
ASD	Anterior segment dysgenesis
A β	Amyloid-β
B-F	1-butanol-soluble fraction
BHA	Butylhydroxyanisole
CDK2	Cyclin dependent kinase 2
CDK4	Cyclin dependent kinase 4
CP	Tetraethyl bis-phosphoric ester of chrysin

CPE	Diethyl chrysin-7-yl phosphate
CSF	Cerebrospinal fluid
DA	Dopamine
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DRG	Dorsal root ganglion
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
EA-F	Ethyl acetate-soluble fraction
EPM	Elevated plus-maze
FAO	Fructus Alpiniae oxyphyllae
FG	Fluoro-Gold
FALS	Familial amyotrophic lateral sclerosis
FOC	Fold of control
Fr.	Fraction
GMP	Good Manufacturing Practice
H_2O_2	Hydrogen peroxide
HCL	Hydrogen chloride
Hcy	Homocysteine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HO	Hydroxyl
i.p injection	intra-peritoneal injection
i.v injection	Intravitreous injection
IOP	Intraocular pressure
KA	Kainic acid
LDH	Lactate dehydrogenas
LDL	Long-term potentiation
MAO	Monoamine oxidases

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MYOC	Myocilin
NADH	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NTG	Normal tension glaucoma
NTP	Neuronal thread protein
O_2	Superoxide
OH	Ocular hypertensive
ONOO-	Peroxynitrite
OPTN	Optineurin gene
PAF	Platelet-activating factor
PBS	Phosphate-buffered saline
PCG	Primary congenital glaucoma
PD	Parkinson disease
PE-F	Petroleum ether-soluble fraction
PNS	Saponins of Panax notoginseng
PS	Penicillin-streptomycin
POAG	Primary open angle glaucoma
Rb	Phosphorylation of Retinoblastoma
RGCs	Retinal ganglion cells
RO	Alkoxy
ROO	Peroxyl
S.E.M.	Standard error of the mean
SALS	Sporadic ALS

SC	Superior colliculi
SD	Sprague-Dawley
SN	Substantia nigra
TBSL	Tris buffered saline
TCM	Traditional Chinese Medicine
TH	Tyrosine hydroxylase
TIGR	Trabecular meshwork-induced glucocorticoid response protein
TLC	Thin layer chromatography
TM	Trabecular meshwork
TSB	Tanshinone IIB
UV	Ultraviolet
W-F	Water-soluble fraction
Y2	Neuropeptide Y

Chapter 1

General Introduction

1.1 Neurodegeneration and neurodegenerative disease

Neurodegeneration is the umbrella term for the progressive loss of structure or function of neurons, including the death of neurons. Neurodegenerative diseases are a heterogeneous group of disorders characterized by gradually progressive, selective loss of anatomically or physiologically related neuronal system. Prototypical examples include Parkinson's Disease (PD), Alzheimer's Disease (AD), Huntington's Disease (HD), amyotrophic lateral sclerosis (ALS) and glaucoma; and all of these diseases occur as a result of neurodegenerative progresses (Lin & Beal 2006). There are nine physiological mediators which are common to all neurodegenerative diseases (Schwartz 2005). These factors including excessive levels of glutamate; oxidative stress; conformational alteration of proteins; presence of inflammation-associated factors; deficiency of neurotrophic and growth factors; metabolic deficits; increase in extracellular matrix proteins; presence of growth-associated inhibitors and malfunction of local immune cells. In the following sections, we will discuss about glutamate and oxidative stress, which are mostly related to our research.

The major risk factor for neurodegenerative diseases is aging. Both of mitochondrial DNA mutations and oxidative stress contribute to aging (Lin & Beal 2006). Many neurodegenerative diseases are late-onset. It means that there are some factors that change as a person ages for each disease. There is one constant factor in each disease – the gradually loss of neuronal function as the disease progresses with age (Rubinsztein 2006).

The prevalence of neurodegenerative diseases increases steadily in recent decades (Cowan & Kandel 2001). Although research to find a cure for these diseases has increased rapidly, most of the clinic trials have yielded mostly disappointing results so far.

1.2 Glutamate

Glutamate is an amino acid, and its chemical structure is shown in Figure 1. The link of glutamate to neurodegeneration was first mentioned by Krebs in 1935. Glutamate was found to increase metabolism in the isolated retina and it was more concentrated in the cerebral gray matter (Krebs 1935). During the following decades, many researches were undertaken in an attempt to reveal the role of glutamate in the CNS (Hayashi 1952; Hayashi 1958; Curtis *et al.* 1959).The role of glutamate in the nervous system has been better understood since 1960s. Today, glutamate is known as the major excitatory neurotransmitter (Fonnum 1984), and is essential for learning and memory. It plays an important role in neuronal survival, synaptic plasticity, as well as in early development of synapses. In addition, glutamate is crucial for the proliferation, migration and differentiation of neuronal progenitors and immature neurons (Guerrini *et al.* 1995; Ikonomidou *et al.* 1999).

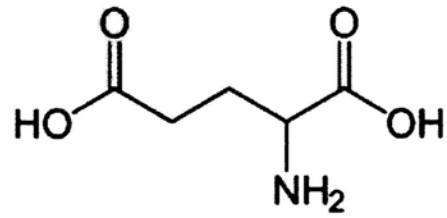


Figure 1.1 Chemical structure of glutamate.

1.2.1 Glutamate synthesis, release and uptake

A crucial requirement for the action of glutamate and other neurotransmitters in the CNS is that their extracellular concentrations be kept low (Nedergaard *et al.* 2002). Glutamate participates in many metabolic pathways (Attwell 2000; Palmer 2001). Glutamine and α-ketoglutarate are thought to be the major precursors of glutamate, which is subsequently packaged into vesicles for future release into the synaptic cleft (Tapiero *et al.* 2002). Glutamine is taken up into the pre-synaptic terminal via an active, Na^+-dependent uptake protein (Anderson & Swanson 2000). It is then transported to mitochondria, where it is converted via phosphate activated glutaminase to glutamate and ammonia. α-Ketoglutarate is also actively taken up into the pre-synaptic terminal, where it is transaminated into glutamate (Anderson & Swanson 2000; Daikhin & Yudkoff 2000). The glutamate in the terminal is then actively taken up into vesicles for future release. Upon release into the cleft, the glutamate either (i) is bound to pre- and post-synaptic receptors, (ii) is actively taken back up via a glutamate transporter and repackaged, (iii) diffuses away from the cleft, or (iv) is internalised by glial glutamate transporters. Five different mammalian glutamate transporters have been cloned (Daikhin & Yudkoff 2000; Platt 2007). Apart from cells in the retina and cerebellum, which express high levels of tissue-specific transporters, the transporters expressed most commonly throughout the brain are GLT-1/EAAT2 in glial cells and EAAC1/EAAT3 in neurons (Attwell 2000).

Once in glial cells, glutamate is metabolised via glutamine synthase into

glutamine or metabolised into α-ketoglutarate by either glutamate oxaloacetate transaminase or glutamate dehydrogenase (Meldrum & Chapman 1999; Anderson & Swanson 2000). The glutamine and α-ketoglutarate are then actively transported out of the glial cells and back into the pre-synaptic terminals for subsequent re-synthesis of glutamate (Meldrum, 1999 #3859}. The extracellular concentration of glutamate is normally very low ($\approx$1 μM) (Anderson & Swanson 2000; Attwell 2000).

Glutamate is released from vesicles in pre-synaptic terminals by a Ca^{2+}-dependent mechanism that involves voltage-dependent calcium channels (Anderson & Swanson 2000; Meldrum 2000). The glutamate concentration within the vesicle is thought to be approximately 100 mmol/L; release of a single vesicle produces an excitatory post-synaptic potential (EPSP) (Meldrum 2000). Glutamate may also be "released" by reverse operation of the glutamate transporters (Anderson & Swanson 2000). This will happen when the Na^+ and K^+ gradient across the membrane is reduced during cerebral ischaemia (Meldrum 2000). The synaptic release of glutamate is controlled by a wide range of pre-synaptic receptors (Anderson & Swanson 2000). These include not only the Group II and Group III metabotropic glutamate receptors (see Section 1.1.2.3) but also cholinergic (nicotinic and muscarinic) receptors, adenosine (A1), j-opioid, c-aminobutyric acid (GABA)B, cholecystokinin and neuropeptide Y (Y2) receptors (Anderson & Swanson 2000; Meldrum 2000).

1.2.2 Glutamate receptors

The release of glutamate is from presynaptic terminals by a calcium-dependent mechanism, and is removed subsequently by uptake into the surrounding glial cells and aminated to glutamate (Fonnum 1984). When glutamate released into the synaptic cleft, it acts at the postsynaptic site on receptors (Hollmann *et al.* 1994). These receptors include the ionotropic glutamate receptors, which couple to ion channels, such as of N-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methylisoxazole-propionate (AMPA), metabotropic glutamate receptors and kainite. The metabotropic receptors are the other kind of glutamate receptor which mediates their actions through G-proteins (Nakanishi 1992).

1.2.2.1 NMDA receptors

NMDA receptor is the predominant molecular device for controlling synaptic plasticity and memory function. It represents tetrameric heteromeric subunit assemblies whose physiological and pharmacological properties depend upon their subunit composition (Li & Tsien 2009). NR1 and NR2 are the two major subunit families of NMDA receptor (Burnashev *et al.* 1992; Monyer *et al.* 1992). NR3 A and B subunits have an inhibitory effect on receptor activity (Chatterton *et al.* 2002; Matsuda *et al.* 2002). Multiple receptor isoforms with distinct brain distributions and functional properties arise by selective splicing of the NR1 transcripts and differential expression of the NR2 subunits. Most of the NMDA receptors in the CNS are formed from NR1 and NR2 subunits. There are eight isoforms of NR2 produced by

alternative splicing of GRIN1 (Stephenson 2006). The NR2 subunit family consists of four individual subunits termed NR2A-D (Monyer *et al.* 1991; Hollmann *et al.* 1994). Different NR2 subunits result in different Ca^{2+} permeability of the NMDA receptor channel, different gating properties and magnesium sensitivity (Monyer *et al.* 1991).

The NMDA receptor is modulated by a number of endogenous and exogenous compounds (Huggins & Grant 2005). Mg^{2+} could block the NMDA channel in a voltage-dependent manner, and is also able to potentiate NMDA-induced responses at positive membrane potentials (Eby & Eby 2006). Ca^{2+} can not only pass through the NMDA receptor channel but also modulate the activity of NMDA receptor (Monyer *et al.* 1991).

Glycine is a coagonist at NMDA receptors. It is also a polyamines positive modulator (McBain & Mayer 1994). Physiological concentrations of glycine reduce the relatively rapid NMDA receptor desensitization. Glycine shows different affinities at NMDA receptor subtypes. This affinity depends on the NR1 isoform and also the NR2 subunit composition of the receptor complex which allosterically influences the glycine recognition site located on the NR1 subunit (Woodward *et al.* 1995) (Monyer *et al.* 1992).

1.2.2.2 AMPA receptors

The AMPA receptor is a non-NMDA-type ionotropic transmembrane receptor for

glutamate that mediates fast synaptic transmission in the CNS. It is found in many parts of the brain and are the most commonly found receptor in the nervous system. There are four subunits of AMPA receptors, GluR1, GluR2, GluR3 and GluR4, also known as GluRA - GluRD (Hollmann *et al.* 1994). They are widely distributed throughout the mammalian CNS and mediate fast glutamatergic neurotransmission. GluR1, GluR4, and GluR2L (a long splice form of GluR2) have a long cytoplasmic carboxy-terminal tail (c-tail), while GluR2, GluR3, and GluR4c (a short splice form of GluR4) have short and structurally similar c-tails. GluR1, -2, and -3 were found in hippocampal pyramidal neurons of mature rats (Zhu *et al.* 2000). Their form channels contain four subunits with a preferred configuration of two identical heterodimer: GluR1/2 and GluR2/3 (Wenthold *et al.* 1996; Mansour *et al.* 2001). Two splice variants for GluR subunits have been described as flip and flop. The different between them is their expression between brain regions and developmental stages. Other non-NMDA receptor subunits are designated GluR5, GluR6, GluR7, KA1 and KA2, and these form high affinity kainate receptors (Herb *et al.* 1992; Hollmann *et al.* 1994). The GluR2 subunit determines Ca^{2+} permeability of AMPA receptor channels. Receptors containing the GluR2 subunit show low Ca^{2+} permeability (Kessels & Malinow 2009).

1.2.2.3 Metabotropic glutamate receptors

Metabotropic glutamate receptors contain eight subunits which are mGluR1–8. The sequence identity of the mGluR-receptor family varies (Pin & Duvoisin 1995).

They are divided into three subclasses (I-III) (Nakanishi 1992). Class I activates phospholipase C, increases phosphoinositide turnover and $Ca2^{+}$ release from internal stores and leads to formation of diacylglycerol which then might activate protein kinase C. Class II and III are coupled negatively to adenylate cyclase and reduce the intracellular amount of cAMP. Class II and III differ in their pharmacological profile against specific agonists (Pin & Duvoisin 1995). mGluR1 and mGluR5 belong to Class I, and mGluR2 and mGluR3 belong to class II, while mGluR4 and mGluR6-8 belong to class III. The amino acid sequence identity is 70% within each class, and 45% between classes.

1.2.3 Glutamate excitotoxicity

Excitotoxicity was first discovered in the 1950s, and was later extensively investigated and characterized in the 1970s and 1980s. It is a process in which an excess of the endogenous excitatory amino acid neurotransmitter glutamate causes overstimulation of glutamate receptors and destroys the central neurons (Platt 2007). Figure. 1.2 depicts the glutamate-induced neuroexcitotoxicity.

In 1957, glutamate excitotoxicity was first described following the observation that systemic administration of monosodium glutamate resulted in degeneration of retinal ganglion cells (RGCs) (Lucas & Newhouse 1957; Olney 1969b; Olney 1969a). Subsequent studies showed that when excitatory amino acid was given to

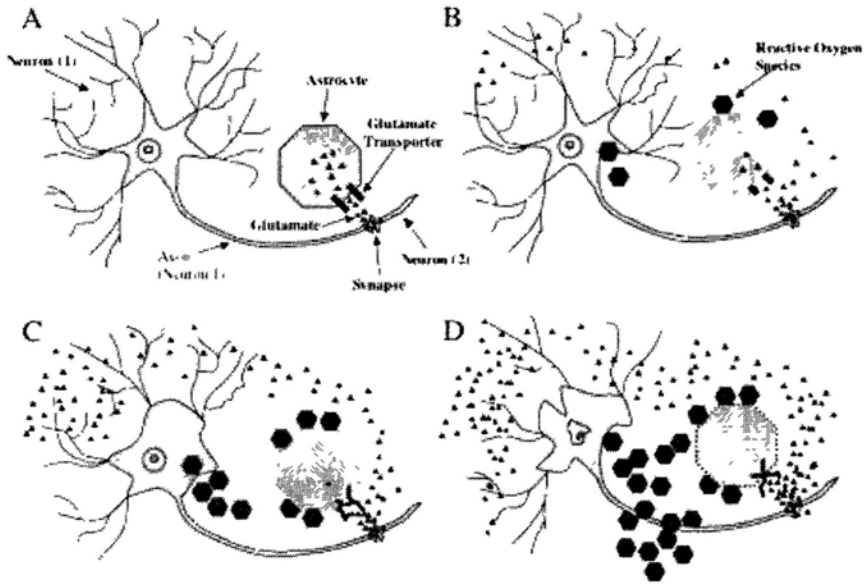


Figure 1.2 Schematic presentation of glutamate neuroexcitotoxicity. (A) As glutamate is released at the end of the axon, some go to the next nerve cell and some are taken up by the astrocytes. (B) Glutamate release by injured neurons causes them to produces reactive oxygen, which damages the glutamate transporter in astrocytes. (C & D) A cycle begins where more glutamate outside causes the neuron to make more reactive oxygen species, which causes more damage to the glutamate transporter, causing damage to all the neurons in its neighbourhood.

infant rodents and primates, it caused neurodegeneration in the brain areas that lack blood-brain barrier (BBB) (Blood *et al.* 1969; Olney *et al.* 1986). In addition, it destroyed the neurons correlated with their excitatory potencies (Olney *et al.* 1972). This interaction was later termed neuroexcitotoxicity.

The neuroexcitotoxicity may be the underlying mechanisms of the CNS disorders. In particular, slow excitotoxicity, triggered by impairment of mitochondrial energy metabolism, has been implicated. It is conceivable that glutamate over-activity caused by exogenous or endogenous factors is an etiological factor for slow progressive death of vulnerable neuronal populations in chronic neurodegenerative diseases or at least contributes to their natural history and progression. Exogenous or endogenous compounds may activate glutamate receptors, increase glutamate synthesis, release or reuptake, interfere with glutamate-linked ion channels or enhance glutamate-linked second messenger systems (Beal 1992).

The acute component of glutamate toxicity is mediated through a massive influx of sodium and chloride ions into the cell (Rothman 1985; Choi 1987). A second component of glutamate neurotoxicity is Ca^{2+}-dependent and mediated through activation of Ca^{2+}-sensitive proteases (Choi 1987; Tymianski *et al.* 1993). Calpains and other proteases that degrade neurofilaments seem to be involved in the late phase of the calcium activated excitotoxic cascade (Manev *et al.* 1991; Bartus *et al.* 1994a; Bartus *et al.* 1994b). Another category of enzymes with potentially self-digesting

properties are the phospholipase A2 and other lipases, also activated by elevations of intracellular calcium. Agents which inhibit phospholipases can partially attenuate some forms of excitotoxic injury (Rothman *et al.* 1993). Similarly, endonuclease and protease C inhibitors have been shown to inhibit glutamate toxicity in cultures (Samples & Dubinsky 1993).

1.3 Oxidative stress

1.3.1 Free radicals

The role of oxygen free radicals and antioxidants in CNS pathology is of major interest. In our body, the brain uses 20% of the oxygen consumed by the resting body while the brain just accounts for 2% of the body weight (Emerit & Michelson 1982).

Oxygen is necessary for life, but paradoxically, as a by-product of its metabolism it produces reactive oxygen species (ROS), which are highly toxic to neurons. Oxygen, because of its bi-radical nature, readily accepts unpaired electrons to give rise to a series of partially reduced species collectively known as ROS, including superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl (HO), peroxyl (ROO), alkoxy (RO), and nitric oxide (NO), until it itself is completely reduced to water (Takeuchi *et al.* 1996).

Free radicals are highly reactive molecules or chemical species capable of independent existence (Singh *et al.* 2004). Generation of highly active ROS is an integral feature of normal cellular function like mitochondrial respiratory chain,

phagocytosis, arachidonic acid metabolism, ovulation, and fertilisation. Their production however, multiplies several folds during pathological conditions. Damage due to free radicals caused by ROS leads to several damaging effects as they can attack lipid, protein/enzyme, carbohudrates, and DNA in cells and tissues. They induce undesirable oxidation, causing membrane damage, protein modification, DNA damage, and cell death induced by DNA fragmentation and lipid peroxidation. This oxidative damage/stress, associated with ROS is believed to be involved in the pathophysiological role in aging (Halliwell & Gutteridge 1984). Postmortem brain tissues from patients with neurodegenerative disorders, including AD, PD and ALS, clearly show increased indices of ROS in affected brain regions (Halliwell & Gutteridge 1986; Brown & Hall 1992; Singh *et al.* 2004).

Most of the superoxide radicals are formed in the mitochondrial and microsomal electron transport chain (Cafe *et al.* 1994). Except for cytochrome oxidase, which retains the partially reduced oxygen intermediates bound to its active site, all other factors in the mitochondrial respiratory chain, such as ubiquinone, transfer the electron directly to oxygen and do not retain the partially reduced oxygen intermediates in their active sites (Hemnani & Parihar 1998). On the internal mitochondrial membrane, the superoxide anion may also be generated by autooxidation of semiquinones. The majority of superoxide radicals generated by mitochondrial electron transport chain are enzymatically dismutated to H_2O_2. The hydroxyl and alkoxy free radicals are highly reactive species and rapidly attack the

macromolecules in cells (Boveris *et al.* 1972). The superoxide anion, lipid hydroperoxides, and nitric oxide are comparatively less reactive. A limited number of enzymes like xanthine-oxidase, tryptophan dioxygenase, and indole-amine dioxygenase produce the superoxide free radical directly (Singh *et al.* 2004). Reactions catalysed by enzymes, such as monoamine oxidase and L-amino acid oxidase, also produce hydrogen peroxide directly (Stohs 1995). Further, nitric oxide free radical can react with superoxide radical to form highly toxic peroxynitrite ($ONOO^-$). When peroxynitrite reacts with human body fluids and tissues, nitrotyrosines are generated, which have been detected in human brain and may be increased in neurodegenerative diseases, especially because glial cells and macrophages generate nitric oxide (Novelli *et al.* 1993; Cafe *et al.* 1994). There has been speculation that nitric oxide is implicated in many of the neurodegenerative disorders.

1.3.2 Antioxidant

1.3.2.1 Endogenous antioxidants

Biologic systems have evolved with endogenous defense mechanisms to help protect against free radical-induced cell damage. Glutathione peroxidase, catalase, and superoxide dismutases are antioxidant enzymes, which metabolize toxic oxidative intermediates. They require micronutrient as cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity and effective antioxidant defense mechanisms (Halliwell & Gutteridge 1990; Halliwell 2001).

SOD, catalase, and glutathione peroxidase are three primary enzymes involved in direct elimination of active oxygen species, such as hydroxyl radical, superoxide radical and hydrogen peroxide. Glutathione reductase, glucose-6-phosphate dehydrogenase, and cytosolic GST are secondary enzymes, which help in the detoxification of ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione (GSH) and NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate (NADP+)) necessary for optimum functioning of the primary antioxidant enzymes (Vendemiale *et al.* 1999; Singh *et al.* 2003).

Glutathione, ascorbic acid, alpha-tocopherol, beta-carotene, bilirubin, selenium, NADPH, butylhydroxyanisole (BHA), mannitol, benzoate, histidine peptide, the iron-bonding transferrin, dihydrolipoic acid, reduced CoQ10, melatonin, uric acid, and plasma protein thiol as a whole play a homeostatic or protective role against ROS produced during normal cellular metabolism and after active oxidation insult (Sies & Stahl 1995). Glutathione is the most significant component which directly quenches ROS such as lipid peroxides and plays major role in xenobiotic metabolism (Meister 1994). When an individual is exposed to high level of xenobiotics, more glutathione is utilized for conjugation making it less available to serve as an antioxidant. It also maintains ascorbate (vitamin C) and alpha-tocopherol (vitamin E), in their reduced form, which also exert an antioxidant effect by quenching free radicals (Anderson

1996).

The most widely studied dietary antioxidants are vitamin C, vitamin E, and beta-carotene. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids, as it is capable of neutralising ROS in the aqueous phase before lipid peroxidation is initiated.

1.3.2.2 Endogenous antioxidants from diet

Vitamin E is a major lipid-soluble antioxidant, and is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Beta-carotene and other carotenoids also provide antioxidant protection to lipid rich tissues (Berry & Kohen 1999). Fruits and vegetables are major sources of vitamin C and carotenoids, while whole grains, such as cereals and high quality vegetable oils, are major sources of vitamin E (Halliwell 1994) (Block *et al.* 1992).

A number of other dietary antioxidants exist beyond the traditional vitamins collectively known as phytonutrients or phytochemicals which are being increasingly appreciated for their antioxidant activity; one example is flavonoids which are a group of polyphenolic compounds (Weisburger 1997; Pietta 2000). These are widely found in plants as glucosylated derivatives. They are responsible for the different brilliant shades such as blue, scarlet, and orange. They are found in leaves, flowers, fruits,

seeds, nuts, grains, spices, different medicinal plants, and beverages such as wine and tea (Gale 2001; Tabet *et al.* 2001).

Flavonoids exhibit several biological effects such as anti-tumour, anti-ischaemic, anti-allergic, anti-hepatotoxic, antiulcerative, and anti-inflammatory activities. These are also known to inhibit the activities of several enzymes, including lipoxygenase, cyclooxygenase, monooxygenase, xanthine oxidase, glutathione-S-transferase, mitochondrial succino-oxidase, and NADH oxidase, phospholipase A2, and protein kinases. Many of the biological activities of flavonoids are attributed to their antioxidant properties and free radical scavenging capabilities. The antioxidant activities of flavonoids vary considerably depending upon the different backbone structures and functional groups. A number of flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron is a potential enhancer of ROS formation as it leads to reduction of H_2O_2 and generation of the highly aggressive hydroxyl radical (Shu 1998; Gale 2001). Free copper mediates low-density lipoprotein (LDL) oxidation and contributes to oxidative damage due to lipid peroxidation. Due to the inefficiency of our endogenous defense systems as well as the existence of some physiopathological situations, such as, cigarette smoke, air pollution, ultraviolet (UV) radiation, inflammation and ischaemia/reperfusion. ROS can be produced in excess, and increasing amounts of dietary antioxidants will be needed for diminishing the cumulative effect of oxidative damage over an individual's life span (Sun *et al.* 2002).

1.3.3 Oxidative stress and neurodegeneration

The oxidative stress is a shift towards the pro-oxidant in the pro-oxidant/antioxidant balance that can occur as a result of an increase in oxidative metabolism. Its increase at the cellular level can come as a consequence of several factors, including exposure to alcohol, cold, medications, trauma, infections, toxins, radiation, strenuous physical activity, and poor diet. Defense against all of these processes is dependent upon the adequacy of various antioxidants that are derived either directly or indirectly from the diet (Gotz *et al.* 1994).

The nervous system is rich in both unsaturated fatty acids and iron. The high lipid content of nervous tissue, coupled with its high aerobic metabolic activity, makes it particularly susceptible to oxidative damage. The high level of iron may be essential, particularly during brain development, but its presence also means that injury to brain cells may release iron ions, which lead to oxidative stress via the iron-catalysed formation of ROS (Bauer & Bauer 1999). In addition, those brain regions that are rich in catecholamines are exceptionally vulnerable to free radical generation. The catecholamine adrenaline, noradrenaline, and dopamine can spontaneously break down to free radicals, or can be metabolized to radicals by the endogenous enzymes such as monoamine oxidases (MAO). One such region of the brain is the substantia nigra (SN), where a connection has been established between antioxidant depletion (including GSH) and tissue degeneration (Perry *et al.* 2002). A number of *in vitro*

studies have shown that antioxidants, both endogenous and dietary, can protect nervous tissue from damage by oxidative stress (Contestabile 2001). Uric acid, an endogenous antioxidant, was found to prevent neuronal damage in rats, both *in vitro* and *in vivo*, from the metabolic stresses of ischaemia, oxidative stress as well as exposure to the excitatory amino acid glutamate and the toxic compound, cyanide. Vitamin E was found to prevent cell death in rat neurons subjected to hypoxia followed by oxygen reperfusion (Yu *et al.* 1998). In the same study it was shown that vitamin E prevented neuronal damage from reactive nitrogen species. Both vitamin E and beta-carotene were found to protect rat neurons against oxidative stress from exposure to ethanol (Copp *et al.* 1999). In an experimental model of diabetes-caused neurovascular dysfunction, beta-carotene was found to protect cells most effectively, followed by vitamin E and vitamin C (Mitchell *et al.* 1999).

Most *in vivo* and clinical studies of the effects of lipid soluble antioxidant supplementation on neurological diseases have focused on vitamin E. A report in 1991 demonstrated that the rate at which PD progressed to the point when the patient required treatment with levodopa was slowed by 2.5 years in patients given large doses of vitamin C and synthetic vitamin E (Prasad *et al.* 1999). Although one study reported that high doses of vitamin E resulted in elevated plasma levels but failed to increase vitamin E levels in cerebrospinal fluid (CSF), a later report demonstrated that high doses of vitamin E did result in elevation of CSF vitamin E levels, and possibly brain vitamin E levels (Higgins *et al.* 2008). Recently it was shown that the protein

responsible for the uptake of vitamin E is in fact present in brain cells of patients suffering from vitamin E deficiency or diseases associated with oxidative stress (Clarke *et al.* 2008). In one study, it was found that the risk for PD was lower for subjects who had higher dietary intakes of antioxidants, particularly vitamin E. It has also reported that a low dietary intake of beta-carotene was associated with impaired cognitive function in a group of persons aged 55-95; no such association was observed for either vitamins C or E (Hellenbrand *et al.* 1996). In another study, serum concentration of vitamin E was found to be significantly associated with cognitive function in adults aged 50 - 75 years measured by a standardised test (Schmidt *et al.* 1998). About 20% of familial amyotrophic lateral sclerosis (FALS) cases are associated with a mutation in the gene for copper/zinc superoxide dismutase, an important antioxidant enzyme, and *in vitro* experiments demonstrated that expression of the mutant enzyme in neuronal cells caused cell death, which could be prevented by antioxidant small molecules such as glutathione and vitamin E (Ferrante *et al.* 1997).

There is substantial evidence that oxidative stress is a causative or at least ancillary factor in the pathogenesis of major neurodegenerative diseases, including PD, AD, and ALS as well as in cases of stroke, trauma, and seizures (Ghadge *et al.* 1997). Decreased levels of antioxidant enzyme activity have been reported in patients with PD (Spina & Cohen 1989; Harish *et al.* 2010). Evidence of increase in lipid peroxidtion and oxidation of DNA and proteins has indeed been seen in the substantia

nigra of patients affected with PD. Similar increase in markers of oxidative stress have also been seen in AD, Huntington's disease, and in both FALS and sporadic ALS (SALS) patients (Boll *et al.* 2008).

1.4 Neurodegenerative diseases

There are many neurodegenerative diseases including PD, AD, HD, ALS and glaucoma, which occur as a result of neurodegenerative changes. In this section, we will mainly discuss on AD and glaucoma.

1.4.1 Alzheimer's disease

AD is a debilitating neurodegenerative disease that directly affects millions of people and indirectly affects the lives of tens of millions of others who must deal with many years of cognitive decline of their loved ones. AD is the most common form of dementia. With an increasing aging population in the world, AD is becoming an ever-increasing social burden for the health care system and national economy in addition to the emotional burden for the immediate family members.

1.4.1.1 Risk factors of AD

Some risk factors are involved in AD. Increasing age is the most important risk factor of AD. Following age, the presence of the apolipoprotein Eε4 (ApoEε4) allele is another major risk factor (Saczynski *et al.* 2010). Other medical conditions such as cardiovascular disease, traumatic brain injury, depression, lower educational

achievement and/or occupational status, parental age at time of birth, smoking, first-degree relative with Down syndrome, low levels of folate and vitamin B12, and elevated plasma and total homocysteine levels are the other putative risk factors of AD (Guo *et al.* 2000; Seshadri *et al.* 2002; Green *et al.* 2003; Qiu *et al.* 2003). In addition, controversy exists regarding the relationship between estrogen and progestin and subsequent development of AD (Zandi *et al.* 2002).

1.4.1.2 Biomarkers of AD

An AD biomarker would provide clinicians with better accuracy in diagnosing the disorder, identifying those individuals at risk in the hopes of employing early therapy, and determining if treatment is effective. Several biomarkers have been investigated thus far, and these include cerebrospinal fluid (CSF) tau, plasma and CSF amyloid-β (Aβ) levels, platelet amyloid precursor protein (APP) isoforms, and neuronal thread protein (NTP) (Davatzikos *et al.* 2010; Wallin *et al.* 2010).

Several studies have shown that CSF levels of $A\beta_{42}$ are reduced in AD relative to normal control subjects (Hu *et al.* 2010). Similarly, CSF tau levels have been shown to be elevated in AD relative to controls (Takeda *et al.* 2010). The simultaneous measurement of CSF $A\beta_{42}$ and tau may be a useful diagnostic biomarker, and studies have indicated that the sensitivities and specificities of these biomarkers are 85% and 87%, respectively (Kasuga *et al.* 2010).

Three major APP isoforms are present in membranes of resting platelets, and

patients with AD show specific alteration of levels of these platelet APP isoforms. Measurement of the ratio of the isoforms has been shown to differentiate AD from normal controls and non-AD dementias (Di Luca *et al.* 1998). It also correlates with decline in cognition in AD and thus may be a biological index of the severity of cognitive loss in AD (Baskin *et al.* 2000). In addition, measurement of the ratio of the isoforms could show the change with administration of cholinesterase inhibitors, such as donepezil, which suggesting that it mat be a marker for pharmacological activity (Borroni *et al.* 2001).

The neural thread proteins are a family of proteins that is normally expressed in the brain and, especially, brains of AD patients (Flirski & Sobow 2005). Increased levels of NTP were found in advanced AD cases and correlated with progression of dementia. NTP can be measured in both spinal fluid and urine and is higher in AD patients than controls (de la Monte & Wands 2004). Furthermore, NTP may distinguish individuals without dementia from those with AD and non-AD pathology (de la Monte & Wands 2001).

1.4.1.3 Treatment of AD

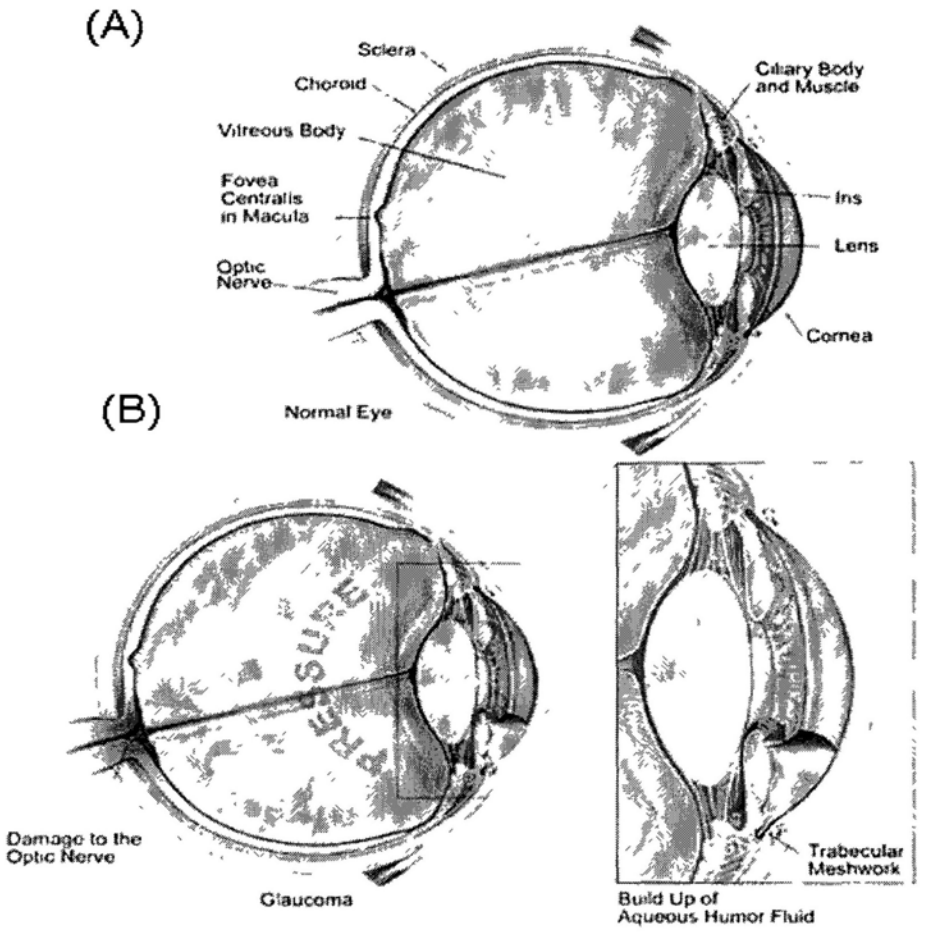
The strategies of pharmacological treatment of AD are disease prevention, symptomatic treatment, as well as disease modification. Current treatments of AD include acetylcholinesterase (AchE) inhibitors, NMDA antagonist and antidepressant.

The use of AchE inhibitors are based on the fact that there is a destruction of

cholinergic neurons and hence decrease cholinergic activity in AD patients. It is believed that this defect would lead to the cognitive impairment in AD. With the use of AchE inhibitors, the concentration of acetylcholine in the synaptic cleft can increase and therefore enhance cholinergic transmission and ameliorate cholinergic deficit. The inhibitors of this kind include galantamine, donepezil and rivastigmine. The NMDA antagonist includes memantine (Molinaro *et al.* 2009). Other treatments are free-radical scavengers, estrogen- or selective estrogen-receptor agonists, anti-inflammatories.

1.4.2 Glaucoma

Glaucoma is a group of diseases of the optic nerve involving the loss of retinal ganglion cells in a characteristic pattern of optic neuropathy (Figure. 1.3).



(www.futurevisionusa.com/)

Figure 1.3 The structure of eye and the occurrence of glaucoma. (A) Structure of eye. To keep it light-weight for rapid movement, the eye is made from soft, but strong tissues. The eye needs to maintain its shape so that it can focus light accurately. This is achieved by keeping the eye firm, like a balloon. Clear fluid (the aqueous humor) is pumped into the eye from the bloodstream carrying oxygen, sugars and other essential nutrients. Circulating around the structures inside the eye, the aqueous is then drained through a meshwork back to the blood to be renewed. Drainage is against resistance, so the eye's pressure kept higher than air pressure, but lower than the blood pressure. (B) Raised intraocular pressure is a significant risk factor for developing glaucoma.

1.4.2.1 Type of glaucoma

1.4.2.1.1 Primary open angle glaucoma (POAG)

Primary open-angle glaucoma (POAG) is a multifactorial optic neuropathy which is chronic and progressive with a characteristic acquired loss of optic nerve fibers (Van Buskirk & Cioffi 1992). POAG is a major health concern worldwide, largely because of its usually silent, progressive nature. It is also one of the leading preventable causes of blindness in the world. Pathophysiologically, the increased pressure of POAG is caused by trabecular blockage which is where the aqueous humor in the eye drains out. The eye adjusts partially to the raised eye pressure and the patient may not notice anything wrong until blood supply of the nerve of sight (the optic nerve) is slowly cut off by the pressure on it, causing blank patches in the field of vision. (Bathija *et al.* 1998)

1.4.2.1.2 Angle-close glaucoma (ACG)

ACG is a rapid or sudden increase in pressure inside the eye. It is caused by contact between the iris and trabecular meshwork (TM), which in turn obstructs outflow of the aqueous humor from the eye. This contact between iris and trabecular meshwork may gradually damage the function of the meshwork until it fails to keep pace with aqueous production, resulting in the pressure rises.

1.4.2.1.3 Secondary glaucoma

Secondary glaucoma is a type of glaucoma that occurs as a complication of

various medical conditions such as eye surgery, advanced cataracts, eye injuries, some eye tumors, uveitis, diabetes or use of corticosteroid drugs.

1.4.2.1.4 Congenital glaucoma

Congenital glaucoma is relatively rare. It presents at birth and most cases are diagnosed during the first year of life. It is characterized by the improper development of trabecular meshwork which leads to an abnormal drainage of the aqueous humor from inside the eye, resulting in higher intraocular pressure.

1.4.2.2 Risk factors for glaucoma

1.4.2.2.1 Intraocular pressure

Increase of the intraocular pressure (IOP) is often considered to be the major risk factor in glaucoma (Leydhecker 1958; Flanagan 1998; Friedman *et al.* 2004). Glaucoma is often called "the sneak thief of sight". This is because in most cases, IOP can build up and destroy sight without causing obvious symptoms. IOP is mainly determined by the coupling of the production of aqueous humor and the drainage of aqueous humor mainly through the trabecular meshwork located in the anterior chamber angle.

The average value of IOP of non-glaucomatous people is 15.5 mmHg with fluctuations of about 2.75 mmHg. The normal pressure can range from 10 to 21 mmHg. The eye pressure has a non-gaussian distribution with a skew towards higher

pressures which means in many people pressure above the 21 mmHg level is not abnormal. On the other hand, it is well recognized that some eyes undergo extensive glaucoma damage even when the pressure is in the normal range, while others suffer no damage with pressure well above 21 mmHg. Despite this, the value of 21 mmHg has been historically accepted as a good way to separate the normal eye pressure from a pressure level which should be viewed with suspicion for glaucoma. In glaucomatous patient, the IOP is greater than 21 mmHg (Kitazawa & Horie 1975; Asrani *et al.* 2000). Moreover, glaucomatous patients with an IOP fluctuations of 6 mmHg or greater are more susceptible to progressive visual field loss (Zeimer *et al.* 1991).

IOP reduction is also the most frequent goal for glaucoma therapy. Lowering IOP is a proven means to slow or halt the disease progression in those who are at high risk of developing glaucoma (Nemesure *et al.* 2003), with mild to moderate glaucoma (Heijl *et al.* 2002) and with more advanced glaucoma (Leske 2003).

1.4.2.2.2 Age

The risk of developing glaucoma increases with age. Chronic glaucoma becomes much more common with increase of age. People who are over 60 years of age are particularly at higher risk of developing glaucoma. People in certain racial or ethnic groups are at higher risk of developing glaucoma at younger ages. Also, the risk is especially high for Asian people and hyperopic patients who tend to have a narrower

anterior chamber angles (Klaver *et al.* 1998a; Klaver *et al.* 1998b).

It is now known that age is closely involved in the incidence and prevalence of POAG and ACG. One early study showed that the prevalence of POAG in white people of 70-79 years old was 3.5 times higher than those who are 40-49 years old (Tielsch *et al.* 1991).

1.4.2.2.3 Positive family history of glaucoma

Wolfs' study showed that the life time risk of developing glaucoma in siblings and children of POAG patients is more than 9 times higher than the matched control (Wolfs *et al.* 1998). In Chinese population, the prevalence of ACG is 6 times higher in patients with a positive family history (Hu *et al.* 1989). The studies suggested that if one has a close relative who has chronic glaucoma then he/she should have an eye test at regular intervals. This is especially important if you are aged over 40, then eye tests should be done every year.

It is clear that genetic plays an important role in both POAG and ACG. The first identified gene associated with POAG was trabecular meshwork-induced glucocorticoid response protein (TIGR), also known as myocilin (MYOC) (Kubota *et al.* 1997; Stone *et al.* 1997). TIGR/MYOC gene mutations are found in ~4% of all POAG patients. More than 40 myocilin mutations have been found to be associated with 2–4% of all POAGs. Almost all of them have been observed in the TIGR/MYOC

third exon, which encodes the olfactomedin homology domain (Alward *et al.* 1998; Fingert *et al.* 1998; Fingert *et al.* 1999). Genetic screening for TIGR/MYOC mutations should be offered to glaucoma families and to close relatives of unrelated patients aware of a family history for glaucoma (Faucher *et al.* 2002). Another gene cytochrome P4501B1 (CYP1B1) was found by Stoilov in 1998 which could interfere with normal function of trabecular meshwork and result in elevation of IOP (Stoilov *et al.* 1998; Rezaie *et al.* 2002). Genetic linkage analysis and mutation studies have identified CYP1B1 as a causative gene in primary congenital glaucoma (PCG), as a modifier gene in POAG, and, on rare occasions, as causative gene in POAG as well as in several anterior segment dysgenesisdisorders (ASD) (Michels-Rautenstrauss *et al.* 2001; Vincent *et al.* 2002; Vasiliou & Gonzalez 2008).

Mutations in the optineurin gene (OPTN) were found in 2002 in a large British pedigree with autosomal dominant normal tension glaucoma (NTG). It is the second gene besides MYOC in which mutations have been identified to be associated with POAG. At the present time, more than 20 distinct mutations have been found in the OPTN coding region, four of which, having close correlation with POAG, are located in exons 4, 5, 6, and 16(Rezaie *et al.* 2002). OPTN mutations have been identified in Hong Kong, Guangzhou and Northeastern China (Leung *et al.* 2003; Xiao *et al.* 2009). OPTN is expressed in trabecular meshwork, nonpigmented ciliary epithelium, retina, and brain. It is believed that OPTN plays a neuroprotective role of the retinal ganglion cells (RGCs)(Sun *et al.* 2006).

1.4.2.2.4 Race

One study showed that glaucoma is 3 to 4 times more likely to occur in Blacks than in Whites. In addition, Glaucoma is 6 times more likely to cause blindness in Blacks than in Whites (Palmisano *et al.* 2000). Another study found that black and white patients with advanced glaucoma respond differently to two surgical treatments for glaucoma. Moreover, Blacks have thinner corneas than Whites (by about 23 microns) and this may well be the factor that put black at a higher risk for glaucoma progression (Judith & Michael 1998). About 80% people with ACG lives in Asia (Quigley & Broman 2006). The prevalence of ACG in Chinese and Eskimo descents was found to be 20 to 40 times higher than in Whites (Alsbirk 1976; Hu *et al.* 1989).

1.4.2.2.5 Suspicious optic nerve appearance

Suspicious optic nerve appearance with increased cupping is considered to be an important risk factor (Nouri-Mahdavi *et al.* 2004; Lim *et al.* 2009). The highest risk patients with glaucoma were identified as having cup to disc ratio more than 0.5.

1.4.2.2.6 Central corneal thickness less than 0.5 mm

Corneal thickness is a strong risk factor for developing glaucoma. Several studies have identified that thinner central corneal thickness was associated with the state of glaucoma damage (Gordon *et al.* 2002; Congdon *et al.* 2006). Patients with a corneal thickness less than 555 microns have a three fold greater risk of developing glaucoma

as compared with those whose cornea are more than 588 microns thick (Kass *et al.* 2002). It is expected to make corneal thickness measurement an important part of the glaucoma work-up.

1.4.2.2.7 Other diseases

Systemic factors may influence glaucoma. Systemic disorders associated with the development of POAG include the diseases of cardiovascular, autonomic nervous and immune systems.

Most studies support a weak association between diabetes and glaucoma. Studies suggest that glaucoma patients with diabetes appear to be more susceptible to nerve damage with resulting visual field loss at a lower intraocular pressure than those patients with glaucoma alone. It has also been found that there is a significant qualitative difference in the pattern of visual field loss between glaucoma patients with and without diabetes. Whether diabetes is a significant risk factor for glaucoma remains controversial (Prahs & Helbig 2009).

Besides diabetes, an association between POAG and other neurodegenerative diseases, such as Alzheimer disease and Parkinson disease, has also been investigated (Pache & Flammer 2006). Chandra and coworkers, who studied all death certificates of the United States since 1978, were the first to describe a high frequency of glaucoma in senile and presenile dementia (Chandra *et al.* 1986). Hinton and coworkers found widespread axonal and retinal ganglion cell degeneration in the optic

nerves of 8 of 10 patients with Alzheimer disease (Hinton *et al.* 1986). Sadun and Bassi showed in 10 patients with Alzheimer disease that this loss was predominant in the largest class of retinal ganglion cells, and they reported a dropout of retinal ganglion cells ranging from 30% to 60% (Sadun & Bassi 1990) (Bassi & Sadun 1990).

1.4.2.3 Models in glaucoma research

The strategic use of different model systems has facilitated major advances in our understanding of glaucoma. Biological basis of glaucoma has been studied by different glaucoma models which allow experiments to be conducted otherwise not possible in human. Moreover, these models, including *in vitro* cell and organ culture and *in vivo* experimental glaucoma, help us establish the proof of principle for potential treatments prior to human testing (Weinreb & Lindsey 2005).

1.4.2.3.1 *In vitro* models

The *in vitro* culture of dissociated cells from animal eyes and post-mortem human eyes allows the study of cell responses in isolation fashion. In addition, it is relatively easy to directly collect cell products produced in the culture medium for further testing.

1.4.2.3.1.1 Cell culture models

Most cell types of the eye have been cultured. These include retinal ganglion cells

(Lindsey & Weinreb 1994), human trabecular meshwork cells (Polansky & Alvarado 1985), ciliary smooth muscle (Weinreb *et al.* 1992), iris melanocytes (Hu *et al.* 1987), Schlemm's canal endothelium (Anthony *et al.* 2001), and lamina cribrosa cells (Hernandez *et al.* 1988). These cell types can be cultured *in vitro* and can be used as a rapid and efficient way for screening experiment within a single study.

1.4.2.3.1.2 Organ culture models

Organ culture is the maintenance of pieces of whole tissues in culture medium for several days up to several months. It also includes the culture of tissue slices generated by using a tissue chopper or vibratome, as well as perfusion cultures of the entire anterior segment of the eye. (Weinreb & Lindsey 2005). Many tissue types of the eye have been studied by organ culture, such as retina (Wizenmann & Bahr 1998; Kretz *et al.* 2004; Kretz *et al.* 2007) and human trabecular meshwork (Johnson & Tschumper 1987). For the organ culture to be successful, it is essential that the tissue is never been disrupted or damaged. In addition, it is also relatively easy to directly collect cell products produced in the culture medium for further biochemical analyses.

1.4.2.3.2 *In vivo* models

The limitations of *in vitro* culture include the possibility of experimental responses that differ from the intact organism, cellular differentiation, tissue reorganization, the variability of post-mortem tissue samples and typically only very small amounts of experimental material can be available from each eye. The value of

animal models as substitutes for the study of human pathology has been recognized by the scientific community for a long time. An experimental model system of pressure-induced optic nerve damage would greatly facilitate the understanding of the cellular events leading to RGC death and of how RGCs are influenced by IOP and other risk factors associated with glaucomatous neuropathy. *In vivo* animal glaucoma models have the advantages of having intact visual system as well as immune system and vascular functions. These models mimic certain physiological responses which more like glaucoma patient's eyes than in cell and organ cultures. Animal models based on increasing IOP have been developed in rabbit, monkeys and rodents (Weinreb & Lindsey 2005). Rats have been widely used in experimental glaucoma models as they have anatomical similarities in the retina and optic nerve with primates.

1.4.2.3.2.1 Episcleral vein cauterization model

After conjunctiva is cut opened, two or three out of four major episcleral venous trunks around the circumference of the rat eye were cauterized. The IOP of experimental eye increased about 1.5 to 1.8 times of the control eyes. The elevation of IOP was proportional to the percentage of venous occlusion (Shareef *et al.* 1995; Laquis *et al.* 1998). The loss of RGCs was at about 4% per week for the first two or three weeks. The surgical techniques are relative easier than the intra-episcleral vein hypertonic saline injection model, but the complete block of 50 to 75% outflow of blood through the episcleral veins can cause congestion in the intraocular vasculature.

The loss of RGCs can be a combined result of vascular congestion and elevation of IOP.

1.4.2.3.2.2 Intra-episcleral vein hypertonic saline injection model

In rat eye, aqueous humor is produced by the ciliary body and collected through the trabecular meshwork into Schlemm cannal that locates in the angle of anterior chamber. It enters the venous plexus through episcleral drainage vein. In this model, a custom-built C-shaped plastic ring is placed behind the limbus of the rat eye occluding all but the superior temporal episcleral veins. Then, a volume of 50 μl of hypertonic saline (~1.75M) was injected into the exposed episcleral vein towards the limbus via a glass microneedle. Because of the occlusion of most episcleral veins by the C-shape plastic ring, the majority of the injected solution is directed into aqueous veins, Schlemm cannal, TM, and the anterior chamber. The hypertonic saline scleroses these aqueous humor drainage apparatus in the anterior chamber inducing inflammation and scarring. After this injection, many rat eyes will develop ocular hypertension (OH) within 7-10 days. A second injection may be required in unsuccessful cases. This method results in an increase of IOP at 7 to 28 mmHg above baseline. The successful elevated IOP is long lasting, and the prolonged OH for up to 200 days has been reported (Morrison *et al.* 1997; Pang & Clark 2007). Characteristic histopathological changes in the optic nerve and loss of RGCs that similar to human glaucoma were observed in the injected eyes. However, the sophisticated procedure for intra-episcleral vein injection is technically challenging, requiring careful training

and practice. In addition, excessive inflammation caused by the hypertonic saline and high injection pressure may affect the retina in the posterior chamber.

1.4.2.3.2.3 Trabecular meshwork laser photocoagulation model

In this model, India ink is injected into the anterior chamber of the rat eye three days prior to laser photocoagulation(Ueda *et al.* 1998; Piri *et al.* 2007). Laser point is focused at the carbon particles deposited at the trabecular meshwork. The operation results in anterior synechia, thinning of the nerve fiber layer and optic atrophy. However, the anterior chamber injection of India ink may cause injury to the anatomical structures such as the iris and the lens. Two different kinds of operations (injection and laser photocoagulation) in this model complicate the evaluation on the loss of RGCs.

1.4.2.3.2.4 Limbal and episcleral vein laser photocoagulation model

In this rat model, the limbal vein and the three radical episcleral aqueous humor drainage vein (superior nasal, superior temporal and inferior temporal) were photocoagulated using an Argon laser (Ji *et al.* 2004; Chan *et al.* 2007; Chiu *et al.* 2007). About 60 laser spots (power, 1000mV; spot size, 50-100 μm; duration,0.1 second) around the limbal vein (except the nasal area) and 15-20 laser spots on each episcleral aqueous humor drainage vein were applied. To maintain high IOP, a second laser treatment at the same settings was applied 7 days laser. The manipulation of the rat eye is limited to the laser burns on the vessels. There is no surgical incision, no

opening of the conjunctiva, no anterior chamber injection and no extra pressure to the limbal vessels. The cause of RGC loss is simplified in this model. The elevation of IOP can be sustained for at least two months after two concessive laser photocoagulation with a 7-day interval, and the RGC loss is about 20% at 2 months (Li *et al.* 2006). This model is widely employed for the investigation of neuroprotective effects on chronic RGC loss (Schori *et al.* 2001; Bakalash *et al.* 2003; Ji *et al.* 2004; Bakalash *et al.* 2005; Bakalash *et al.* 2007; Chan *et al.* 2007). When the small size of the rat eyes is taken into consideration, this procedure necessitates very steady hands in practice to get consistent results.

1.4.2.3.3 Post-mortem human eye models

Post-mortem human eye tissues (eyes obtained soon after the death of a person) have been used to investigate the physical properties of ocular tissues such as scleral strength and permeability, optic nerve head compliance, and the contraction of isolation ciliary muscle and iris tissue (Zeimer & Ogura 1989; Olsen *et al.* 1995). The usefulness of these models is limited by variations in the eye tissue preservation procedure after death, the disease history and the cause of donor death.

1.4.2.4 Physiological mediators in glaucoma

As a kind of aging-associated neurodegenerative disease, glaucoma shares the similar modes to other neurodegenerative diseases, such as AD and PD. There are nine physiological mediators which are common to all neurodegenerative diseases,

including glaucoma (Schwartz 2005). These factors including excessive levels of glutamate; oxidative stress; conformational alteration of proteins; presence of inflammation-associated factors; deficiency of neurotrophic and growth factors; metabolic deficits; increase in extracellular matrix proteins; presence of growth-associated inhibitors and malfunction of local immune cells. Two of these factors will be discussed in this section.

1.4.2.4.1 Excessive levels of glutamate

Glutamate is a major excitatory neurotransmitter in the CNS. If excessive amounts of glutamate are released or if glutamate clearance is insufficient, neuronal death will result in a process known as excitotoxicity (Lucas & Newhouse 1957; Thoreson & Witkovsky 1999) (Fang *et al.* 2010). Glutamate release has been implicated as a mechanism of RGC death in glaucoma (Guo *et al.* 2006) (Osborne *et al.* 1999; Salt & Cordeiro 2006) and inhibition or blockade of glutamate activity - in particular, modulation of the NMDA-type receptor has been advocated to be an important strategy for neuroprotection in glaucoma although its exact role is controversial (Hare *et al.* 2004) (Chaudhary *et al.* 1998; Guo *et al.* 2006). In glaucoma, the initial damage to RGCs produce a massive release of glutamate from dying cells and the excessive glutamate subsequently kills the neighboring RGCs. Evidence showed that vitreal glutamate levels are elevated in dog (Brooks *et al.* 1997) and humans with primary glaucoma (Dreyer *et al.* 1996).

1.4.2.4.2 Oxidative stress

The retina is particularly susceptible to oxidative stress because of its high consumption of oxygen, its high proportion of polyunsaturated fatty acids, and its direct exposure to light. Some studies have described that there are some oxidative agents presence in the aqueous humor, such as superoxide anion and hydrogen peroxide, superoxide dismutase (SOD), catalase, and glutathione peroxidase (Marshall 1997; Behndig *et al.* 1998). SOD can be found in all eye tissues, and three SOD isoenzymes (Cu-Zn SOD, Mn SOD, and EC-SOD) have been reported to be present in aqueous humor. It was reported that high levels of extra-cellular SOD in the cornea may be related to the exposure of the eye to visible and ultraviolet light (Behndig *et al.* 1998). It is also known that oxidative stress has been implicated in the possible pathophysiology of different ocular diseases, such as retinopathy of prematurity, macular degeneration, and uveitis (Halliwell & Gutteridge 1989)

1.4.2.5 Treatment of glaucoma

1.4.2.5.1 Medical management

Current medical treatments of glaucoma focus on the lowering of IOP to decrease the likelihood of disease progression. The most common treatments for glaucoma are eye drops and rarely, pills. There are a number of various categories of eye drops, but all are used to either decrease the amount of aqueous humor in the eye or improve its outward flow. These medications include alpha-adrenergic agonists (e.g. apraclonidine, epinephrine), beta-adrenergic antagonists (e.g. timolol, betaxolol),

carbonic anhydrase inhibitors (e.g. dorzolamide, brinzolamide), cholinergic agents (e.g. pilocarpine, echothiophate), prostaglandin analogs (e.g. latanoprost, bimatoprost) and the combined use of several eye drops (e.g. dorzolamide and timolol, latanoprost and timolol).

1.4.2.5.2 Surgical management

There are several forms of surgical or laser therapy for glaucoma which are designed to reduce the IOP either through increasing the rate of aqueous outflow or decreasing aqueous production. These operations include laser trabeculoplasty, laser iridectomy, laser pupilloplasty and other laser procedures for the iris. Incisional surgeries include incisional iridectomy, incisional trabeculotomy, goniotomy and filtering surgery (Allingham *et al.* 2005).

1.4.2.6 Neuroprotection in glaucoma

Both medical and surgical treatment of glaucoma can effectively lower IOP to elicit the protective effect of the optic nerve from progressive glaucomatous optic neuropathy in most glaucoma patients. However, even if IOP is markedly reduced, some glaucoma patients still show progressive visual field loss. This is because RGC death is a common final pathology that causes patients to lose their visual in glaucoma. Evidence suggests that glaucoma treatment might benefit by preventing the death of RGC and maintaining neural function, which is neuroprotection (Sena *et al.* 2010) (Cheung *et al.* 2008).

A number of drugs have been shown to have neuroprotective effect in animal models of retinal and optic nerve injury (Hare & Wheeler 2009). However, to date several clinical trials have been conducted to evaluate the neuroprotection in neuronal disease of the visual system, and none has shown efficacy. One of these drugs is memantine, which is an uncompetitive antagonist of the NMDA glutamate receptor. It is approved only for the treatment of moderate-to-severe AD. Clinical trials have explored the role of memantine in glaucoma. Nevertheless, even the study showed that the progression of disease was significantly lower in patients receiving the higher dose of memantine compared to patients receiving the low-dose of memantine, there was no significant benefit compared to patients receiving placebo. Therefore, the study failed to meet its primary endpoint and to sufficiently replicate the results of the first Phase III trial. (http://agn.client.shareholder.com/releasedetail.cfm?ReleaseID_290764. Accessed: May 2, 2009.) (Danesh-Meyer & Levin 2009; Osborne 2009)

There are still some other agents that have undergone clinical investigation, even on a much smaller scale, such as brimonidine (Kandarakis *et al.* 2010; Park *et al.* 2010), which has been studied in three clinical trials of optic nerve disease. But it is still not known whether this drug showed a neuroprotective effect.

There are several criteria for identifying a drug as potential glaucoma

neuroprotective agent (Weinreb 2007): 1) The drug must reach the retina or optic nerve in pharmacologically effective concentrations; 2) It should have a special receptor target in the retina or optic nerve; 3) Evidence must be obtained in animal models that activation of the target triggers pathway increases neuronal survival; 4) The neuroprotective activity must be demonstrated in human trials.

1.5 Neurodegenerative disease and Chinese medicine

Growing studies indicated that neuroprotective strategies to rescue neurons from progressive death are valuable therapeutic pathways worth exploring. Among different treatment paradigms, Chinese herbal medicine has shown potential clinical benefits in attenuating the progression of neurodegenerative disease in human beings. The development of anti-aging drugs from Chinese medicine herbs may be one of the possible interventions (Chen *et al.* 2007).

Chinese herbal medicine may present a valuable resource in prevention as well as treatment of some CNS diseases, especially incorporating with a healthy lifestyle including good dietary habits and moderate physical activity. Chinese herbal medicine is a part of traditional Chinese medicine (TCM) system, which is one of the world's oldest documented medical systems (Iriti *et al.* 2010). *Yin*, *Yang* and *Qi* are the typical three concepts of TCM. They represent a term for collectively describing both the mental and physical energy, which is known as "vital energy" (Ody 2000). It is believed that these three factors are essential for growth, daily activities, reproduction,

and cognitive functions. In addition, the unbalance of *Yin* and *Yang* would break the body's harmony predisposing to diseases.

Ancient Chinese medical literature ascribes the location of neurodegenerative diseases to the brain, which is closely related to the functions of kidney, as well as the spleen, heart and liver. According to Chinese medical theories, neurodegenerative diseases are characterized by asthenia of marrow, or *Sui* in Chinese. *Sui* is divided into brain, spinal cord and bone marrow. They are related to each other closely, and are produced from the kidney essence, which is called *Jing*. *Jing* is the Chinese designation for the essential fluid of our physical body. The traditional Chinese physician Zhang Xi-chun of the Qing dynasty proposed that our brain takes charge of all the vital movements, eventually produces consciousness and controls its corresponding behaviours. If *Jing* in the kidney is plentiful, and the *Sui* of the body is rich, our body remains healthy. In contrast, if our kidney is lack of *Jing*, the sea of *Sui* will become empty, and not enough essence would supply for our brain. As a result, abnormal behaviours may develop.

To restore the harmony is the principle for treating illnesses in TCM. Aging is considered a progressive decline of vital energy in our body, and anti-aging herbs are able to restore the imbalance of vital energy (Ou *et al.* 2003; Ho *et al.* 2010a). In recent decades, TCM has presented therapeutic wisdom for Western medicine as in case of anti-aging herbs (pharmacopoeia 1985; Jiang 2005). On the other hand, herbal

medicine has been widely investigated for drug development because it has fewer side effects and is much safer to be used than that of synthetic compounds (Wong *et al.* 1994). The treatments of neurodegenerative diseases in TCM mainly focus on the kind of Chinese herbs belonging to tonifying herbs. This category of herbs can help boosting up the level of vital energy in body. As deficiency of vital energy is thought to be the cause of aging, this property is especially important. Anti-aging herbs which are described to have general tonifying functions are therefore being investigated for their actions in the brain as anti-dementia drugs. In addition, anti-aging herbs are often multi-targets and can be used in a number of diseases. They also achieve their therapeutic effects through modulating multiple pathological aspects. In order to elaborate the properties of anti-aging herbs, we summarised the Chinese herbs and prescriptions commonly used to treat neurodegenerative diseases in Table 1.1 and Table 1.2, respectively.

Table 1.1 Chinese herbs commonly used to treat neurodegenerative diseases.

Plant species	Compounds	Effects	Mechanism	References
Herba Erigerontis (燈盞花)	Unkown	Inhibit the aggregation of Aβ	Unknown	(Zhu *et al.* 2009)
Ramulus Uncaria cum Uncis (鉤藤)	Rhynchophylline Isorhynchophylline	Reduce oxidative damage	Unkown	(Mahakunakorn *et al.* 2005)
Radix Salviae Miltiorrhizae (丹參)	Salvianolic acid B (Sal B) Tanshinone IIB (TSB)	Inhibit Ca^{2+} overload	Unknown	(Chen & Liu 2006)
Radix Notoginseng (三七)	Rb1 Rg1 R1	Decrease neuronal apoptosis and necrosis; reduce intracellular free Ca^{2+} concentration	Unkown	(Zhou *et al.* 2007)
Semen Cassiae (決明子)	Unknown	Against NMDA-induced Ca^{2+} dysregulation; inhibit 3 -nitropropionic acid-induced cell death	Unkown	(Drever *et al.* 2008)

Flos Chrysanthemi (菊花)	Unknown	Reduce oxidative damage	Unkown	(Wang *et al.* 2001)
Flos Buddlejae (密蒙花)	Unknown	inhibitory effects on aldose reductase of the unpurified rat lens aldose reductase	Unkown	(Matsuda *et al.* 1995)
Radix et Rhizoma Tripterygii (雷公藤)	Cynandione A	Against H_2O_2, glutamate, kainite -induced degeneration of neurons	Unknown	(Lee *et al.* 2000)
Radix Peucedani (前胡索)	Decursinol , decursin	Decrease of glutamate-induced Ca^{2+} influx; retention of the glutathione defense system	Unknown	(Kang *et al.* 2005; Kang & Kim 2007)
Huperzia serratum (蛇足石杉)	Huperzine A	Against H_2O_2, Aβ, glutamate, ischemia and staurosporine -induced cytotoxicity and apoptosis	Inhibition of AChE	(Wang *et al.* 2006)
Folium Ginkgo (銀杏)	Ginkgolide B	Scavenge free radicals and non-specifically inhibit seryl and aspartyl proteases	Antagonistic action on PAF* receptor	(Maclennan *et al.* 2002)

Radix Polygalae (遠志)	unknown	Inhibition on NMDA-induced elevation of Ca^{2+}	Unknown	(Lee *et al.* 2004)
Rhizoma Polygoni Cuspidati (虎杖)	emodin-8-O-β-d-glucoside	Against cerebral ischemia - reperfused injury and glutamate induced neuronal damage	Unknown	(Wang *et al.* 2007a)
Fructus Lycii (枸杞子)	Polysaccharides	Inhibition of Aβ toxicity; modulation on glutamate toxicity; attenuation of homocysteine toxicity	Modulation of phosphorylations of JNK and PKR	(Ho *et al.* 2007) (Ho *et al.* 2010b)
Rhizoma Phragmitis (蘆根)	p-Coumaric acid	Against $A\beta_{1-42}$, glutamate, or H_2O_2 induced neurotoxicity	Unknown	(Shin *et al.* 2007)
Radix Sanguisorbae (地榆)	gallic acid	Inhibition of $A\beta_{(25-35)}$-induced injury	Unknown	(Ban *et al.* 2008)

* PFA = platelet-activating factor

Table 1.2 Some Chinese prescriptions commonly used to treat neurodegenerative diseases.

Prescriptions	Compounds	Effects	References
Nao Tong Compound	Radix Gineng,、Radix Astragali,、Radix paeoniae, Radix puerariae, Cortex Phellodendri, Rhizoma Cimicifugae, Radix Glycyrrhizae, et al.	Improve the learning and memory ability of rats, decrease the activity of AChE	(Xiao *et al.* 2010)
Nao Li Zhi Bao	Carapax et Plastrum Testudinis, Fructus Lycii, Rhizoma Polygonati, Rhizoma Acori Tatarinowii, Semen Persicae, Radix Angelicae Sinensis, Radix Polygalae, Flos Carthami, et al.	Promote the learning and memory ability of mice; Strengthen immunity; clean free radical and regulate the center cholinergic and monoamine systems	(Bao *et al.* 2000)
HT008-1	Radix ginseng, Radix et Caulis Acanthopanacis Senticosi, Radix Angelicae Sinensis, Radix Scutellariae.	Down-regulated the COX-2 and OX-42 expression in the penumbra region; reduce damage in the cortex	(Bu *et al.* 2010)

As herbal medicine contain complex mixture of active components (phytochemicals), including phenylpropanoids, isoprenoids and alkaloids, it is often difficult to determine which component(s) of the herb(s) are responsible for the biological activity (Suk 2005). Even though several herbal medicines have already been proven to protect neurons, the discovery of the active component(s) needs further investigation.

1.6 Objective of this study

Natural herbs have long been used in Asian societies for treating neurodegenerative disorders. However, for many of them the active compounds are still unknown. In the present work, we aimed to:

1. Find the most effective neuroprotective Chinese herbal extract from the selected Chinese medicines by using a glutamate-induced neurotoxicity on cultured rat cortical neurons model;
2. Identify the active compound(s) from the herbal extract by using bioassay-guided isolation;
3. Conduct the *in vitro* model of glutamate-induced neuronal cell death to further examine the protective effects; and
4. Apply an *in vivo* rat model of ocular hypertension to explore whether the active compound is beneficial for glaucoma.

Chapter 2

Selection and Screening of Chinese Herbal Medicines for Neuroprotective Activities

2.1 Introduction

Medical and surgical methods have already been used to treat neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and glaucoma. Current researches suggest that development of anti-aging drugs from Chinese medicinal herbs may be one of the possible interventions (Bastianetto & Quirion 2002) (Chang 2001). Natural herbs have long been used in Asian societies for treating these diseases and widely investigated for drug development because they have fewer side effects and are safer to be used than that of synthetic compounds (Garai & Lorand 2009). Twelve Chinese herbs were selected for this project based on their clinical application and pharmacological data showing their potential usage in the treatment of neurodegenerative disorders. The general description, preclinical studies and clinical applications of these selected Chinese herbal medicines in the treatment of neurodegenerative disorders will be reviewed in the following sections.

2.1.1 Fructus Alpinae oxyphyllae (益智仁)

Fructus Alpinae oxyphyllae (FAO) is the fruit of *Alpinia oxyphylla* Miq. which belongs to the *Alpinia Roxb* genus and Zingiberaceae family. It is widely distributed in Hainan and Guangdong provinces of China. In the ancient Chinese herbal literature, FAO was recorded as a kind of Chinese herbal medicine which can improve the resistance and health of human body. It could be used as a general tonic, an aphrodisiac, an anti-salivation and anti-diarrhea agent as well as treatment for dementia (Li 1967). Studies have been performed to investigate the different

biological effects of FAO, such as anti-dementia and learning function enhancment (Kubo *et al.* 1995), anti-tumor (Lee *et al.* 1998). One early research has reported that, during the treatment of Wind-heat syndrome and heat-phlegm, the water extract of FAO is beneficial for cerebrovascular lesion and aphasia (Lee 1986). In another study, Lee demonstrated that the pharmacological mechanism for FAO was related to its anti-aging and sexual-reinforcing activities in *in vitro* and *in vivo* experimental systems ((Chen 1992; Lee & Chung 1998). One recent study shows that the water extract of FAO could protect the Aβ-induced neuronal cell death in a dose-dependent manner *in vitro* (Koo *et al.* 2004). The water extract of FAO may exert its neuroprotective actions by reducing the NO-mediated formation of free radicals or antagonizing their toxicity, On the other hand, oral administration of water extract of FAO in mice could prevent ischemia-induced learning disability and rescued hippocampal CA1 neurons from lethal ischemic damage by counting synapses in the hippocampal CA1 region (Koo *et al.* 2004). The ethanol extract of FAO shows strong GSK-3β-dependant inhibition of tau protein phosphorylation at sites T231, T202 and S396. Its inhibitory effect was also shown in differentiated and non-differentiated neuroblastoma SH-SY5Y cell line (Wong *et al.* 2004).

Protocatechuic acid (PCA) is one of the active constituent isolated from FAO. It is able to significantly protect PC12 cells against MPP^+-induced neurotoxicity (An *et al.* 2006; Guan *et al.* 2006) as well as the hydrogen peroxide (H_2O_2) -induced oxidative PC12 cell death both *in vivo* and *in vitro* (Shi *et al.* 2006; Shui *et al.* 2006).

2.1.2 Ramulus Uncaria cum Uncis (鉤藤)

Ramulus Uncaria cum Uncis is the dry stem of *Uncaria rhynchophylla* (Miq.) Jacks.. It is a kind of traditional Chinese medicine which has been long used as an antipyretic, anti-hypertensive and anticonvulsant medication for treatment of headache, vertigo and epilepsy (Li 1967; Tang & Eisenbrand 1992; Xu *et al.* 2007). Fujiwara (Fujiwara *et al.* 2006) suggested that Ramulus Uncaria cum Uncis may be a new class of therapeutic and preventive drug for AD through regulation of the formation and clearance of senile plaques. In addition, it was reported that Ramulus Uncaria cum Uncis has neuroprotective effects both *in vitro* and *in vivo* by reducing oxidative damage to neurons (Dohi *et al.* 2003; Mahakunakorn *et al.* 2004; Mahakunakorn *et al.* 2005). Also, the total alkaloids of Ramulus Uncaria cum Uncis are able to alleviate the acute hypoxia-induced change of sodium currents of rat hippocampal neurons which may be one of the mechanisms for its protective effects on cells (Liu *et al.* 2006).

Rhynchophylline and isorhynchophylline are two major active components of Ramulus Uncaria cum Uncis (Nozoye 1958; Zhang *et al.* 2004b; Jung *et al.* 2006). It was reported that rhynchophylline could protect NT2 neurons from undergoing apoptosis induced by dopamine (DA) (Shi & Kenneth 2002) and it has antiepileptic effect in kainic acid (KA)-induced seizures (Hsieh *et al.* 2009). Both rhynchophylline and isorhynchophylline exhibited anti-hypertensive, free radical scavenging and

antiexcitotoxic effects (Kang *et al.* 2002; Shi *et al.* 2003; Watanabe *et al.* 2003; Kang *et al.* 2004b; Matsumoto *et al.* 2005; Chen *et al.* 2009).

2.1.3 Herba Erigerontis (燈盞花)

Herba Erigerontis is a traditional Chinese herbal medicine derived from *Erigeron breviscapus* which is commonly used to treat stroke, paralysis and other central nervous system (CNS) diseases. Injection of Herba Erigerontis to Japanese rabbits can inhibit nerve cell apoptosis by decreasing the contents of caspase-9 and apoptosis-induced factor (AIF) in cytoplasm and serum (Cheng *et al.* 2009). Scutellarin, a major flavone found in Herba Erigerontis, showed inhibitory effects on the aggregation of Aβ *in vitro* and prevented the cell death mediated by Aβ when applied to cultured neuronal PC12 cells (Zhu *et al.* 2009).

2.1.4 Semen Cuscutae (菟絲子)

Semen Cuscutae (Dodder Seed) is the ripe dried seed of *Cuscuta chinessis* Lam.. It is a traditional herbal medicine used to invigorate the kidneys and consolidate kidney essence, nourish the liver, improve eyesight, arrest diarrhea and soothe an unborn fetus (Quan 2000; Kim *et al.* 2007b). There are no existent studies about this herbal medicine and neuroprotection.

2.1.5 Radix Salviae Miltiorrhizae (丹參)

Radix Salviae Miltiorrhizae, also known as Red Sage, Chinese Sage and Danshen, is the dried root of *Salvia Miltiorrhiza* Bge. and belongs to the genus *Salvia*. In traditional Chinese medicine, Danshen has been used to treat coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrheal and neurasthetic insomnia (Kang *et al.* 2004a). Danshen could alleviate pain and inhibit calcium overload by modulating hyperpolarization-activated current channels in dorsal root ganglion (DRG) neurons of rats (Chen & Liu 2006). The aqueous extracts of Danshen blocked the NMDA-evoked currents in cerebrocortical neurons *in vitro* (Sun *et al.* 2005).

Salvianolic acid B (Sal B) is a major active constituent of Danshen. Consecutive administration of 10 mg/kg Sal B once a day by ip injection could stimulate neurogenesis process both in sub-granular zone and sub-ventricular zone after brain ischemia, and could also alleviate neural cells loss and improve motor function recovery in rats (Zhong *et al.* 2007). Tanshinone IIB (TSB), another main active constituent of Danshen, was able to significantly inhibit the cytotoxicity and apoptosis induced by staurosporine in rat cortical neurons through suppressing the elevated Bax protein and decrease bcl-2 and caspase-3 protein. In addition, TSB was effective in reducing stroke-induced brain damage by ip injection into the experimentally-induced stroke rats (Yu *et al.* 2007). The other known active constituents in Danshen that have neuroprotective effect include tanshinone IIA (Xia *et al.* 2005; Meng *et al.* 2006; Xiao *et al.* 2006; Wang *et al.* 2007c) and cryptotanshinone (Mei *et al.* 2009; Zhang *et al.*

2009) (Wong *et al.* 2010).

2.1.6 Radix Notoginseng (三七)

Radix Notoginseng is the root of *Panax notoginseng* (Buck.) F.H. Chen. (Arialiaceae), which is highly prized in China owing to its therapeutic abilities for haemostasis, regulating circulation and general tonic action. The total saponins of Radix Notoginseng (PNS), mainly containing four active saponins ginsenosides Rg1, Rb1, Re and notoginsenoside R1, are regarded as the principal components for various pharmacological activities (Han & Zhong 2002). PNS plays a protective role in pathological lesion of cholinergic neuron in AD rat model (Zhong *et al.* 2005). Zhu *et al* found that PNS could significantly decrease the percentage of neuronal apoptosis and necrosis, and reduce intracellular free calcium concentration in cultured rat hippocampal neurons (Zhu *et al.* 2003). In one study, they used blind whole-cell voltage-clamp recordings to detect the effects of PNS on long-term potentiation (LTP) in the CA1 region of the hippocampus, and investigated the electrophysiological mechanisms underlying potentiating effects of PNS on learning and memory. The results showed that PNS at the concentration of 0.04-0.05 g/L could facilitate LTP in the CA1 region of the hippocampus which may contribute to its promoting effects on learning and memory (Zhou *et al.* 2007).

Recent studies have shown that both Rb1 and Rg1 protect spinal neurons from excitotoxicity by glutamate or KA, as well as oxidative stress by H_2O_2 *in vitro* (Liao

et al. 2002). Similarly, Rb1 and Rg1 significantly increase the number and length of neurites of surviving dopaminergic cells exposed to glutamate exposure (Radad *et al.* 2004). R1 may preferentially protect neurons from glutamate excitotoxicity mediated by NMDA receptor composed of an NR1/NR2B subunit assembled in the brain (Gu *et al.* 2009). Repetitive ip injection of ginsenosides was shown to prevent behavioral, biochemical and morphological abnormalities induced by KA in rats (Shin *et al.* 2009).

2.1.7 Semen Cassiae (决明子)

Semen Cassiae is the dried seed of *Cassia tora* L.. It is commonly drunk as a roasted tea. The traditional use of Semen Cassiae is to treat dizziness and headache, and benefit the eye by nourishing the liver *yin* and anchoring *yang* (Chen 2001). Recent studies have reported the neuroprotective effects of Semen Cassiae in brain disease models. It improves learning and memory impairment induced by scopolamine and by transient cerebral hypoperfusion in mice that mediated the inhibition of acetylcholinesterase activity (Kim *et al.* 2007a). Moreover, it is able to protect against NMDA-induced calcium dysregulation, and 3-nitropropionic acid-induced cell death in mouse hippocampal cultures (Drever *et al.* 2008). Furthermore, Semen Cassiae protects transient cerebral global ischemia in mice based on the demonstrated anti-inflammatory action and neurotrophic factor release (Kim *et al.* 2009a). It is also reported that Semen Cassiae can protect DA neurons against the toxicities involved in PD (Ju *et al.* 2010).

2.1.8 Flos Chrysanthemi (菊花)

Flos Chrysanthemi is the dry flower of *Chrysanthemum morifolium* Ramat.. It has the functions of eliminating evil-heat and detoxification. Pharmacological experiments have shown this herb to possess direct inhibitory activity against inflammation (Cheng *et al.* 2005). Wang *et al.* reported that the water extract of Flos Chrysanthemi could restrain the produce of free radicals and lipid per-oxidation induced by free radicals on heart and brain *in vivo* and *in vitro* (Wang *et al.* 2001).

2.1.9 Rhizoma Rhodiolae (紅景天)

Rhizoma Rhodiolae is the dry root and stem of *Rhodiola rosea* L.. Diabetic peripheral nerve injury is possibly related to inhibitory effect of high glucose on activity and proliferative capability of Schwann cells (Sango *et al.* 2006). One study has demonstrated that Rhizoma Rhodiolae could markedly improve high glucose-induced inhibitory effect on both activity and proliferative capability of Schwann cells (Wu *et al.* 2009).

2.1.10 Rhizoma Polygonati (黃精)

Rhizoma Polygonati is the dry root of *Polygonatum sibiricum* Red.. It has been used traditionally as a tonic in Asia which was mainly used for reducing blood glucose (Ed. 1978) (Kato *et al.* 1994). The water extract of Rhizoma Polygonati was studied for its hypoglycemic effect in diabetic mice and rats (Kim *et al.* 2009b) (Chen

et al. 2001) (Kato & Miura 1993).

2.1.11 Folium Ginkgo (銀杏叶)

Folium Ginkgo is the leave of *Ginkgo biloba*, which has been cultivated and held sacred for its health-promoting properties (Jacobs & Browner 2000). Substantial scientific evidence has accumulated to suggest that concentrated and partially purified extracts of Folium Ginkgo afford protective activities against several kinds of neural and vascular damage (Haan *et al.* 1982; Kleijnen & Knipschild 1992; Maclennan *et al.* 2002).

EGb-761 is a patented extract from Folium Ginkgo which has been well investigated to have neuroprotective effects (Ramassamy *et al.* 2007). It has been demonstrated that EGb-761 exhibits anti-depressant activity in neurons and hippocampus of double TgAD mice (Hou *et al.* 2010). In addition, the pretreatment of EGb-761 could protect against cerebral ischemia in different animal models (Domorakova *et al.* 2006) (Domorakova *et al.* 2009). EGb-761 is able to attenuate degeneration of dopamine neurons and symptoms caused by the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) *in vitro* and *in vivo* (Chen *et al.* 2007). Intraperitoneal injection of EGb-761 could enhance the anti-oxidative ability of retina and partially inhibited the apoptosis of photoreceptors, thus exerting a protective effect on photoreceptors (Xie *et al.* 2007).

2.1.12 Flos Buddlejae (密蒙花)

Flos Buddlejae is the flowers and buds of *Buddleja officinalis* Maxim.. In the ancient Chinese herbal literature, Flos Buddlejae is used to treat conjunctival congestion, clustered nebulae, as well as being used as an antiseptic. Previous studies on Buddleja species have led to the isolation of various natural products, such as triterpenoid glycosides, phenylethanoids, flavonoids and diterpenes (Li *et al.* 1997) (Lin *et al.* 1998) (Mensah *et al.* 2000) (Piao *et al.* 2003). One research found that a 70% methanolic extract of Flos Buddlejae and 4 kinds of flavonols or their glycosides of Flos Buddlejae exhibited inhibitory effects on aldose reductase of the unpurified rat lens aldose reductase (Matsuda *et al.* 1995). Flos Buddlejae has also been reported to have inflammatory activity (Lee *et al.* 2005) (Liao *et al.* 1999).

In general, these twelve selected traditional Chinese medicines have been demonstrated to possess certain level of neuroprotective activities in both *in vitro* and *in vivo* studies. However, the effectiveness in treating neurodegenerative disease, such as glaucoma, as well as the protective action against glutamate-induced neuronal cell death by these selected medicines has not been systematically investigated. Thus, the first aim of the present project was to investigate the neuroprotective effect of the selected Chinese herbs in cultured rat cortical neurons *in vitro*. It is hoped that through this screening programme, herbs with potent cytotoxic effect on cultured cortical neurons would be identified.

2.2 Materials and Methods

2.2.1 Preparation of ethanolic extracts of the selected Chinese medicinal herbs.

All the herbal materials used in the current project were purchased from Zhi-xin Herbal Company, Hong Kong. Their identities were authenticated by comparing the authentic specimens stored in the Herbarium of the School of Chinese Medicine, Faculty of Science, The Chinese University of Hong Kong. In preparation of the extracts, the twelve herbs, i.e. Fructus Alpinae Oxyphyllae (益智仁), Ramulus Uncaria cum Uncis (鉤藤), Herba Erigerontis (燈盞花), Semen Cuscuta (菟絲子), Radix Salviae Miltiorrhizae (丹參), Radix Notoginseng (三七), Semen Cassiae (決明子), Flos Chrysanthemi (菊花), Rhizoma Rhodiolae (紅景天), Rhizoma Polygonati (黃精), Folium Ginkgo (銀杏) and Flos Buddleajae (密蒙花), were first ground to powder or small pieces using an electrical blender. One hundred gram of each ground powder were dissolved in 80% ethanol for 24 h and then extracted under reflux with 80% aqueous ethanol for 2 h and extracted for 3 times. The ethanolic crude extracts were filtered. The filtrates were concentrated in a rotary evaporator under negative pressure, followed by freeze dried. The resultant extracts were weighted and stored at -20°C until subsequent bioassay testing. Figure. 2.1 shows the schematic flow of the extraction methods. Table 2.1 depicts the extract yields of the herbs.

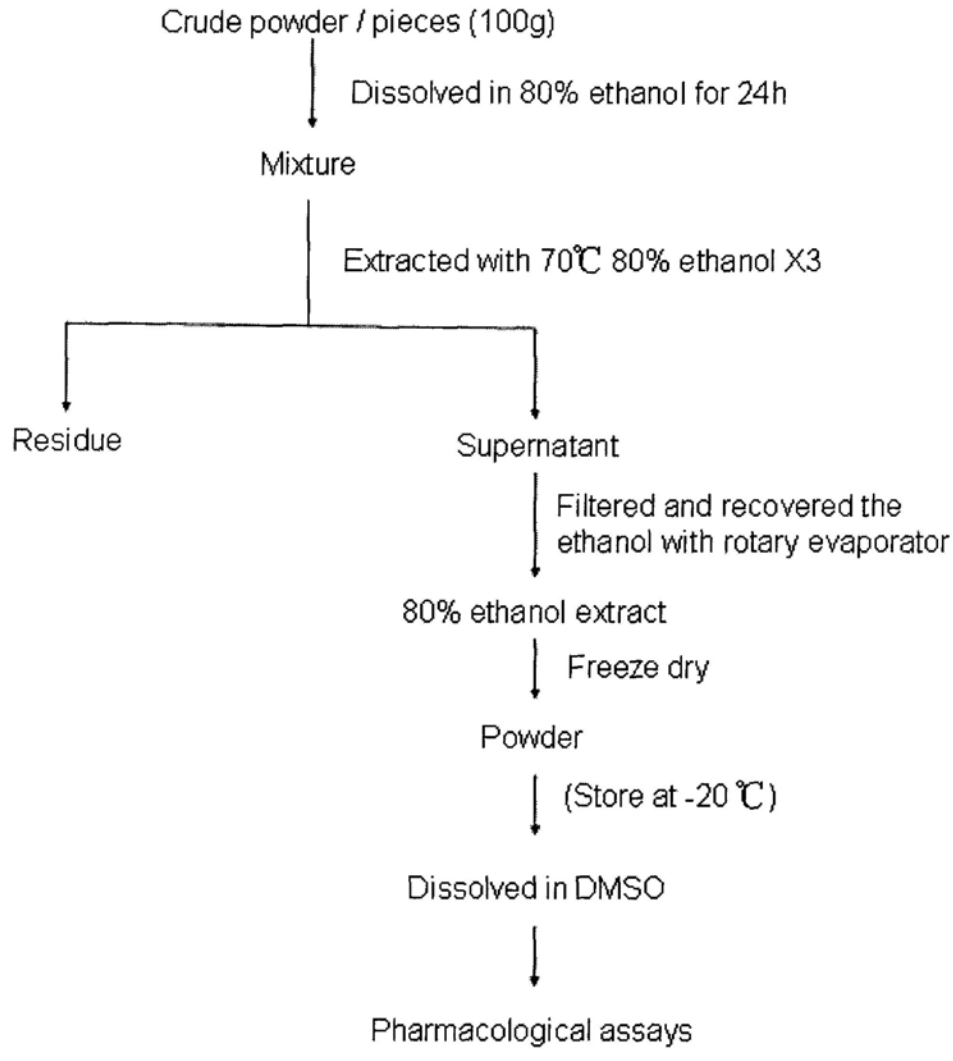


Figure. 2.1. The extraction method of the herbal extracts.

Table 2.1 The extract yields of the selected herbs.

Chinese medicines	Yields (g)
Fructus Alpinae oxyphyllae (益智仁)	32.2
Ramulus Uncaria cum Uncis (鉤藤)	46.2
Semen Cuscuta (菟絲子)	9.1
Herba Rhodiolae (紅景天)	72.6
Herba Erigerontis (燈盞花)	36
Radix Salviae Miltiorrhizae (丹參)	54.3
Flos Chrysanthemi (菊花)	47.7
Radix Notoginseng (三七)	49.9
Rhizoma Polygonati (黃精)	51.2
Folium Ginkgo (銀杏)	44.3
Flos Buddleajae (密蒙花)	26.8
Semen Cassiae (決明子)	6.2

2.2.2 General cell culture methods

Primary neuronal cultures were prepared from day 17 embryonic Sprague-Dawley rats (Laboratory Animal Unit, The University of Hong Kong) according to the previously described methods (Lai *et al.* 2006; Ho *et al.* 2010b). Briefly, cerebral cortices were micro-dissected from the brains of rat fetuses in PBS with glucose (18 mM). After removal of the meninges, the cortices were minced and seeded onto poly-L-lysine- (25 μg/ml) coated 6-well plates at a density of $1x10^6$ cells/well. Neurons were cultured with Neurobasal™ medium supplemented with L-glutamate (2mM) (Gibco Laboratories), penicillin (50U/ml) and streptomycin (50 μg/ml) (Gibco Laboratories) and 2% B27 supplement. Neurons were maintained in a 5% CO_2 incubator at 37°C for 14 days prior to treatments. Half of the medium in each well was replaced with fresh medium twice a week. On day 7, the cultured cells were treated with different dosages of herbal extracts or glutamate.

The herbal extracts were reconstituted in dimethyl sulfoxide (DMSO) at 300 mg/ml concentration, centrifuged at 9000 × g for 10 min, and passed through a 0.2 μm filter for a stock solution. In order to find out whether these Chinese herbal medicines could exert neuroprotective effects, the stock was then diluted with culture medium to various concentrations for cell culture experiments. The final concentrations of the extracts are 0 (drug-free control), 0.1, 1, 10 and 100 μg/ml. To minimize potential solvent effects on cell growth, the final concentrations in all culture wells were made sure to be less than 0.05% DMSO. At the low concentration, the solvent did not exhibit any toxic effects on neurons (data not shown). We also first evaluated the cytotoxic effects of glutamate on cultured neurons and also determined optimal concentration of glutamate for inducing neuron cytotoxicity. Glutamate (purchased

from Sigma, Saint Louis) was dissolved in 1N hydrogen chloride (HCl) to make a stock solution of 500 mM, and then diluted with culture medium to different concentrations (0, 10, 30, 60 and 80 μM).

2.2.3 Cytotoxicity assay

Neurons were treated with different dosages of extracts for 1 h, and then exposed to glutamate (30 μM) for 24 h. The general cytotoxicity in various treatment groups was determined by the lactate dehydrogenase (LDH) assay as described elsewhere (Yu *et al.* 2005; Lai *et al.* 2006; Yu *et al.* 2006). In brief, equal volume of the supernatant of cell free culture medium was collected into a 96-well-plate and incubated with the assay buffer for 30 min in dark at room temperature. LDH cytotoxicity assay kit was from Roche Diagnostics (Mannheim, Germany). Leakage of LDH into the culture medium was determined by measuring the absorbance at 492 nm wavelength. Results were expressed as the fold of control (Fold of control was calculated as follow: $\text{absorbance}_{\text{of glutamate-treated/drug -treated}}/\text{Absorbance}_{\text{of control}}$).

2.2.4 Caspase-3-like activity assay

Apoptotic cell death was determined by measuring the caspase-3-like activity as described elsewhere (Suen *et al.* 2003; Ho *et al.* 2007; Cheung *et al.* 2009). Proteins of treated cells were extracted from cortical neurons using an ice-cold lysis buffer containing 5 mM dithiothreitol (DTT), 0.1 mM EDTA, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) and Triton X-100 (0.2%) without protease inhibitors. Fifty micrograms (μg) of cellular proteins were incubated with caspase-3 substrates (Ac-DEVE-p-NA) (Calbiochem, La Jolla, CA, USA), and then, were transferred to a 96-well-plate for another 2h incubation at

37°C. During the reaction, a yellow product (pNA) was cleaved from substrate (i.e. DEVD cleavage) and its absorbance was measured at 405 nm for caspase-3 activity. Specific activity of caspase-3 was then calculated (s.a., unit = pmol/min/μg) from the absorbance readings. Results were expressed as fold of control.

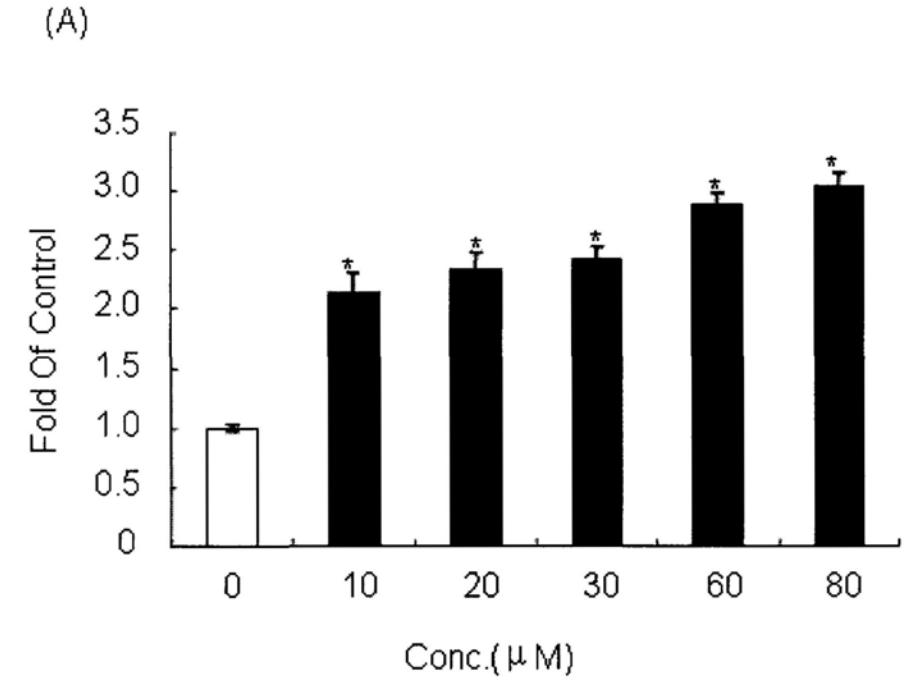
2.2.5 Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM) from at least three independent tests. Data for multiple comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Student-Newman-Keuls test was used as post hoc test using the statistical program SigmaStat (Jandel Scientific, Chicago)

2.3 Results

2.3.1 Glutamate induced neurotoxicity on neurons

The toxicity of glutamate on cultured cortical neurons was examined by both LDH assay and caspase-3 assay. Figure 2.2. shows that different concentrations of glutamate exerted different degrees of toxic effects on the cultured cortical neurons, and the cytotoxic effect of glutamate on the neurons clearly followed a dose-dependent manner. From the results, it was evidenced that glutamate at 30 μM could induce 2.4 fold of change in LDH release and 2 fold of change in caspase-3 activity when compared to the control.


(A)
Fold Of Control
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
60
80
Conc.(μM)

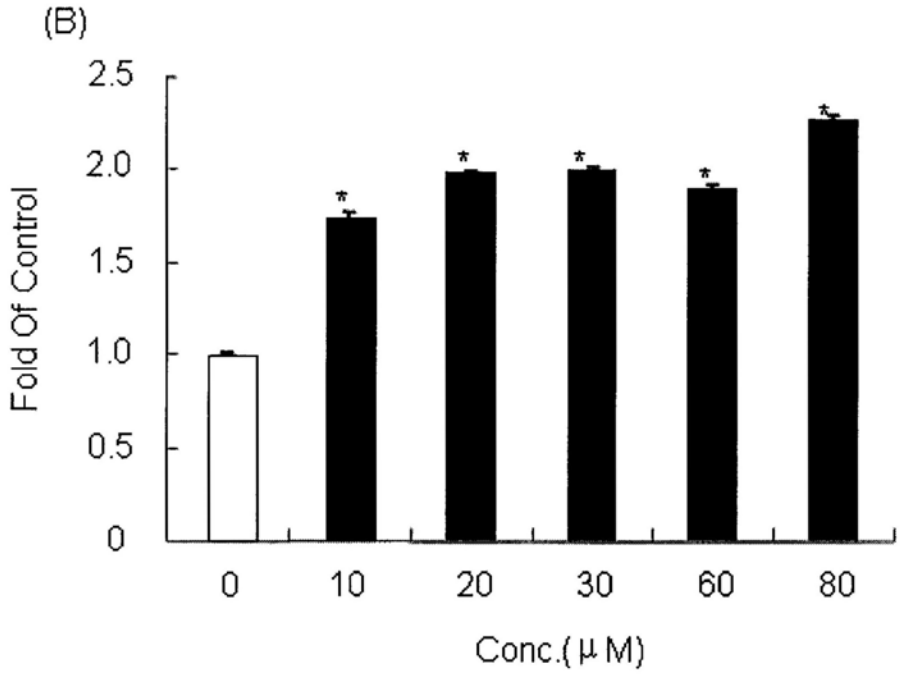

(B)
Fold Of Control
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
60
80
Conc.(μM)

Figure 2.2. Cytotoxic effect of glutamate on cultured cortical neurons. The neurons were treated with different dosages of glutamate for 24 h. Neurotoxicity was assessed by (A) LDH assay and (B) caspase-3 assay. Results are expressed as means ± SEM from at least three independent experiments. *, $p<0.001$ vs control.

2.3.2 Glutamate induced morphological changes on cultured cortical neurons

The morphological changes of neurons were shown in Figure 2.3. Neurons in control group (Figure 2.3A) displayed much fasciculation of neurites and intact cell bodies, suggesting that they had good survival and were interconnected to each others. Upon exposure to glutamate, the number of cell body was markedly reduced and the network of neurites had been retracted. Surviving neurons had much shorter neurites than that of control (Figure 2.3B). These morphological changes indicate massive neuronal damage in the presence of glutamate.

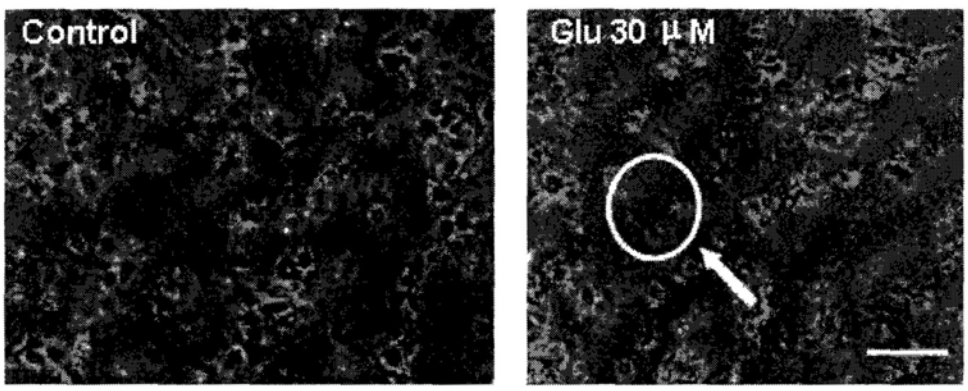


Figure 2.3. The morphological alterations of the glutamate-treated neurons. The neurons were treated with glutamate (30 μM) for 24 h. (A) control; (B) glutamate (30 μM) treatment for 24 h. Scale bar represents 5 μm.

2.3.3 Cytotoxic effects of the twelve selected herbal extracts on neurons *in vitro*.

Table 2.1 summarizes the neuroprotective effects of the twelve extracts on the glutamate-induced neuron damage as assessed by LDH assay. Each herbal extract shows different degree of attenuating the LDH release on neurons. By comparing the ability of reducing LDH release among the twelve extracts, it is clear that FAO extract exhibited the most potent neuroprotection on neurons. At the concentration of 100 μg/ml, FAO extract could confer as much as 67.2% neuroprotection when compared to the non-treated control. The successful identification of FAO as the most potent neuroprotective agent *in vitro* would pave the way to elucidate the underlying mechanism of actions and identify the active components in FAO extract responsible for the observed biological activity.

Table 2.2. Neuroprotective effects of the twelve selected Chinese herbal extracts on glutamate-induced toxicity.

Chinese medicines	Neuroprotective effect (%)*			
	0.1 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
Fructus Alpinae oxyphyllae (益智仁)	13.4±0.1	17.3±0.2	30.1±0.5	67.2±0.7
Ramulus Uncaria cum Uncis (鉤藤)	38.9±0.9	42.3±0.4	28±0.6	35.2±0.3
Semen Cuscuta (菟絲子)	44.3±0.4	44.9±0.9	28.6±0.8	40.6±0.3
Herba Rhodiolae (紅景天)	21.99±0.5	2.11±0.8	5.6±0.1	-18.29±02.
Herba Erigerontis (燈盞花)	26.9±0.3	37.7±0.7	32.5±0.2	29.3±0.3
Radix Salviae Miltiorrhizae (丹參)	25.34±0.2	6.56±0.4	21.45±0.2	30.69±0.4
Flos Chrysanthemi (菊花)	9.61±0.5	13.63±0.5	29.08±0.3	30.07±0.2
Radix Notoginseng (三七)	-6.04±0.3	-4.49±0.1	-8.83±0.5	-10.94±0.1
Rhizoma Polygonati (黃精)	12.07±0.4	33.42±0.7	28.36±0.7	41.14±0.8
Folium Ginkgo (銀杏)	23.24±0.7	8.12±0.9	6.49±0.6	8,85±0.6

Flos Buddleajae (密蒙花)	13.43±0.1	1.39±0.8	-6.86±0.6	5.78±0.4
Semen Cassiae (决明子)	21.01±0.5	8.12±0.2	6.49±0.3	8.85±0.4

* The neurons were pretreated with the extracts for 1 h, followed by exposure to glutamate at 30 μM for 24 h. Neurotoxicity was assessed by LDH assay. Neuroprotective effect % = (absorbance $_{\text{of glutamate-treated/drug -treated}}$ /Absorbance $_{\text{of control}}$) ×100%

2.4 Discussion

To find out the potential neuroprotective Chinese herbal medicine, the screening model we used here is glutamate-induce neurotoxicity on cultured rat primary cortical neurons. Primary tissue culture is a very useful experimental method that is commonly applied to study various areas of the central nervous system, including cerebral cortex, hippocampus, retina, cerebellum, spinal cord, hypothalamus, striatum, mesencephalon and lower brain stem (Thomas 1986). Glutamate is the major excitatory transmitter in the brain and the eye. In the eye, glutamate is released from injured retinal ganglion cells as well as from adjacent cells, and the subsequent increase in intracellular as well as extracellular glutamate is associated with programmed cell death (Dkhissi *et al.* 1999). A number of *in vitro* studies have indicated that glutamate is a potent neurotoxin capable of destroying neurons (Froissard & Duval 1994) (Davis & Maher 1994) (Zhang & Bhavnani 2005). In the present herbal screening experiment, glutamate is used as a neurotoxin to induce the cortical neuron death.

The quantification of neuronal cell death is achieved using a variety of *in vitro* assays with different techniques. One commonly used method of assaying loss of membrane integrity is LDH assay (Korzeniewski & Callewaert 1983). It is a simple and effective method for determining cell death (Koh & Choi 1987) (Lobner 2000). LDH, a cytoplasmic enzyme, is released into the cell medium upon neuronal injury and/or death. It is assessed by measuring the pyruvate-depend oxidation of

nicotinamide adenine dinucleotide (NADH).

Our study demonstrated that glutamate-induced neurotoxicity on primary neuronal cells is suitable for testing the potential neuroprotective Chinese herbal medicine. Among twelve herbal extracts tested, FAO exhibited the most potent protective effects. FAO at the concentration of 100 μg/ml could attenuate glutamate-induced LDH release by 67.2% when compared to glutamate-treated neurons. The results indicate that FAO is a promising herb which possesses potent neuroprotective effects on neurons. The ensuing parts of the project will be focus on further confirmation of the neuroprotective effect of FAO and its active ingredients in mediating the neuroprotection of neurons. The current experimental results not only provide experimental evidence to support the use of this Chinese herb in neurodegenerative diseases treatment in Chinese medicine practice, but also open up a new vista for further identification of active ingredients in this medicinal plant with neurodegenerative activity.

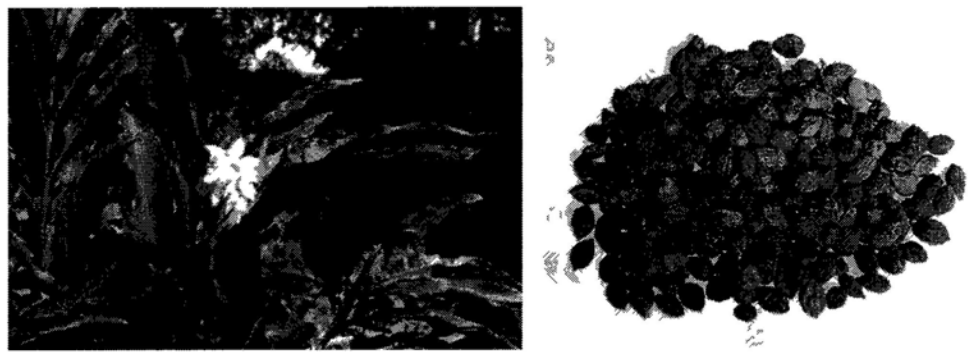
Chapter 3

Bioassay-guided Isolation of Chemical Constituents from Fructus Alpinae oxyphyllae

3.1 Introduction

Chinese medicinal herbs contain many active compounds. Up to now, a number of crude extracts prepared from traditional Chinese medicinal herbs have been reported to have neuroprotective effect *in vitro* against glutamate excitotoxicity, such as extract of Acori graminei rhizome (Cho *et al.* 2000). Fructus Alpinae oxyphyllae (FAO), known as *Yi zhi ren* in Chinese, is a very important traditional Chinese medicinal herb. It is the fruit of *Alpinia oxyphylla* Miq. which belongs to the *Alpinia Roxb* genus and Zingiberaceae family. It is widely found in the Hainan and Guangdong provinces of China. The original plant of Fructus Alpinae oxyphyllae is shown in Figure. 3.1.

(A) (B)



(http://www.cdutcm.edu.cn/)

Figure 3.1 (A) Original plant of *Alpina oxyphylla* Miq.; (B) Dried Fructus Alpinae oxyphyllae (FAO)

The chemical components studies on FAO began since 1920s in Japan. Until recently, there are some systematic studies of FAO. Up to now, several chemical constituents have been isolated from FAO. These constituents can be classified into four classes: (i) terpenes, mainly sesquiterpenes, such as nootkatol (Shoji *et al.* 1984; Xie *et al.* 2009) and volatile oils, such as P-Cymene (44.87%), valencene (9.13%), linalool (4.39%) and myrteng (3.9%) (Luo *et al.* 2001; Morikawa *et al.* 2002; Fang *et al.* 2003); (ii) flavonoids, such as tectochrysin and chrysin; (iii) diarylheptanoids, such as yakuchinone A, yuakuchinon B, neonootkatol and oxyphyllacinol (Chun *et al.* 1999); and (iv) other compounds of various structures,including amino acid, fatty acid, vitamins and micronutrients (Xu *et al.* 2009).

FAO has been widely used as a tonic, an aphrodisiac, an anti-salivation and anti-diarrhea agent. Owing to its broad spectrum of biological activities, FAO is now commonly applied in Chinese medicine for a number of medical conditions including enuresis, excessive salivation in toddles and elder people, diarrhea and impotence. A number of recent investigations using animals, isolated tissues and cultured cells have demonstrated that FAO possesses different biological properties, such as anti-dementia and learning function-improving (Kubo *et al.* 1995), and anti-tumor effects (Lee *et al.* 1998). The neuroprotective effects of FAO has been reviewed in Section 2.1.1 in Chapter 2.

Certain medicinal plants containing anti-inflammatory and anti-oxidative

substances can exert chemopreventive effects. Yakuchinone A and yakuchinone B were two compounds isolated from FAO. Both of these compounds have strong inhibitory effects on the synthesis of prostaglandins and leukotrienes *in vitro* (Chun *et al.* 2002a). Chun et al. also demonstrated that the methanolic extract of FAO suppressed mouse skin tumor growth and induced apoptosis in cultured human promyelocytic leukemia cells (Chun *et al.* 2002b).

The 80% aqueous acetone extract and the ethyl acetate-soluble portion from FAO were found to show inhibitory effects on nitric oxide production in lipopolysaccharide-activated macrophages and antigen-induced degranulation in RBL-2H3 cells (Morikawa *et al.* 2002).

In the process of screening for naturally occurring substances with neuroprotective effects against glutamate-induced excitotoxicity in primary cultured cortical neurons, we have discovered that the ethanol extract of FAO exhibited significant neuroprotective activity. However, the chemical constituents presented in FAO responsible for the observed biological activity have not been identified.

Bioassay-guided isolation approach integrates the processes of biological testing, separation of compounds in a mixture, and various analytical methods leading to the identification of biologically active components. This methodology precludes overlooking novel compounds that are often missed in studies that only

identify those compounds with which the investigator is familiar. Moreover, the possibility of discovering an unknown molecular site of action is maximized (Duke *et al.* 2000). The process begins with testing a herb extract to confirm its biological activity, followed by crude separation of the compounds in the matrix and testing the crude fractions (Figure. 3.2). Further fractionation is carried out on the fractions which are determined to be active part, at a certain concentration threshold, whereas the inactive fractions are set aside or discarded. The process of fractionation and biological testing is repeated until pure compound(s) are obtained. Structural identification of the pure compound then follows (Rimando *et al.* 2001).

Although there have been attempts to identify several chemicals from FAO to date, no laboratory has attempted to conduct bioassay-directed isolation of FAO-derived chemicals. In this study, we used the bioassay-guided isolation approach to find out the most effective neuroprotective compound(s) from FAO.

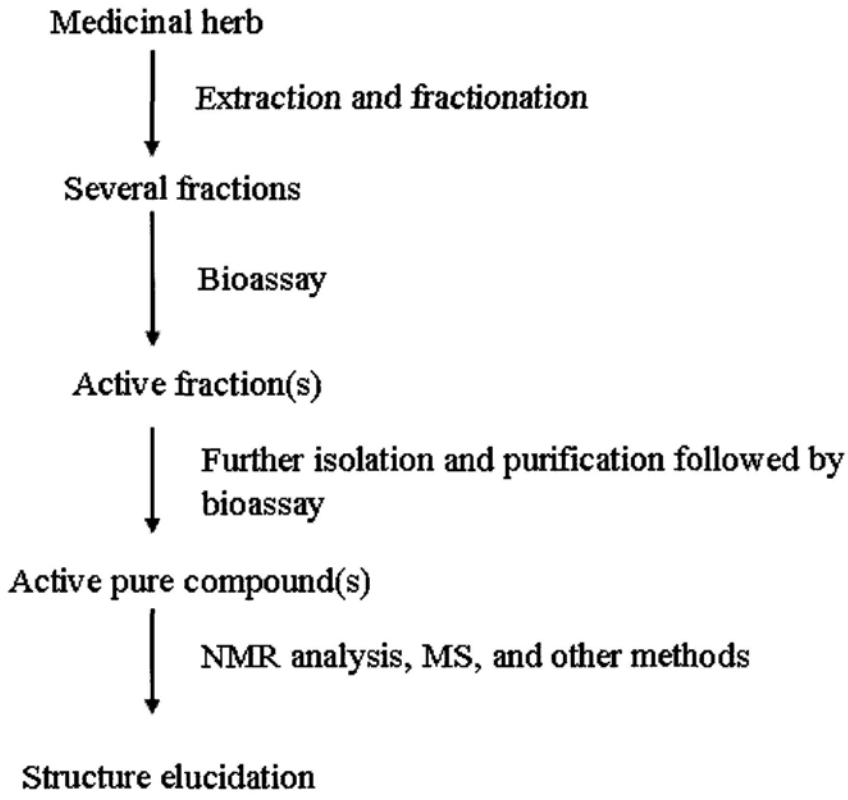


Figure 3.2 Schematic presentation of bioassay-guided isolation process.

3.2 Materials and Methods

3.2.1 Chemicals

All reagents and solvents used were of analytical grade. Chrysin (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol was of chromatographic grade (Fisher, Fair Lawn, NJ, USA). Water was purified by Milli-Q academic purification system (Millipore, USA), and was used to prepare standard solutions, dilutions, and blanks. Methanol, petrol ether and butanol used were from Merck (Darmstadt, Germany). Thin layer chromatography (TLC) analysis was carried out on aluminum-coated silica gel (Aldrich) and cellulose F254 plates from Merck. All glassware used was left with sulfonitric solution overnight and then washed with ultrapure water.

3.2.2 General cell culture method

The general cell culture method was conducted using the same protocol as stated in Section 2.2.2.

3.2.3 Treatment of neurons

The test fractions were reconstituted in DMSO at a concentration of 300 mg/ml, centrifuged at 9000 x g for 10 min, and passed through a 0.2 μm filter for a stock solution ((10 μg/ml for less pure fractions; 0.1, 1 and 10 μg/ml for pure fractions).). To find out which fraction could exert neuroprotective effects, the stock was then diluted with culture medium to various concentrations for cell culture experiments.

To minimize potential solvent effects on cell growth, the final concentrations in all culture wells were made sure to be less than 0.05% DMSO. At this low concentration, the solvent did not exhibit any toxic effects on neurons (data not shown). We also evaluated the cytotoxic effect of glutamate (30 μM) on the cultured cortical neurons (Sigma, Saint Louis). Glutamate was dissolved in 1 N hydrogen chloride (HCl) to make a stock of 500 mM, and then diluted with culture medium to 30 μM.

3.2.4 Preparation of ethanolic extracts from FAO

The method of preparation of ethanolic extract of FAO was conducted following the same protocol as stated in Section 2.2.1.

3.2.5 Determination of general cytotoxicity

The determination of general cytotoxicity method was conducted using the same protocol as stated in Section 2.2.3.

3.2.6 Caspase-3-like activity assay

The caspase-3-like activity assay was conducted employing the same protocol as stated in Section 2.2.4.

3.2.7 Chemical identification

TLC analysis was done on silica gel plates. Column chromatography (CC) was performed on columns packed with normal phase silica gel (using the mixtures of

CH_2Cl_2 and MeOH as mobile phase) and reverse phase silica gel (C18) (eluted with the mixtures of H_2O and MeOH). Identification of pure compound was carried out by means of nuclear magnetic resonance (NMR) and mass spectroscopy analyses.

An Agilent 1100 LC-MSD-Trap-SL system (Agilent Technologies, Palo Alto, CA, USA) consisted of a degasser, an autosampler, a column compartment, a quaternary pump, a diode-array detector, and an atmospheric pressure chemical ionization (APCI) ion trap mass spectrometer. The HPLC analysis was performed on a Prevail C18 column (250mm×4.6mm I.D., 5 μm, Alltech) with a guard column (12.5mm×4.6mm I.D., 5 μm, Alltech).

3.2.8 Preparation of standard solution of chrysin

A standard stock solution of chrysin was prepared in methanol at a concentration of 1 mg/mL. The stock solutions were stored at -20 ℃.

3.2.9 HPLC-MS conditions

The flow rate of HPLC was 10 mL/min. The mobile phase consisted of formic acid (A) and 0.1% formic acid (B) with a gradient elution of a ratio from 50:50 to 75:25 (v/v) in 0-35 min. The column temperature was set at 25 °C. The injection volume was 10 μl and the detection wavelength was set at 267 nm. The effluent was directly introduced to the APCI-MS.

3.2.10 Statistical analysis

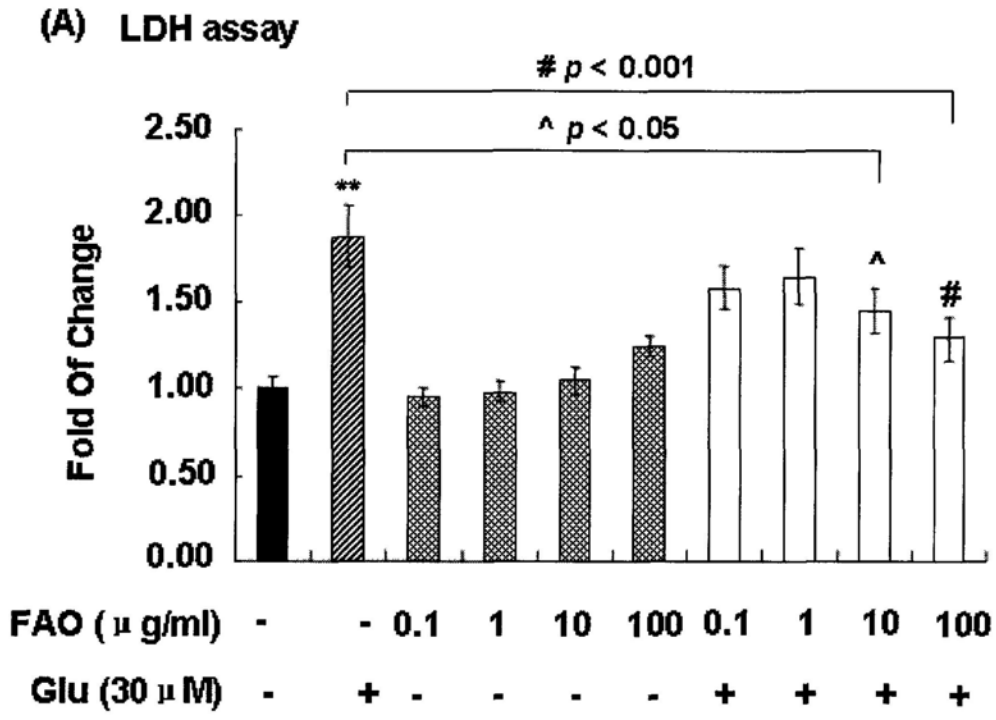
Results were expressed as the mean ± standard error of the mean (SEM) from at least three independent tests. Data for multiple comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Student-Newman-Keuls test was used as post hoc test using the statistical program SigmaStat (Jandel Scientific, Chicago)

3.3 Results

3.3.1 FAO attenuated glutamate-induced neurotoxicity

In Chapter 2, we have demonstrated that, compared with other herb extracts, FAO showed significantly protective effects on cortical neurons in culture as determined by LDH assay. As shown in Figure. 3.3A, the level of LDH release in the control group were found to be 0.256 ± 0.06. This baseline level reflected normal turnover of neurons and were set as 1 fold of control during normalization of data. The neurons exposed to glutamate significantly increased the level of LDH release to 1.8 ± 0.2 fold of control. FAO at the concentrations of 0.1, 1, 10 and 100 μg/ml was not toxic to neurons. When neurons were treated with 10 or 100 μg/ml FAO, release of LDH was significantly decreased to 1.4 ± 0.7 or 1.2 ± 0.1 fold of control, respectively. The results indicated that FAO was able to reduce glutamate-induced neuronal cell death.

By using caspase-3 like activity assay, we demonstrated that incubation of cortical neurons with 30 μM glutamate for 24 h led to neuronal cell apoptosis. The level of caspase-3 activity in the control group was found to be 0.064 ± 0.03 pmol/min/μg of protein. This baseline level reflected normal turnover of neurons and was set as 1 fold of control during normalization of data. As shown in Figure. 3.3B, when the neurons were exposed to glutamate, activity of caspase-3 was elevated to 1.8 ± 0.3 fold of control. FAO at 1, 10 and 100 μg/ml significantly reduced glutamate-triggered activity of caspase-3 to 0.2 ± 0.3, 0.3 ± 0.4, and 1.3 ± 0.2 fold of control, respectively.


(A) LDH assay
# p < 0.001
^ p < 0.05
Fold Of Change
2.50
2.00
1.50
1.00
0.50
0.00
**
^
#
FAO (μ g/ml) - - 0.1 1 10 100 0.1 1 10 100
Glu (30 μ M) - + - - - - + + + +

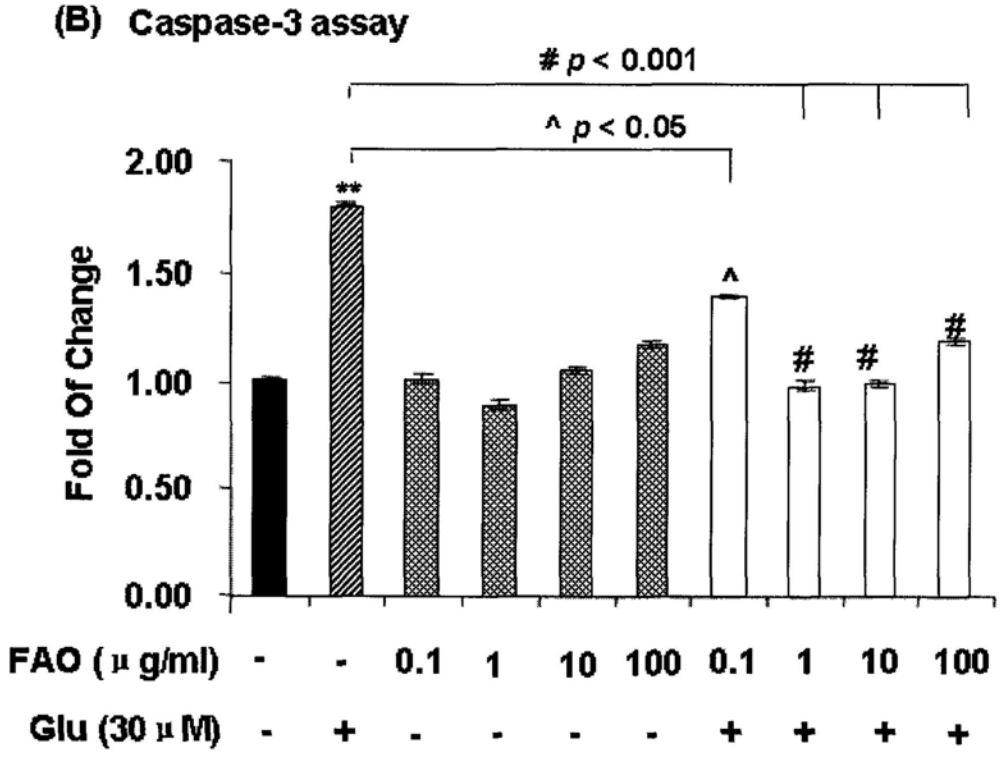

(B) Caspase-3 assay
# p < 0.001
^ p < 0.05
Fold Of Change
2.00
1.50
1.00
0.50
0.00
**
^
#
#
#
FAO (μ g/ml) - - 0.1 1 10 100 0.1 1 10 100
Glu (30 μ M) - + - - - - + + + +

Figure 3.3 Neuroprotective effects of FAO on glutamate-induced toxicity. The neurons were treated with FAO (0.1, 1, 10 and 100μg/ml) for 1 h, followed by exposure to 30 μM Glutamate for 24 h. Neurotoxicity was assessed by (A) LDH assay; (B) caspase-3-like assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control; #, $p < 0.001$ vs Glu; ^, $p < 0.05$ vs Glu by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

3.3.2 FAO preserved the fasciculation of neurites in glutamate-treated cultured neurons

Protective effects of FAO on glutamate-induced neuronal damage were also examined by studying the morphological changes of the neurons in different treatment groups. As shown in Figure. 3.4A, the neurons in control group showed much fasciculation of neurites and intact cell bodies, suggesting that they had good survival and were interconnected to each others. When exposure to glutamate (Figure. 3.4B), the number of cell body was markedly reduced and the network of neurites had been retracted. Surviving neurons had much shorter neurites than that of control. These morphological changes indicate massive neuronal damage induced by glutamate. The presence of FAO at either 10 or 100 μg/ml markedly preserved the number of neurons and fasciculation of neurites even though neurons had been stressed by glutamate (Figure. 3.4C&D).

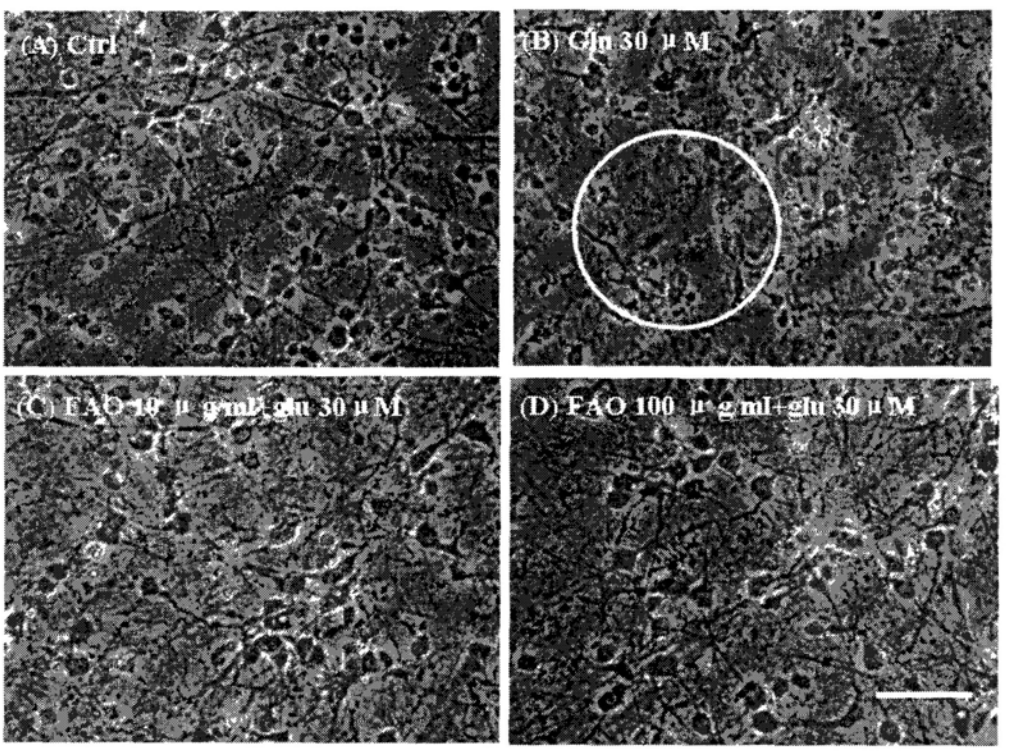
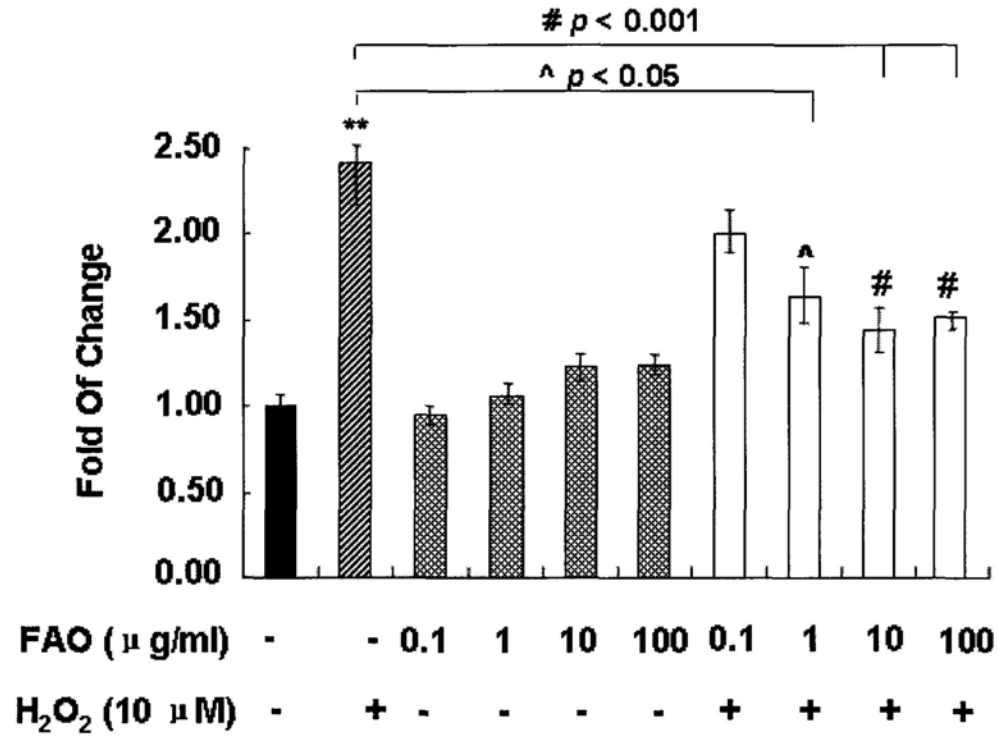


Figure 3.4 Morphology of the neurons treated with FAO. The neurons were treated with FAO for 1 h, followed by exposure to 30 μM Glutamate for 24 h. (A) control, (B) glutamate stimulation for 24 h alone, (C) the neurons treated with FAO 10 μg/ml, and (D) 100 μg/ml for 24 h and then together with glutamate 30 μM for 24 h. Scale bar represents 5 μM.

3.3.3 The anti-oxidative effects of FAO on H_2O_2-induced neurotoxicity

Oxidative stress is a major factor in glutamate excitotoxicity. To investigate any potential anti-oxidative effects of FAO, we conducted an indirect approach to examine if FAO could protect neurons against hydrogen peroxide. The neurons were treated with FAO for 1 h prior to exposure to H_2O_2 at 10 μM for 24 h. The release of LDH and caspase-3 activity were examined. As shown in Figure. 3.4, H_2O_2 could induce release of LDH and activation of caspase-3 to 2.4 ± 0.3 and 1.8 ± 0.3 fold of control, respectively. We found that FAO at the concentrations of 10 and 100 μg/ml significantly protected the neurons from H_2O_2-induced LDH release to 1.4 ± 0.2 and 1.5 ± 0.7 fold of control (Figure. 3.5A). FAO could also dramatically attenuate caspase-3 activity at the concentrations of 10 and 100 μg/ml (Figure. 3.5B). The results showed that FAO has an anti-oxidative effect on the neurons.

(A) LDH assay



(B) Caspase-3 assay

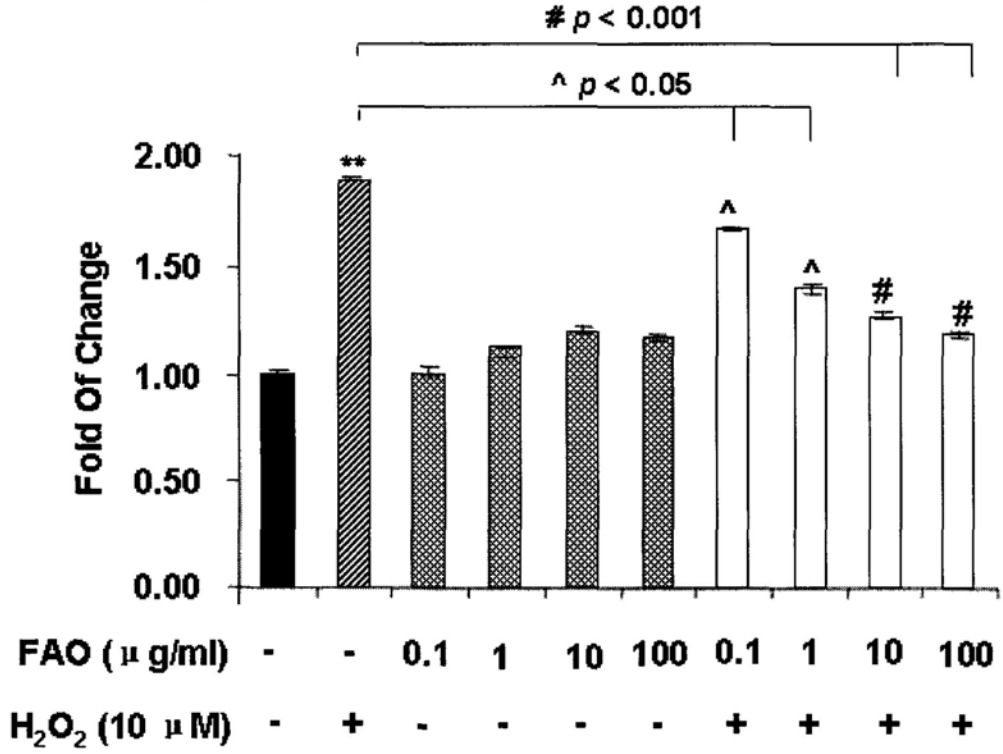


Figure 3.5 Effects of FAO on H_2O_2-induced toxicity. The neurons were treated with FAO for 1 h, followed by incubation with 10 μM H_2O_2 for 24 h. Neurotoxicity was assessed by (A) LDH assay and (B) caspase-3-like assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control; $^{\#}$, $p < 0.001$ vs H_2O_2; ^, $p < 0.05$ vs H_2O_2 by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

3.3.4 Bioassay-guided isolation of FAO

The ethanolic extract of FAO was first suspended in water, and then partitioned in turn with petroleum ether, ethyl acetate, and 1-butanol, respectively to obtain petroleum ether-soluble, ethyl acetate-soluble, 1-butanol-soluble, and water-soluble fractions. The glutamate-induced neuronal cell death model was applied here to test the activity of the fractions. Our data showed that the petroleum ether-soluble-fraction was the most active part against glutamate-induced LDH release at 10 μg/ml, whereas the others (ethyl acetate-soluble and 1-butanol-soluble fractions) were less active or had no activity (water-soluble fraction) (Table 3.1).

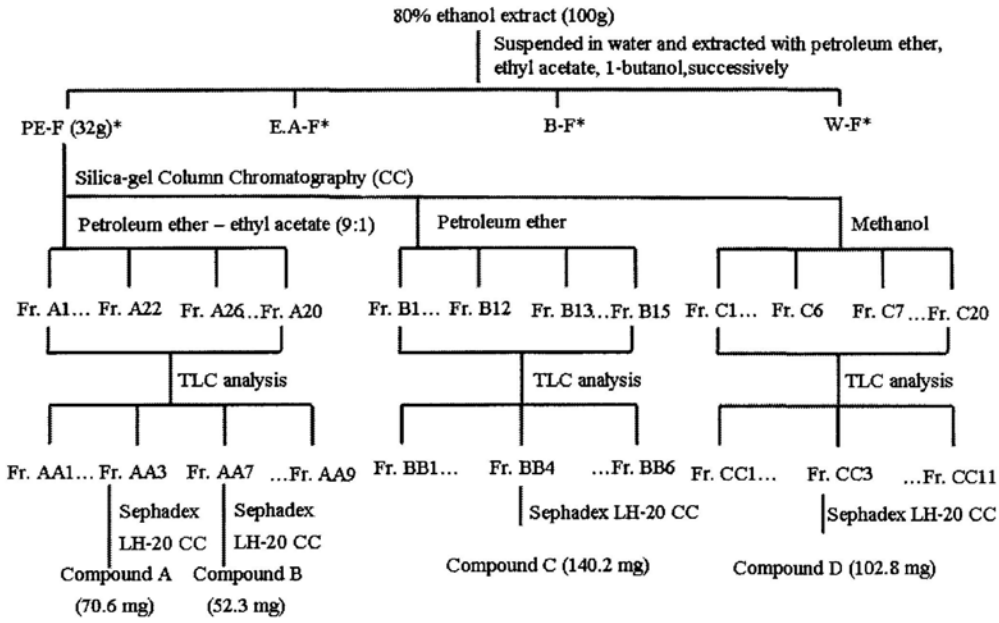
The petroleum ether-soluble fraction was subjected to a normal phase silica gel chromatography, and eluted with petroleum ether-ethyl acetate (9:1), petroleum ether and methanol, successively. Twenty fractions (Figure 3.6., Fr. A1 - A20) were eluted out by petroleum ether-ethyl acetate (9:1) and then combined into nine fractions (Fr. AA1 - AA9) on the basis of TLC analysis. Testing of these fractions on the neuroprotective effects on cortical neuronal cells showed that Fr. AA3 and Fr. AA7 had a similar protective effect on the neurons (Table. 3.3). These two fractions were further purified by Sephadex LH-20 column chromatography leading to the isolation of compounds A and B.

Under the elution of petroleum ether, 20 fractions (Figure 3.6., Fr. B1-B15) were collected. They were further combined into six fractions (Fr. BB1-BB6) based

on the TLC analysis. These fractions were then tested by LDH assay on the cultured neurons. The fraction BB4 was found to be active whereas the others were not (Table. 3.2). The fraction BB4 was then further purified on a Sephadex LH-20 column chromatography eluted with methanol to yield pure compound C.

Finally, the silica gel column was eluted with methanol. 20 fractions (Figure 3.6., Fr. C1 - C20) were collected and then combined into eleven fractions (Fr. CC1 - CC11) by TLC analysis. Testing on the neuroprotective effect in culture showed that Fr. CC3 was most active compound among all the fractions (Table 3.4). Further purification of Fr. CC3 was performed on a Sephadex LH-20 column chromatography to obtain pure compound D.

Through the use of the bioassay-guided fractionation, we successfully obtained four pure chemical compounds from the ethanolic extract of FAO. Further biological testing using a wide range of concentrations i.e. 0.1, 1, 10 and 100 μg/ml was performed on cortical neuronal cells in culture to determine the neuroprotective activities of these compounds. As shown in Table 3.5, the results indicate that compound A has the highest potency in attenuating glutamate-induced neuronal cell death.



* PE-F: petroleum ether-soluble fraction
* EA-F: ethyl acetate-soluble fraction
* B-F: 1-butanol-soluble fraction
* W-F: water-soluble fraction

Figure 3.6 Schematic presentation of the bioassay-guided fractionation of the ethanolic extract of FAO.

Table 3.1 Protective effect of different fractions of FAO on cultured cortical neurons as measured by LDH assay*.

Fractionate (yield)	Neuroprotective effect (%) Conc. = 10 μg/ml
Petrol ether (32.2g)	46.2±0.1
Ethyl acetate (27.7g)	7.4±0.6
Butanol (28.6g)	6.1±0.7
Water (52.3g)	- 9.6±0.1

*The neurons pretreated with the extracts for 1 h, followed by exposure to glutamate 30 μM for 24 h. Neurotoxicity was assessed by LDH assay.

Neuroprotective effect % = (absorbance $_{\text{of glutamate-treated/drug-treated}}$ /Absorbance $_{\text{of control}}$) x 100%

Table 3.2 Protective effects of fraction AA1 – AA9 on cultured cortical neurons*.

Fraction	Neuroprotective effect (%) Conc. = 10μg/ml
AA1	0.8 ± 0.2
AA2	5.6 ± 0.2
AA3	37.7 ± 0.2
AA4	8.6 ± 0.9
AA5	-2.2 ± 0.3
AA6	11.65 ± 0.9
AA7	44.6 ± 0.8
AA8	5.6 ± 0.1
AA9	2.1 ± 0.3

*The neurons pretreated with the extracts for 1 h, followed by exposure to glutamate 30 μM for 24 h. Neurotoxicity was assessed by LDH assay.

Neuroprotective effect % = (absorbance $_{\text{of glutamate-treated/drug-treated}}$ /Absorbance $_{\text{of control}}$) x 100%

Table 3.3 Protective effects of fraction BB1 – BB6 on cultured cortical neurons*.

Fraction	Neuroprotective effect (%) Conc. = 10 μg/ml
BB1	- 3.3 ± 0.9
BB2	2.1 ± 0.2
BB3	6.1 ± 0.7
BB4	48.8 ± 0.1
BB5	9.1 ± 0.9
BB6	2.7 ± 0.8

*The neurons pretreated with the extracts for 1 h, followed by exposure to glutamate 30 μM for 24 h. Neurotoxicity was assessed by LDH assay.

Neuroprotective effect % = (absorbance $_{\text{of glutamate-treated/drug-treated}}$ /Absorbance $_{\text{of control}}$) x 100%

Table 3.4 Protective effects of fraction CC1 - CC11 on cultured cortical neurons*.

Fraction	Neuroprotective effect (%) Conc. = 10μg/ml
CC1	6.7 ± 0.8
CC2	5.4 ± 0.9
CC3	32.7 ± 0.2
CC4	9.9 ± 0.2
CC5	12.1 ± 0.8
CC6	4.2 ± 0.2
CC7	7.0 ± 0.5
CC8	3.2 ± 0.7
CC9	-9.2 ± 0.7
CC10	- 10.9 ± 0.8
CC11	0.1 ± 0.5

*The neurons pretreated with the extracts for 1 h, followed by exposure to glutamate 30 μM for 24 h. Neurotoxicity was assessed by LDH assay.

Neuroprotective effect % = (absorbance $_{\text{of glutamate-treated/drug-treated}}$ /Absorbance $_{\text{of control}}$) x 100%

Table 3.5 Protective effects of compound A, B, C and D on cultured cortical neurons.

Compounds	Yields (mg)	Neuroprotective effect (%)		
		0.1 μg/ml	1 μg/ml	10 μg/ml
A	70.6	-20.9 ± 0.9	-10.8 ± 0.1	-7.3 ± 0.7
B	52.3	6.2 ± 0.2	7.9 ± 0.8	5.2 ± 0.1
C	140.2	5.2 ± 0.4	22.6 ± 0.9	43.4 ± 0.4
D	102.8	1.1 ± 0.9	-9.7 ± 0.3	-10.0 ± 0.7

The neurons pretreated with the four compounds for 1 h, followed by exposure to glutamate 30 μM for 24 h, respectively. Neurotoxicity was assessed by LDH assay.

Neuroprotective effect % = (absorbance $_{\text{of glutamate-treated/drug-treated}}$ /Absorbance $_{\text{of control}}$) x 100%

3.3.5 Identification of compounds A, B, C and D

Compound C was obtained as yellow crystals. Its APCIMS displayed a quasi-molecular ion peak at *m/z* 255 $[M+H]^+$. Its ^1H NMR spectroscopic data indicated the presence of the flavonoid skeleton. The ^1H NMR spectrum (DMSO-d_6) displayed a pair of *meta*-coupled aromatic protons at δ 6.223 (1H, d, J = 2.4 Hz) and δ 6.528 (1H, d, J = 2.4 Hz), one olefinic proton at δ 6.977 (1H, s), three aromatic protons at δ 7.600 (3H, m), two aromatic protons at δ 8.073 (2H, m), and two hydroxyl groups at δ 10.921 and δ 12.829. Based on the available data and the structural characteristics of flavonoid, compound C was identified to be chrysin (5,7-dihydroxy-flavone) (Luo *et al.* 2000). The result was further confirmed by comparison of compound C and commercial source of chrysin purchased from Sigma using LC-MS method.

Compouds A, B, and D were identified to be nootkatol, yakuchinone A, and valencene, respectively. The identifications were mainly based on the comparisons of their MS data and NMR spectroscopic data with reported data in literature (Luo *et al.* 2000). The summary of all isolated compounds was shown in Table 3.6 and Figure 3.7.

Table 3.6 Identification of compound A, B, C and D.

Compounds	Identity of compound
A	nootkatol
B	yakuchinone A
C	chrysin
D	valencene

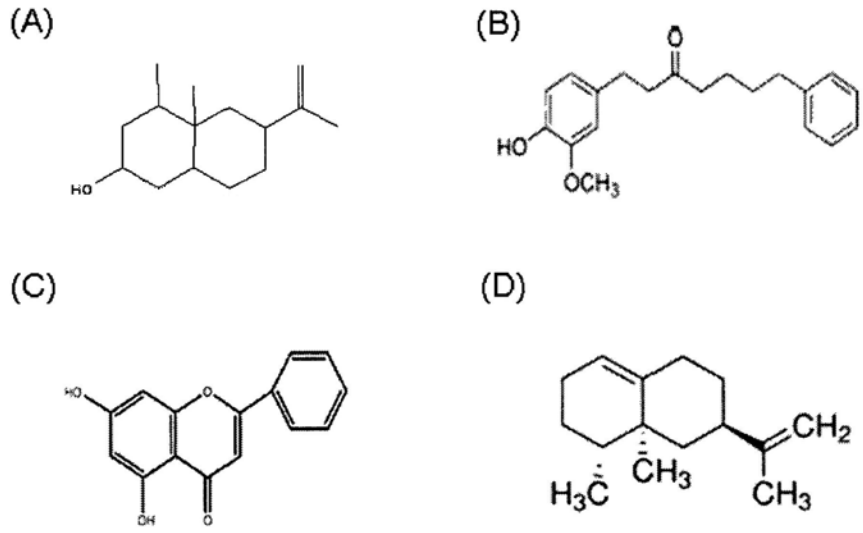


Figure 3.7 Structures of compounds A, B, C and D. (A) nootkatol; (B) yakuchinone A; (C) chrysin; (D) valencene.

3.4 Discussion

Our previous findings have demonstrated that FAO could protect the neurons from glutamate-induced LDH release and caspase-3 activity. Moreover, it could protect the neurons from H_2O_2-induced oxidative stress. The results suggest that FAO is a potential neuroprotective agent against glutamate-induced toxicity. Using bioassay-guided fractionation and isolation techniques, we were able to obtain four chemical compounds from the ethanolic extract of FAO. Chrysin, a known compounds presented in FAO, showed a strong protective effect on glutamate-induced LDH release. None of the other isolated compounds (nootkatone, yakuchinone A and valencene) showed neuroprotection against the glutamate-induced LDH release in cultured cortical neurons.

To carry out bioassay-guided isolation of FAO, several important factors must be considered. First, the FAO to be extracted has to be chosen with care to make sure that the neuroprotection is a likely explanation of the effects on seeds which is found in the field and laboratory. The FAO we used in the current experiments was purchased from Zhixin Pharmaceutical Company, a Good Manufacturing Practice (GMP) herbal supplier based in Guangdong, China. Moreover, the identity of the herbal material was carefully authenticated at our School by Ms. Zong Yuying, an expert in herbal identification and authentication using microscopic and macroscopic methods. FAO is commonly found in Hainan and Guangdong provinces of China.

Second, an appropriate and sensitive bioassay method needs to be employed to eliminate nonactive fractions and prevent giving too many false positive results. As has been discussed in Chapter 2, LDH assay is a simple and effective method for determining cell death (Koh & Choi 1987) (Lobner 2000), and this assay effectively helped us to identify the active compounds.

Thirdly, the target herbal extract to be tested in the bioassay is an important factor to make sure that the laboratory screening actually targets herb of interest (Rimando *et al.* 2001). As a traditional Chinese herbal medicine, FAO has been found to be a potential neuroprotective agent for treating neurodegenerative diseases. Recently, several studies have been published on neuroprotective effects of FAO and the possible active compounds derived from this herb (Koo *et al.* 2004; An *et al.* 2006). The findings from our previous experiments demonstrated that the ethanolic extract of FAO had a significantly neuroprotective effect on glutamate-triggered LDH release, as well as the activation of caspase-3 (Figure. 3.3). Moreover, the ethanolic extract of FAO at the concentrations of 10 and 100 μg/ml was able to attenuate the H_2O_2-triggered LDH release and caspase-3 activity. Our experimental results unambiguously corroborate the neuroprotective action of FAO. Further isolation and identification of the active ingredients responsible for its neuroprotective activity ensued. The bioassay-directed isolation methods used in our project led to the successful isolation of the active compound chrysin, which has

a strong neuroprotective effect. In addition, the fractions of FAO containing this strong neuroprotective compound showed a better activity than others. The neuroprotective activity of chrysin will be further tested and confirmed in the ensuing chapters.

Chapter 4

Protective Effects of Chrysin on Glutamate-induced Neurotoxicity in Cultured Rat Cortical Neurons

4.1 Introduction

Progressive loss of neurons is the major problem of neurodegenerative diseases, such as AD, PD and glaucoma. The excitatory neurotransmitter glutamate can be a potent neurotoxin, especially when its concentrations in synapse increase abnormally (Choi 1988; Sucher *et al.* 1991). Neurons exposed to glutamate *in vitro* can undergo both apoptosis and necrosis. The glutamate-mediated excitotoxicity is caused by intracellular Ca^{2+} overload via the N-methyl-D-aspartate receptor (NMDAR). The increased levels of Ca^{2+} lead to production of radical oxygen species (ROS) (Reynolds & Hastings 1995; Chinopoulos & Adam-Vizi 2006; Bano & Nicotera 2007). Consequently, high ROS levels contribute to the development of neurological disorders such as ischemia, AD and glaucoma (Choi 1988; Meldrum & Garthwaite 1990; Hayashi *et al.* 2006).

To achieve multi-targets of neuroprotection by reducing glutamate excitotoxicity and oxidative stress, herbal medicine may be an ideal source for neuroprotective drug development. Herbal medicines, such as *Ginkgo biloba* extract, *Lycium barbarum*, have been used to treat neurological disorders for a long time and have been observed to have good clinical results, (Zhou *et al.* 2006).In recent studies, it has been found that the water extract of FAO could protect neurons from ischemic damage by reducing the NO-mediated formation of free radicals and by antagonizing their toxicity (Koo *et al.* 2004). Although the report showed the anti-oxidative effects of water extract from FAO, no active components have been identified to be responsible for the observed antioxidant effect (Koo *et al.* 2004). Our earlier work has already shown that FAO had potent neuroprotective effects

against glutamate-induced neurotoxicity (Chapter 3, Section 3.3). In the subsequent phytochemical fractionation and isolation, we successfully isolated chemical compound chrysin, a flavonoid, from FAO.

Chrysin is a kind of natural and bioactive flavonoids present not only in FAO, but also in honey, propolis and *Passiflora incarnata* (Rapta *et al.* 1995; Williams *et al.* 1997; Volpi & Bergonzini 2006; Jaganathan & Mandal 2009). It possesses potent anti-inflammatory, anti-oxidant properties, as well as ability to promote cell death, and alter cell cycle progression.

The anti-cancer effects of chrysin have been studied (Weng *et al.* 2005). Chrysin was shown to inhibit the C6 glioma cell proliferation after 24, 48 and 72 hours. Flow cytometry analysis was conducted to confirm this result. It has been found that the levels of phosphorylation of retinoblastoma (Rb) protein in C6 glioma cells decreased after treatment with chrysin. Moreover, in chrysin-treated cells, cyclin dependent kinase inhibitor ($p21^{Waf1/Cip1}$) levels were increased significantly without the change in p53 protein level. Furthermore it was shown that proteosome activity, cyclin dependent kinase 2 (CDK2) and 4 (CDK4) were also inhibited by chrysin. These results suggested that chrysin exerts its growth-inhibitory effects either through activating p38-MAPK leading to the accumulation of $p21^{Waf1/Cip1}$ protein or through mediating the inhibition of proteosome activity (Weng *et al.* 2005).

Another study by Woo *et al.* demonstrated that chrysin mediated apoptosis in U-937 cancer cell lines through the Akt-signaling pathway (Woo *et al.* 2004).

Furthermore, Zhang *et al.* synthesized 13 derivatives of chrysin and tested them for anticancer effects against human gastric adenocarcinoma cell line (SGC-7901) and colorectal adenocarcinoma (HT-29) cells. These derivatives were formed mainly through alkylation, halogenation, nitration, methylation, acetylation and trifluoromethylation of chrysin. MTT assay revealed that 5, 7-dimethoxy-8-iodochrysin and 8-bromo-5-hydroxy-7-methoxychrysin have the strongest activities against SGC-7901 and HT-29 cells respectively. Moreover, diethyl chrysin-7-yl phosphate (CPE: C(19)H(19)O(7)P) and tetraethyl bis-phosphoric ester of chrysin (CP: C(23)H(28)O(10)P(2)) could inhibit proliferation and induce apoptosis in cultured human HeLa cell line. It was concluded that CP and CPE were new potential therapeutic candidates for human cervical cancer (Zhang *et al.* 2004a).

The anti-inflammatory effect of chrysin was also investigated. Chrysin was shown to inhibit Iκ-Bα degradation and nuclear translocation of NF-κB in TNF-α-stimulated intestinal epithelial cells. Furthermore, chrysin alleviated the symptoms of Dextran sodium sulfate (DSS)-induced colitis and improved colitis DAI scores by reducing the production of various inflammatory mediators, such as

NO, inflammatory cytokines, PGE2, and chemokines. It was concluded that chrysin has good potential as a useful pharmacological agent with anti-inflammatory effect through suppression of NF-κB activation (Shin *et al.* 2009).

In addition, chrysin could affect inflammation-associated COX-2 expression and PGE2 formation in Raw 264.7 cell (Harris *et al.* 2006). It suppressed the lopopolysaccharide (LPS)-activated COX-2. It was also shown to reduce LPS-induced hydroxyl radical and superoxide formation relative to positive control. The nuclear factor for IL-6_was identified as responsible for the chrysin-mediated COX-2 downregulation (Woo *et al.* 2005) (Lin *et al.* 2006; Wang *et al.* 2007b) (Cho *et al.* 2004).

In recent years, many studies have been focused on identifying chemical compounds with free radical scavenging activity. The identification of such compounds may be useful in the prevention of diseases such as atherosclerosis, heart diseases, cancer and arthritis in which reactive oxygen species or free radicals seem to be implicated (Urquiaga & Leighton 2000).

Certain flavonoids are known to elicit anti-oxidative effects (Lapidot *et al.* 2002). This is because they have their classical anti-oxidant properties. The properties are defined mainly by the presence of a dihydrogenated B-ring which is capable of readily donating hydrogen (electron) to stabilise a radical species

(Rice-Evans *et al.* 1996; Bors & Michel 1999; Bors *et al.* 2001). The other features important for anti-oxidant nature include the presence of 2,3 unsaturatation in conjugation with a 4-oxo-function in C-ring, as well as the presence of functional groups capable of binding transitional metal ions (Rice-Evans *et al.* 1996) (Figure 4.1). Being with a structure of flavonoid, chrysin has been studied for its anti-oxidative effect.

Primary mesencephalic cultures were sensitive to oxidative insults which produced concentration-dependent decreases in cellular viability across an apoptotic-necrotic continuum of injury. Chrysin was shown to protect mesencephalic cultures from injury by MPP^+, as shown by DNA fragmentation studies and tyrosine hydroxylase (TH) immunocytochemistry of DA neurones (Mercer *et al.* 2005). Chrysin was also found to inhibit calcium-induced contraction of the isolated rat aorta and inhibit superoxide radical generation in a cell-free system in the presence of xanthine/xanthine oxidase. In addition, chrysin has been found to be an effective vasodilator and anti-oxidant due to its ability to prevent vascular reperfusion injury (Woodman & Chan 2004).

Moreover, the anti-aging effect of chrysin has been investigated. Chrysin has the potential for clinical and therapeutic applications against the physiological and biochemical effects of aging. Chrysin has been found to concentration-dependently inhibit neuroblastoma SH-SY5Y cell death. It attenuated tunicamycin-induced

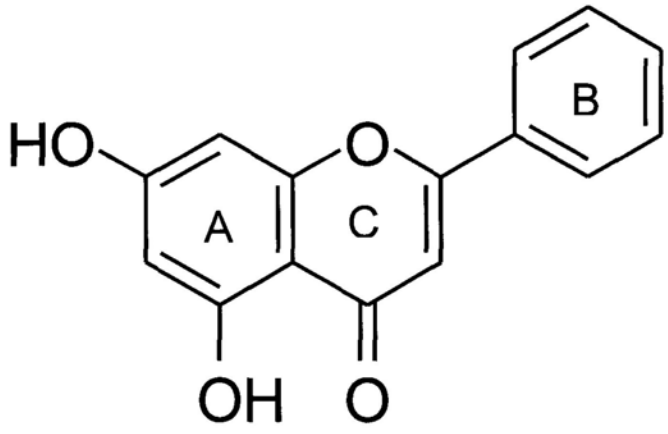


Figure 4.1 Structure of chrysin showing features important in defining the classical antioxidant potential of flavonoids. The most important of this structure is the presence of unsaturation in the C-ring (shaded green) and the presence of a 4-oxo function in the C-ring (shaded yellow) is also the other feature.

activation of caspase-3, and this effect was through a mitochondria-dependent pathway and inhibited $\Delta\Psi_m$ depolarization as well as cytochrome c release. In addition, chrysin could inhibit staurosporine-induced cell death (Izuta *et al.* 2008).

The anxiolytic effect of chrysin has also been studied. The elevated plus-maze (EPM) was used to evaluate the behavioural component of anxiolysis, and catecholamine and corticosterone assays were performed to measure the neurohormonal effects of anxiety. Chrysin was able to decrease anxiety via interaction with the GABA(A) receptor in rats as demonstrated by EPM, corticosterone, and catecholamine assays.

Chrysin has also been shown to have anti-viral activity. Chrysin was found to be a potent inhibitor of the enzyme aromatase (Sanderson *et al.* 2004). It inhibited human immunodeficiency virus (HIV) activation in models of latent infection (Critchfield *et al.* 1996). Moreover, it could modulate GABAA and GABAC receptors through binding to the benzodiazepine site located on the GABAA receptor (Goutman *et al.* 2003).

Furthermore, chrysin was able to inhibit HIV expression in TNF-alpha-treated OM-10.1 culture. It also inhibited HIV expression in response to PMA in OM-10.1 cells, in ACH-2 cells stimulated with either TNF-alpha or PMA, and in 8E5 culture. It is a potential candidate for a therapeutic strategy aimed at maintaining a cellular state of HIV-1 latency (Critchfield *et al.* 1996).

This part of the project aimed to investigate the neuroprotective effects of chrysin against glutamate-induced neurotoxicity using primary cultures of cortical neurons.

4.2 Materials and methods

4.2.1 Chemicals

Glutamate and chrysin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and the purity of chrysin was > 98%.

4.2.2 General cell culture method

The general cell culture method was conducted under the same protocol as stated in Section 2.2.2.

4.2.3 Treatments of neurons

Stock solution of chrysin for the *in vitro* study in cultured cortical neurons was prepared in 100% DMSO at a concentration of 300 mg/ml. The stock solution of chrysin was then centrifuged at 9000 x g for 10 min, and passed through a 0.2 μm filter and stored at -20°C until cell culture experiments. Working solutions were obtained by spiking the required amount of stock solution in culture medium to afford the final concentrations of 10, 1 and 0.1 μM, respectively. The final concentration of DMSO in culture was made sure to be ≤ 1%. In such this concentration, it exerted neither cytotoxic not proliferative effect on cultured cortical neurons (data not shown).

To find out whether chrysin could exert neuroprotective effects, the cultured neurons were treated with different dosages of chrysin for 1 h, and then exposed to glutamate (30 μM) for 24 h. For post-treatment experiments, neurons were exposed to

glutamate (30 μM) for 1 h and then chrysin was added into the cultures for co-incubation of another 23 h. For 'wash-out' experiments, neurons were treated with chrysin for 1 h; afterwards, chrysin-containing medium was removed and the neurons were then washed with PBS prior to exposure of glutamate (30 μM) for 24 h.

4.2.4 Determination of general cytotoxicity

The possible general cytotoxicity induced by chrysin was evaluated using the same protocol previously described in Section 2.2.3.

4.2.5 Caspase-3-like activity assay

The caspase-3-like activity induced by chrysin was assayed using the same protocol previously described in Section 2.2.4.

4.2.6 α-Tubulin staining

The neurons were grown on coverslips for the staining of α-tubulin, a protocol similar to that described previously (Lai *et al.* 2009). After treatments, the neurons were washed with tris buffered saline (TBS), followed by fixing with 5% paraformaldehyde in TBS for 20 min at room temperature. After this, neurons were rinsed with TBS three times, and permeabilized with 0.1% Triton X-100 solution containing TBS for 5 min and washed with TBS three times. The neurons were subsequently blocked with 10% BSA for 1 h at room temperature. Afterwards, the fixed neurons were incubated with mouse anti-α-tubulin antibody at 1:2000 dilution (Sigma) for 2 h at room temperature. Then, the neurons were incubated with F $(ab')_2$ fragment of goat anti-mouse IgG (H+L) secondary antibody at 1:500 dilution for 1 h

at room temperature. After removal of the antibody solution, the fixed neurons were mounted with antifade reagent (Invitrogen, Eugene, Oregon, USA). The fluorescence image was recorded by LSM510meta multiphoton confocal microscope (Carl Zeiss, Germany).

4.2.7 NBT assay

Three hundred μl NBT (1.0 mg/ml) was added to the medium after the treatment and incubated for 2 h at 37°C according to previously described procedures (Chao *et al.* 2008; Ho *et al.* 2009) (Ho *et al.* 2009). The neurons were then harvested in 100 μl DMSO. The lysates were then dissolved in 100 μl KOH (2 M) and were further sonicated for breaking down the cells. The samples were then centrifuged for 5 min. The absorbance of the supernatant was measured using a microplate reader at a reference wavelength of 570 nm. The results were expressed as the fold of control.

4.2.8 Statistical analysis

The experimental results were expressed as the mean ± standard error of the mean (SEM) from at least three independent tests. Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Student-Newman-Keuls test was used as post hoc test using the statistical program SigmaStat (Jandel Scientific, Chicago).

4.3 Results

4.3.1 Chrysin attenuated glutamate-induced LDH release

To explore and characterize neuroprotective properties of chrysin, we set up an *in vitro* model of glutamate excitotoxicity using primary cultures of cortical neurons. By using LDH assay, we demonstrated that incubation of cortical neurons with 30 μM glutamate for 24 h led to significant neuronal cell death. The levels of LDH release in the control group were found to be 0.243 ± 0.05. This baseline level reflected normal turnover of neurons and were set as 1 fold of control during normalization of data. As shown in Figure. 4.2, exposure of the neurons to glutamate significantly increased the levels of LDH release to 1.9 ± 0.4 fold of control. When the neurons were treated with 1 or 10 μM chrysin, release of LDH was significantly decreased to 1.6 ± 0.1 or 1.5 ± 0.2 fold of control, respectively, suggesting a reduction of neuronal cell death.

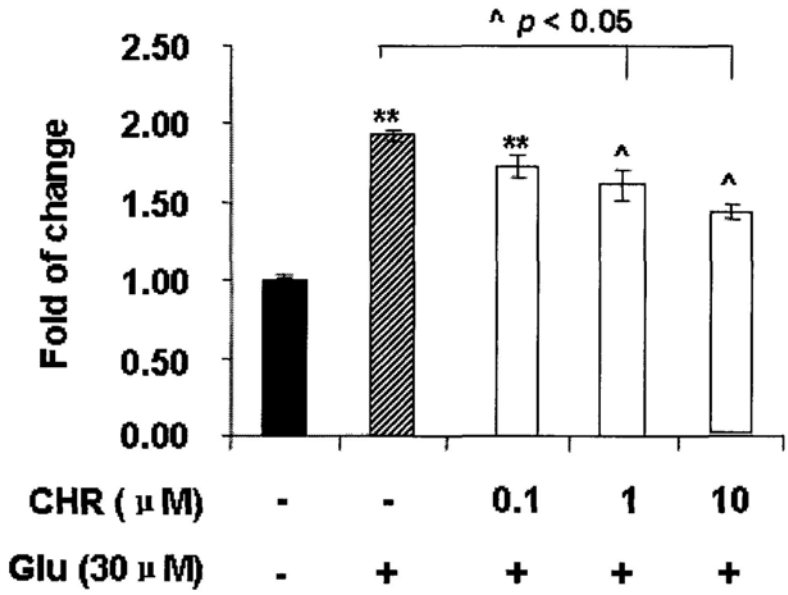


Figure. 4.2 Neuroprotective effects of chrysin on glutamate-induced toxicity in cultured cortical neurons. The neurons were treated with chrysin for 1 h, followed by exposure to 30 μM Glutamate for 24 h. Neurotoxicity was assessed by LDH assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control; $^{\#}p < 0.001$ vs Glu; ^, $p < 0.05$ vs Glu. Statistical analyses: one-way ANOVA for multiple comparisons and Student–Newman–Keuls test as post hoc test.

4.3.2 Chrysin attenuated glutamate-induced caspase-3 activity

By using caspase-3 like activity assay, we demonstrated that incubation of cortical neurons with 30 μM glutamate for 24 h led to neuronal cell apoptosis. The level of caspase-3 activity in the control group was found to be 0.053 ± 0.02 pmol/min/μg of protein. This baseline level reflected normal turnover of neurons and was set as 1 fold of control during normalization of data. As shown in Figure 4.3, when the neurons were exposed to glutamate, activity of caspase-3 was elevated to 2.7 ± 0.3 fold of control. Chrysin at 1 and 10 μM significantly reduced glutamate-triggered activity of caspase-3 to 1.6 ± 0.2 and 1.5 ± 0.2 fold of the control, respectively.

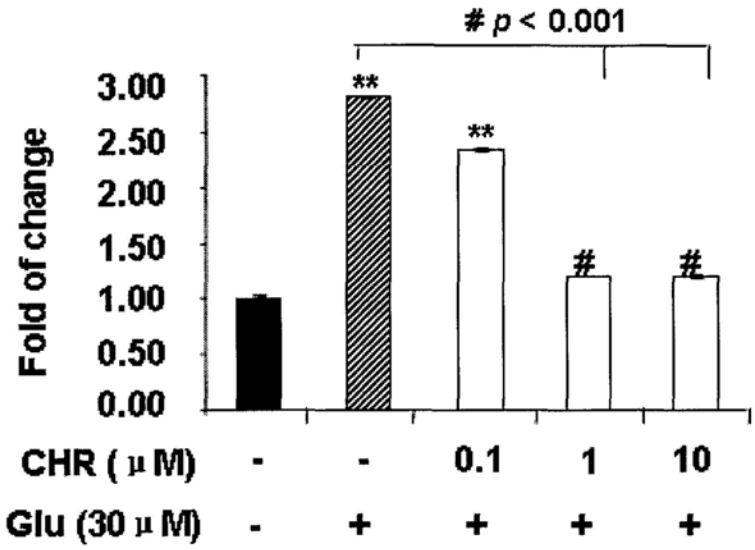


Figure. 4.3 Neuroprotective effects of chrysin on glutamate-induced caspase-3 expression in cultured cortical neurons. The neurons were treated with chrysin for 1 h, followed by exposure to 30 μM Glutamate for 24 h. Neurotoxicity was assessed by caspase-3-like assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control; #, $p < 0.001$ vs Glu; ^, $p < 0.05$ vs Glu by one-way ANOVA for multiple comparisons and Student–Newman–Keuls test as post hoc test.

4.3.3 Chrysin preserved the fasciculation of neurites in glutamate-treated cultured neurons

Protective effects of chrysin on glutamate-induced neuronal damage were further confirmed by studying the morphological changes of the neurons in different treatment groups. Staining with α-tubulin allows clear visualization of neuronal structure and fasciculation under fluorescent microscope. As shown in Figure 4.4 A, the neurons in control group displayed many fasciculation of neurites and intact cell bodies, suggesting that they had good survival and were interconnected to each others. Upon exposure to glutamate (Figure. 4.4 B), the number of cell body was markedly reduced and the network of neurites had been retracted. The surviving neurons had much shorter neurites than that of control. These morphological changes indicate massive neuronal damage induced by glutamate. The presence of chrysin at either 1 or 10 μM markedly preserved the number of neurons and fasciculation of neurites even though neurons had been stressed by glutamate (Figure 4.4 C&D).

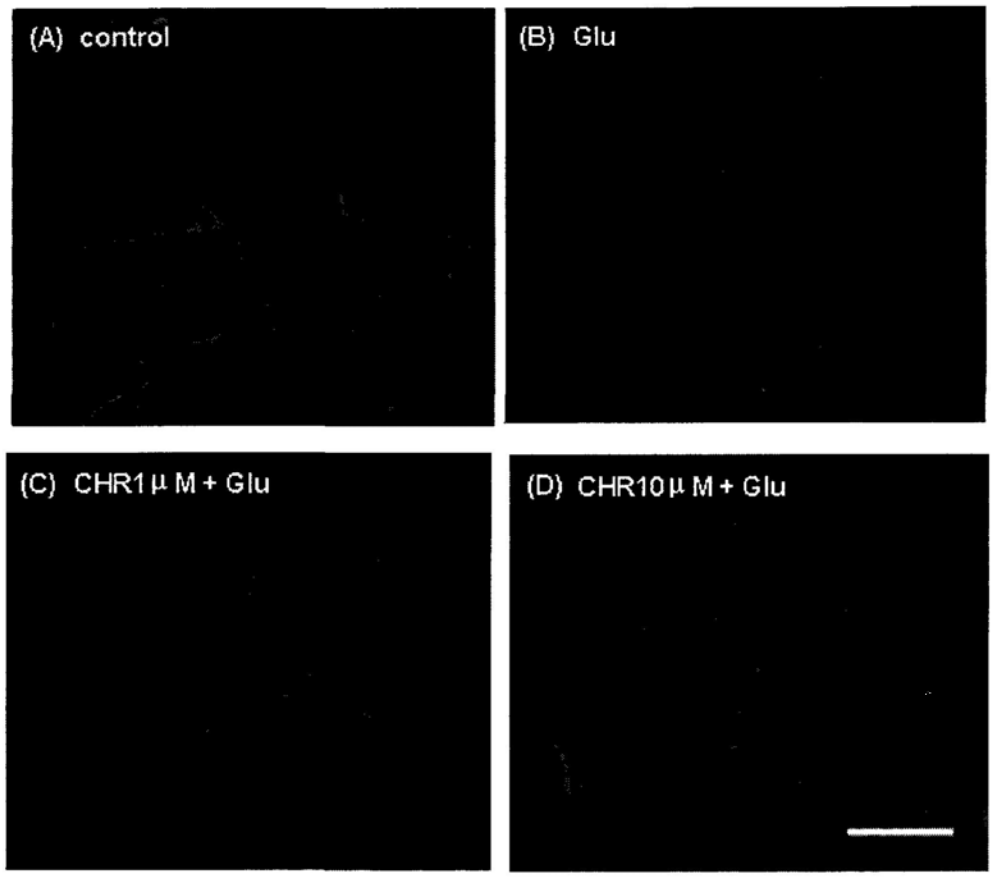


Figure 4.4 Immunocytochemical analysis of α-tubulin-stained neurons in the absence and presence of chrysin. The neurons were treated with chrysin for 1 h, followed by exposure to 30 μM glutamate for 24 h. (A) control; (B) glutamate treatment for 24 h alone; (C) the neurons treated with chrysin at 1 μM, and (D) 10 μM for 24 h and then incubated together with glutamate 30 μM for 24 h. Scale bar represents 5 μM.

4.3.4 Reduction of intracellular ROS by chrysin

As glutamate could trigger production of ROS during the initiation phase of excitotoxic injury, we aimed to investigate if chrysin would protect neurons through an anti-oxidative mechanism. The effects of chrysin on intracellular ROS were investigated by NBT assay. As shown in Figure 4.5, oxidative stress was induced by glutamate and there was a nearly two-fold increase of intracellular ROS. Chrysin significantly decreased ROS level at the doses of 1 and 10 μM by 60 ± 1% and 71 ± 0.7%, respectively, when compared with glutamate-treated group. The results imply that chrysin exhibited anti-oxidative effect at these concentrations to protect the neurons from glutamate-induced excitotoxicity. Chrysin at 0.1 μM did not significantly elicit anti-oxidative effect, however.

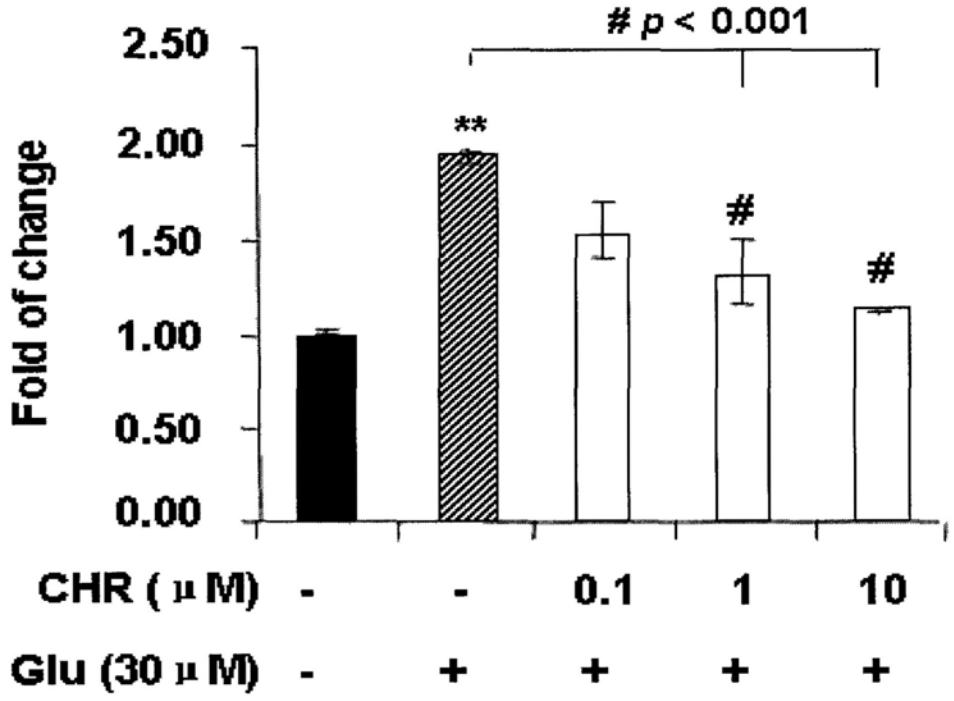
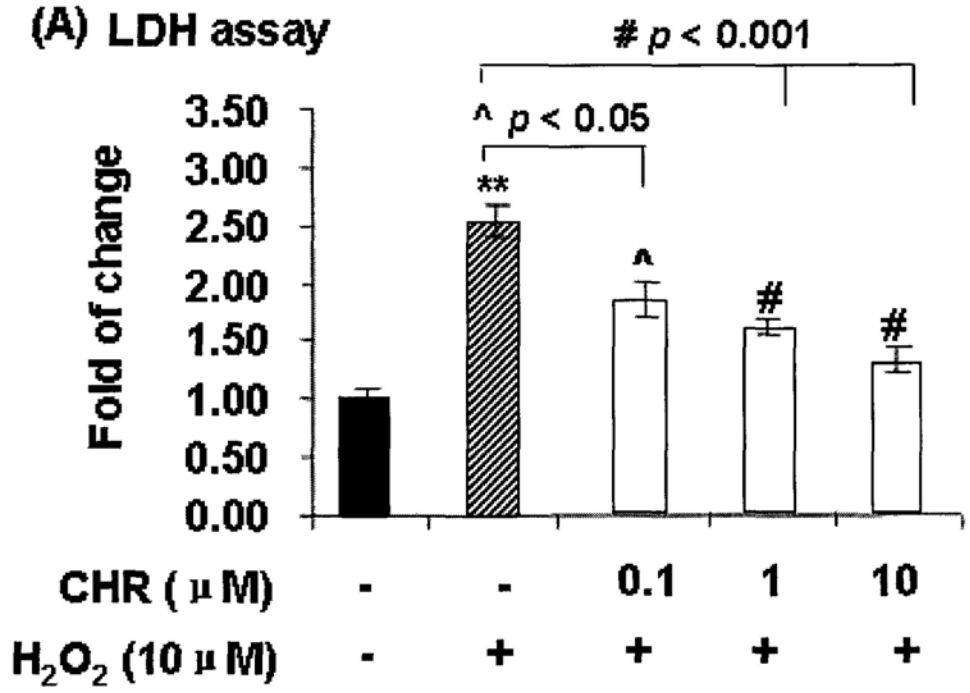


Figure 4.5 Chrysin attenuated the production of intracellular ROS induced by glutamate. The neurons were treated with chrysin for 1 h, followed by incubation with 30 μM glutamate for 24 h. Production of ROS was detected by NBT reduction assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control; $^{\#}$, $p < 0.001$ vs Glu; ^, $p < 0.05$ vs Glu by one-way ANOVA for multiple comparisons and Student–Newman–Keuls test as post hoc test.

4.3.5 Examination of the anti-oxidative effects of chrysin on H_2O_2-induced toxicity

Oxidative stress is a major factor in glutamate-induced excitotoxicity. To further investigate any potential anti-oxidative effects of chrysin, we adopted an indirect approach to examine if chrysin could protect the neurons from damages induced by hydrogen peroxide. The neurons were treated with chrysin for 1 h prior to exposure to H_2O_2 at 10 μM for 24 h. The release of LDH and caspase-3 activity were examined. As shown in Figure. 4.6, H_2O_2 could induce release of LDH and activation of caspase-3 to 2.5 ± 0.3 and 2.0 ± 0.1 fold of control, respectively. We found that chrysin at all tested dosages (0.1, 1 and 10 μM) significantly protected the neurons from H_2O_2-induced neuronal damage. Chrysin at the concentration of 10 μM could significantly attenuate LDH release to 1.3 ± 0.2 fold of the control (Figure. 4.6A). While 0.1 μM chrysin did not induce anti-oxidative effects as shown in Figure. 4.5, it was noted that chrysin at this concentration exert significant protection by attenuating caspase-3 activity (Figure. 4.6B). Protective effects of chrysin against H_2O_2-induced activation of caspese-3 did not follow a dose-response curve, however.


(A) LDH assay
# p < 0.001
^ p < 0.05
Fold of change
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
^
#
#
CHR (μ M) - - 0.1 1 10
H2O2 (10 μ M) - + + + +

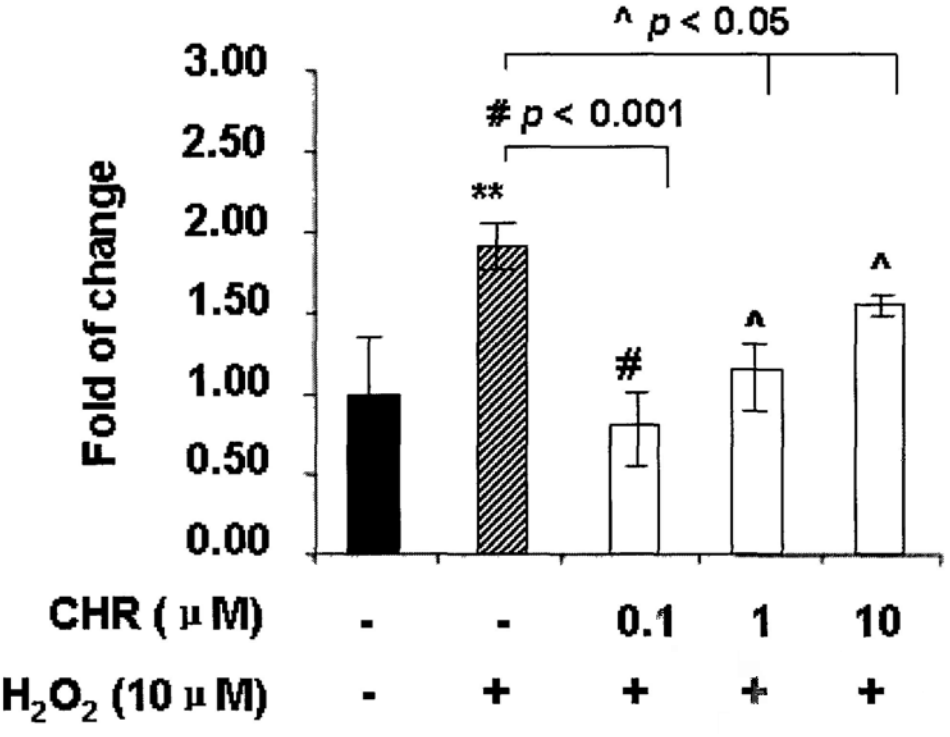
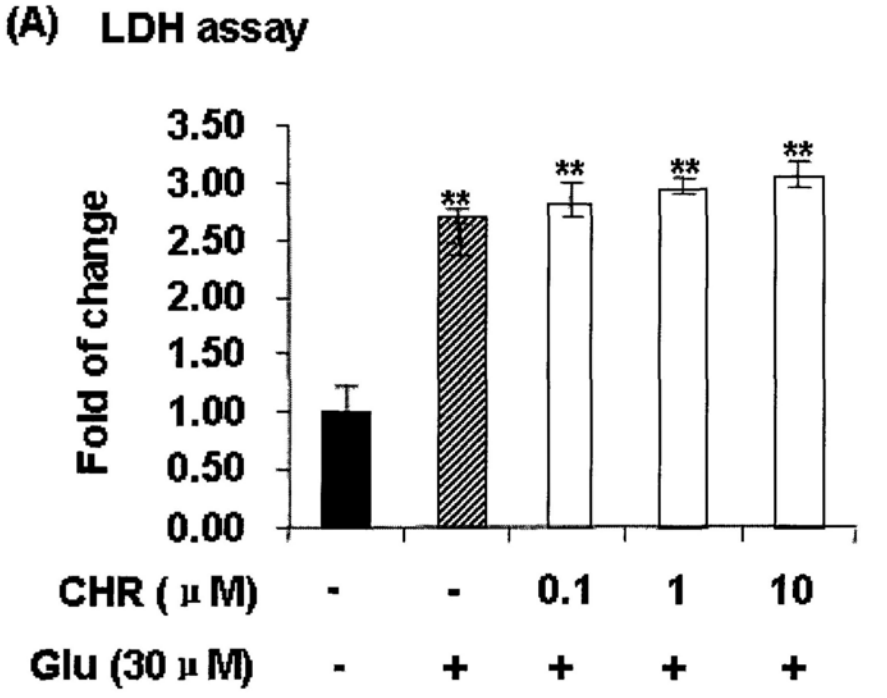

(B) Caspase-3 assay
^ p < 0.05
# p < 0.001
Fold of change
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
#
^
^
CHR (μ M) - - 0.1 1 10
H2O2 (10 μ M) - + + + +

Figure 4.6 Protective effects of chrysin on H_2O_2-induced neurotoxicity. The neurons were treated with chrysin for 1 h, followed by incubation with 10 μM H_2O_2 for 24 h. The neurotoxicity was assessed by (A) LDH assay and (B) caspase-3-like assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control; #, $p < 0.001$ vs H_2O_2; ^, $p < 0.05$ vs H_2O_2 by one-way ANOVA for multiple comparisons and Student–Newman–Keuls test as post hoc test.

4.3.6 Effects of chrysin in wash-out experiments

Although chrysin exhibited anti-oxidative properties which may account for its protective effects against glutamate-induced cellular damage, the results of 0.1 μM chrysin against H_2O_2-induced neurotoxicity led us to consider that neuroprotective effects of chrysin may not be accomplished simply by its anti-oxidative effects. It is also possible for chrysin to protect neurons via other mechanisms such as modulation of intracellular signaling pathways (Williams *et al.* 2004) and function as ligand for receptors to interfere their activities (Medina *et al.* 1990). In order to investigate neuroprotective mechanisms of chrysin, the wash-out experiment was performed. As shown in Figure 4.7 A and B, when chrysin was removed by wash-out 1 h after incubation, there were no protective effects against the subsequent glutamate challenge. No reduction in both glutamate-induced LDH release and activation of caspase-3 could be found in all tested dosages of chrysin. It was surprised to note that chrysin at 1 and 10 μM significantly increased the caspase-3 activity induced by glutamate (Figure. 4.7 B).


(A) LDH assay
Fold of change
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
CHR (μ M) - - 0.1 1 10
Glu (30 μ M) - + + + +

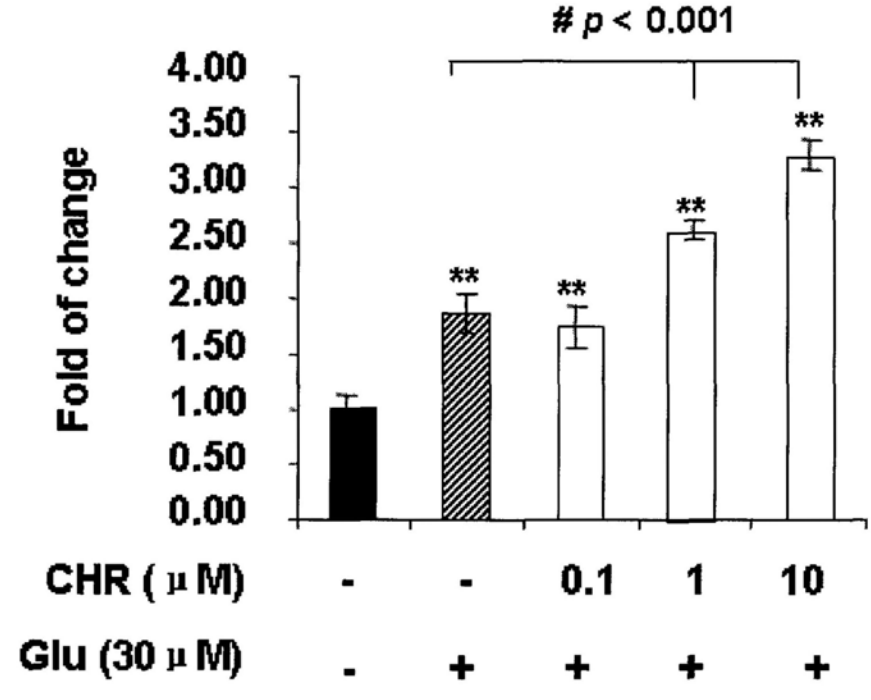
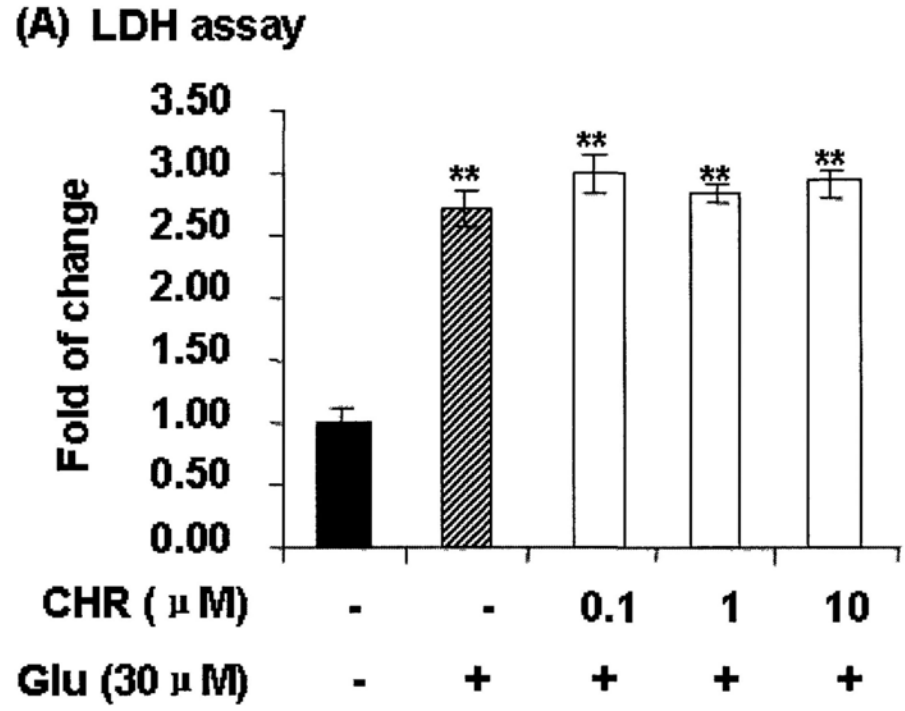

(B) Caspase-3 assay
# p < 0.001
Fold of change
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
CHR (μ M) - - 0.1 1 10
Glu (30 μ M) - + + + +

Figure 4.7 Protective effects of chrysin on glutamate-induced neuronal damage in wash-out experiments. The neurons were treated with chrysin for 1 h and the chrysin-containing medium was removed and the neurons were rinsed with PBS before exposure to 30 μM glutamate for another 24 h. The neurotoxicity was assessed by (A) LDH assay and (B) caspase-3-like assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control by one-way ANOVA for multiple comparisons and Student–Newman–Keuls test as post hoc test.

4.3.7 Effects of chrysin in post-treatment experiments

Similar results were obtained in the post-treatment experiment. When chrysin was added 1 h after the exposure of the neurons to glutamate, it could not significantly reduce glutamate-induced LDH release (Figure 4.8A). The results of caspase-3 activity assay had specifically drawn our attention as it showed loss of neuroprotection by 1 and 10 μM of chrysin in post-treatment but some protective effects exhibited by 0.1 μM (Figure 4.8B). At this concentration, chrysin was found to be the least effective in attenuating ROS generation as shown in Figure 4.4. Our results suggest that the anti-oxidative effects of chrysin cannot fully explain its neuroprotective actions.


(A) LDH assay
Fold of change
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
**
**
**
CHR (μ M) - - 0.1 1 10
Glu (30 μ M) - + + + +

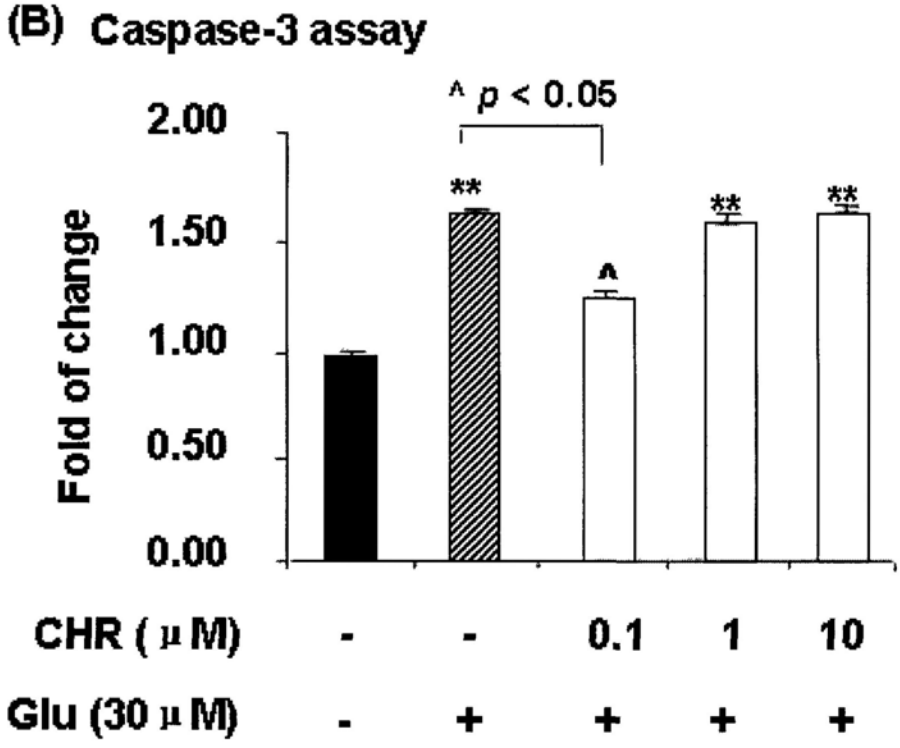

(B) Caspase-3 assay
^ p < 0.05
Fold of change
2.00
1.50
1.00
0.50
0.00
**
^
**
**
CHR (μ M) - - 0.1 1 10
Glu (30 μ M) - + + + +

Figure 4.8 Protective effects of chrysin on the glutamate-induced neuronal damage in post-treatment experiments. The neurons were exposed to 30 μM glutamate for 1 h and then co-cultured with chrysin for another 23 h. The neurotoxicity was assessed by (A) LDH assay and (B) caspase-3-like assay. Results are expressed as means ± SEM from at least three independent experiments. **, $p < 0.001$ vs control; ^, $p < 0.05$ vs Glu by using one-way ANOVA for multiple comparisons and Student–Newman–Keuls test as post hoc test.

4.4 Discussion

In the present study, we have further evaluated the neuroprotective effects of chrysin on glutamate-induced neuronal death *in vitro*. We have found that chrysin could protect cultured cortical neurons from glutamate-induced excitotoxicity through attenuating the release of LDH and caspase-3-like activity. In addition, it could mitigate H_2O_2-triggered LDH release and caspase-3-like activity, as well as intracellular ROS at high concentrations. While chrysin has been considered to exert neuroprotection by its anti-oxidative effect, low concentration of chrysin (i.e. 0.1 μM) appeared to elicit neuroprotection via an anti-oxidant-independent process. Our results suggest that small molecule of flavonoid chrysin has potential to be developed as neuroprotective agent against aging-associated neurodegenerative diseases.

In our study, we found that chrysin was able to protect neurons against glutamate-induced neurotoxicity. Exposure of chrysin at 1 or 10 μM significantly attenuated glutamate-induced LDH release and apoptosis in a dose-dependent manner. Since oxidative stress is one of the key mediators in glutamate-mediated neurotoxicity. We also performed NBT assay to access the effects of chrysin on glutamate-induced ROS. Our data were in agreement with previous reports showing that chrysin could elicit anti-oxidative effects and had cytoprotective action against insults such as hydrogen peroxide, nitric oxide and lipids (Mercer *et al.* 2005; Weng *et al.* 2005).

However, does the anti-oxidative property of chrysin accounts for its neuroprotective effects in our *in vitro* models? Our data presented in Figure. 4.6 and 4.8 actually did not support this hypothesis in which chrysin simply functions as an

anti-oxidant to protect neurons against glutamate-induced damages. Chrysin at 0.1 μM was the most effective dose in attenuating H_2O_2-induced caspase-3 activity as shown in Figure 4.6. Similarly, in the post-treatment experiments, only 0.1 μM chrysin was able to attenuate glutamate-induced caspase-3 activity (Figure. 4.8). Furthermore, only this concentration could safeguard neurons from glutamate-induced caspase-3 activity but not LDH release. All these results suggest that anti-oxidant mechanism cannot fully explain the protective effects of chrysin.

In addition to anti-oxidative effects, chrysin may elicit neuroprotection by the following possible mechanisms: (i) functioning as ligand for glutamate receptor to interfere with their activities (Medina *et al.* 1990); (ii) affecting glutamate receptor activity; iii) interacting with glutamate to affect its binding affinity. The wash-out and post-treatment experiments were performed to investigate the underlying mechanisms. Our results demonstrated that chrysin did not show any protective effects in the wash-out experiments, which suggest that it is unlikely for chrysin to interact with glutamate to affect its binding affinity (iii). On the other hand, chrysin was able to attenuate glutamate-induced neurotoxicity in the pretreatment experiment and also decrease the caspase-3 activity in the post-treatment experiments. Therefore, both (i) and (ii) are possible mechanisms to explain how chrysin protects neurons. Further examination is required to clarify the exact neuroprotective mechanism(s).

Another possibility is that chrysin is able to modulate intracellular signaling

pathways. By using an endoplasmic reticulum (ER)-stress inducer, tunicamycin, chrysin elicited neuroprotection by concentration-dependently inhibit tunicamycin-induced SH-SY5Y cell death (Izuta *et al.* 2008). There are two main pathways through which ER-stress can trigger apoptosis. One is mitochondria-dependent (Csordas *et al.* 2006) pathyway, and the other is impairment of ER functions (Breckenridge *et al.* 2003). Chrysin has been found to protect neuroblastoma SH-SY5Y cell from ER-stress responses by a mitochondria-dependent pathway, inhibition of $\Delta\Box_m$ depolarization and cytochrome *c* release (Izuta *et al.* 2008). Therefore, chrysin may affect a wide array of signaling pathways.

Taken together, we have demonstrated that chrysin was able to exert neuroprotection against glutamate-induced damage in rat cortical neurons. The mechanisms of neuroprotection may be partly related to its anti-oxidative activity when chrysin is at high concentrations. Chrysin could also elicit neuroprotection at low concentration via a mechanism independent of its anti-oxidative effects. In view of its effective neuroprotection, it may have good potential to be further developed into therapeutic agent for aging-associated neurodegenerative diseases.

Chapter 5

Neuroprotective Effects of Chrysin in a Rat Model of Chronic Ocular Hypertension

5.1 Introduction

As discussed in the previous chapter, progressive loss of neurons is the major problem of many neurodegenerative diseases, such as AD, PD and glaucoma. Glaucoma is defined as an optic neuropathy with damage to retinal ganglion cells (RGCs) and their axons, resulting in visual field changes and even loss of vision in the late stage of the disease (Foster *et al.* 2002; Dahlmann-Noor *et al.* 2010). In 2002, epidemiological statistics gathered by WHO showed that glaucoma was the second leading cause of blindness worldwide, after cataract (Resnikoff *et al.* 2004). Glaucoma accounts for 12.3% of 37 million people affected by blindness, and 82% of whom were 50 years or older. It has been estimated that among people aged 40 or older worldwide, there will be 60.5 million with glaucoma, and over 8.4 million will be bilaterally blind from primary glaucoma by 2010. The number of glaucoma will increase to 79.6 million and the resulting blindness will increase to 11.1 million by 2020 (Quigley & Broman 2006). In China, there were about 9.4 million people with glaucomatous optic neuropathy (Foster & Johnson 2001). There will be about 15.8 million glaucoma patient by 2010 and the patient number will increase to 21.8 million by 2020 (Quigley & Broman 2006). With the global increase in the aging population, it is foreseeable that glaucoma will have increasing impact on both patients and the society.

No experimental evidence is available so far to indicate that chrysin could protect RGCs under the elevated IOP in rat model. Increasing lines of evidence support the idea that oxidative stress plays an important role in the pathogenesis of RGC loss in

glaucoma (Tezel & Wax 2000). Free radicals are able to damage the trabecular meshwork (Tamm *et al.* 1996; Sacca *et al.* 2005; Sacca & Izzotti 2008) leading to alteration of the aqueous humor outflow pathway. Consequently, all these events will increase IOP and damage RGCs (Izzotti *et al.* 2003).

Apart from free radicals, other pathological factors such as glutamate excitotoxicity are also involved in glaucoma (Komara *et al.* 1986; Lazzarino *et al.* 1992). Glutamate excitotoxicity can induce neuronal damage. These injured or dead neurons can in turn release glutamate to further affect the survival of neighboring neurons. It has been reported that glutamate induces progressive degeneration of RGCs, in which the modes of degeneration share lots of similarity with that in AD (Quigley & Broman 2006) (Sucher *et al.* 1991; Neufeld *et al.* 1999; McKinnon *et al.* 2002). Therefore, it is essential to find neuroprotective agent to prevent the damage from the above pathological factors (Simonian & Coyle 1996; Ritch 2000; Mozaffarieh & Flammer 2007).

In our study, we have already proved that chrysin could attenuate glutamate-induced neurotoxicity in rat cortical neurons. We hypothesized that chrysin may also protect RGCs. In this study, we aimed to investigate whether chrysin could protect RGCs under the elevated IOP in rat model of ocular hypertension.

5.2 Materials and Methods

5.2.1 Animals

Adult female Sprague-Dawley (SD) rats (weights 250-280 g) were used in the present experiments. They were housed in temperature-controlled animal room with a 12-h light/12-h dark cycle, and sufficient food and water supply at the Laboratory Animal Unit of The University of Hong Kong.

Before surgery and measuring the IOP, animals were anesthetized with intra-peritoneal injection of a ketamine/xylazine mixture (80 mg/kg ketamine and 8 mg/kg xylazine). Proparacaine hydrochloride (Alcon 0.5% Alcaine®) was also applied as topical anaesthetic. All operations were performed under an operating microscope (Olympus OME, Tokyo, Japan). After surgery, ophthalmic ointment (3% tobramyxin, Alcon-Couvreur, Belgium) was applied topically to prevent inflammation. All experimental protocols and procedures for animals followed the tenets of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

5.2.2 Measurement of IOP

IOP was measured with a Tonolab (Mentor®, Norwell, USA) before the induction of ocular hypertension and every subsequent week until the rats were euthanized. Before each measurement, one drop of proparacaine hydrochloride (0.5% alcaine, Alcon-Couvreur, Belgium) was applied to the eyes as a topical anesthetic. To avoid diurnal variation and effect of anesthesia, all IOP measurements were taken at 10 o'clock in the morning and within 15 to 30 minutes after anesthesia using ketamine

and xylazine mixture (i.p.). An average of ten measurements was used to determine the IOP of the eye.

5.2.3 Ocular hypertensive (OH) model

OH was induced in the right eye of each animal by cauterizing the episcleral veins according to previous publications (Shareef *et al.* 1995; Laquis *et al.* 1998). Briefly, the three radical episcleral aqueous humor drainage veins (superior nasal, superior temporal and inferior temporal) were exposed after conjunctiva incision. Then the episcleral veins were cauterized using a cautery device (Bovie Medical Corporation, St. Petersburg, FL, USA) (Figure 5.1). The contralateral (left) eyes were untouched and used as controls for measuring IOP. After each operation, ophthalmic Tobrex ointment was applied topically to prevent infection.

5.2.4 Intravitreous injection of immune stimulant

Chrysin was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Figure 5.2 shows the schedule of IOP measurement, induction of OH, intravitreous injection, SC labeling and sacrifice of the animals.

Two groups of six rats each (group 1 & 2) were used as normal controls for the 2- or 4-week time points, respectively. Forty-eight rats with high IOP in the right eye were divided into 7 groups (n = 6-7 per group) for 2- or 4-week time points after the cauterization. Group 3 & 4 animals did not receive any intravitreous injections and

served as OH control. For the rest of the groups, two microliters of different drug solutions were given intravitreously: (1) two groups (group 5 & 6) of rats received phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and were used as solvent control for 2- and 4-week; (2) two groups of rats (group 7 and 8) received an intravitreous injection of 10 or 0.1 μM chrysin dissolved in 2 μl PBS and were kept for monitoring for 2-week time point while another group of rats (group 9) received 0.1 μM chrysin and were kept for monitoring for 4 weeks. Table 5.1. summarizes the groupings of the experimental animals.

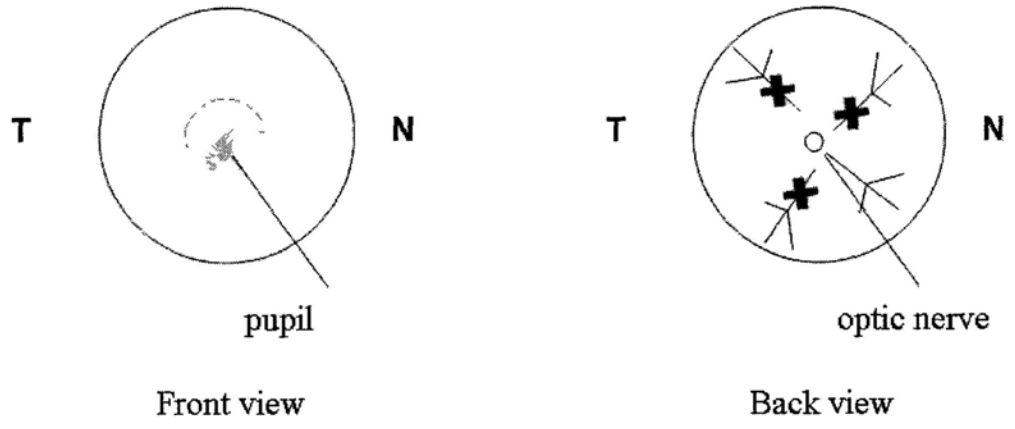


Figure 5.1 Episcleral vein cauterization model. Three radical episcleral aqueous humor drainage veins (superior nasal, superior temporal and inferior temporal) of the right eye of the rat were cauterized using a cautery device. (A) shows the front view (observed from the corneal side); (B) shows the back view (observed from the optic nerve side). T, temporal; N, nasal.

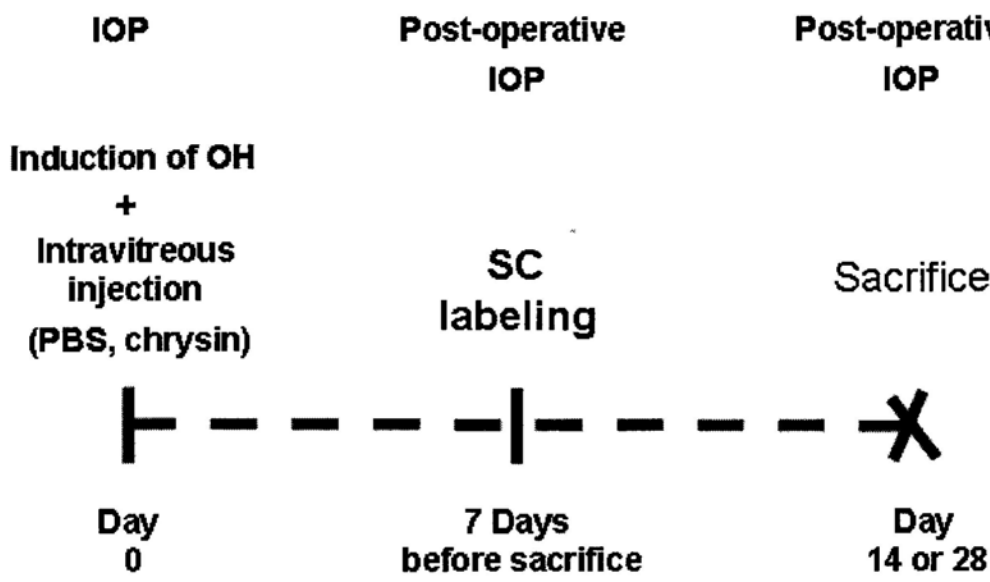


Figure 5.2 Treatment schedule of IOP measurement, induction of OH model, intravitreous injection and SC labeling in the 2- and 4-week studies. IOP was measured before inducing the OH model and before sacrifice (post-operative). Within 1 min of induction of the OH model, 2 μl sterilized PBS and chrysin was injected intravitreously. SC labeling was done 7 days prior to termination of experiment (14 or 28 days). IOP, intraocular pressure; OH, ocular hypertension; PBS, phosphate buffered saline; SC, superior colliculus.

Table 5.1 Grouping of the experimental animals.

Treatment (OH in the right eye)	Survival time after cauterization		Designation
	2 weeks	4 weeks	
Normal rats	6 (group 1)	6 (group 2)	Normal control
OH	6 (group 3)	7 (group 4)	OH Model control
OH + PBS i.v.	6 (group 5)	7 (group 6)	Solvent control for drug treatment
OH + CHR 0.1μM i.v	7 (group 7)		Effects of chrysin dose-response
OH + CHR 10 μM i.v.	8 (group 8)	7 (group 9)	Effects of chrysin time course study

For the intravitreous injection {Chan, 2007 #17}, 2 μl of different solutions were injected into the vitreous of the right eye right after cauterization of the vortex veins. The site of injection was at the superior temple region and just below the limbus of the cornea, which provides minimal possibility of injury to the retina. The needle tip was inserted into the vitreous body without touching the iris or the lens. The injection was performed within one minute and the needle was kept in place for an additional 2 minutes before being gently removed (Figure 5.3). Rats with cataract, intraocular bleeding, retinal detachment or non-elevated IOP were excluded from this study (~ 15% of the experimental animals).

5.2.5 Retrograde labelling of RGCs

Surviving RGCs were retrogradely labeled by applying gelatin sponge soaked with Fluoro-Gold (FG) over the entire surface of bilateral superior colliculi (SC) seven days prior to sacrifice (Chiu *et al.* 2008) (Figure 5.4). Briefly, the cerebral content that lies over the dorsal surface of the superior colliculus was carefully removed by the vacuum pump till four edges of SC can be observed directly under microscope. Then, a thin layer of gelatin sponge (Upjohn, Kalamazoo, MI, USA) pre-soaked with 6% FG (Fluorochrome, Denver, CO, USA) was placed on the surface of SC. FG was taken up by the axon terminals of RGCs and bilaterally transported retrogradely to their somas in the retina. After SC labeling, bupreorphine (100 mcg/kg), an analgesic, was orally administered for 5 days to relieve pain caused by the operation.

5.2.6 RGCs counting

At two or four weeks after the induction of OH, after taking the IOP, the rats were euthanized by an over-dose of a mixture of ketamine/xylazine, the rats were transcardially perfused with 0.9% saline for 5 min. Both eyes of each animal were enucleated and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 60 min. Then the eyes were cut horizontally into superior and inferior eyecups. The superior eyecups with intact optic nerves were fixed overnight and processed to make paraffin blocks. Retinas from the inferior eyecups were dissected from the underlying sclera, and flattened with the vitreal side up and mounted using fluorescent mounting medium (Dako Corporation, Carpentaria, CA, USA). The FG-labeled RGCs (FG particles in the cytoplasm) were visualized at 40x magnification using a fluorescent microscope with a UV-385 filter (Nikon, Kawasaki, Japan) (excitation wavelength = 330-380 nm). The number of RGCs was quantified under an eyepiece grid of 200×200 μm^2 along the midline of each quadrant, from the optic disc to the border at 500 μm intervals. Eight microscopic fields for each quadrant were counted according to a method preciously described (Chan *et al.* 2007) (Figure 5.5). The data were expressed as the loss of cells compare to the normal control.

Changes in the density of RGCs were expressed as a percentage loss of RGCs by comparing the OH right eye and normal control eye (Chiu *et al.* 2010):

$$\left[\frac{\text{RGC density in the normal eye} - \text{RGC density in the right eye with OH}}{\text{RGC density in the normal eye}}\right] \times 100\%$$

Percentage loss of RGCs in different treatment groups was compared using one-way analysis-of-variance (ANOVA) followed Student–Newman–Keuls test as post hoc test.

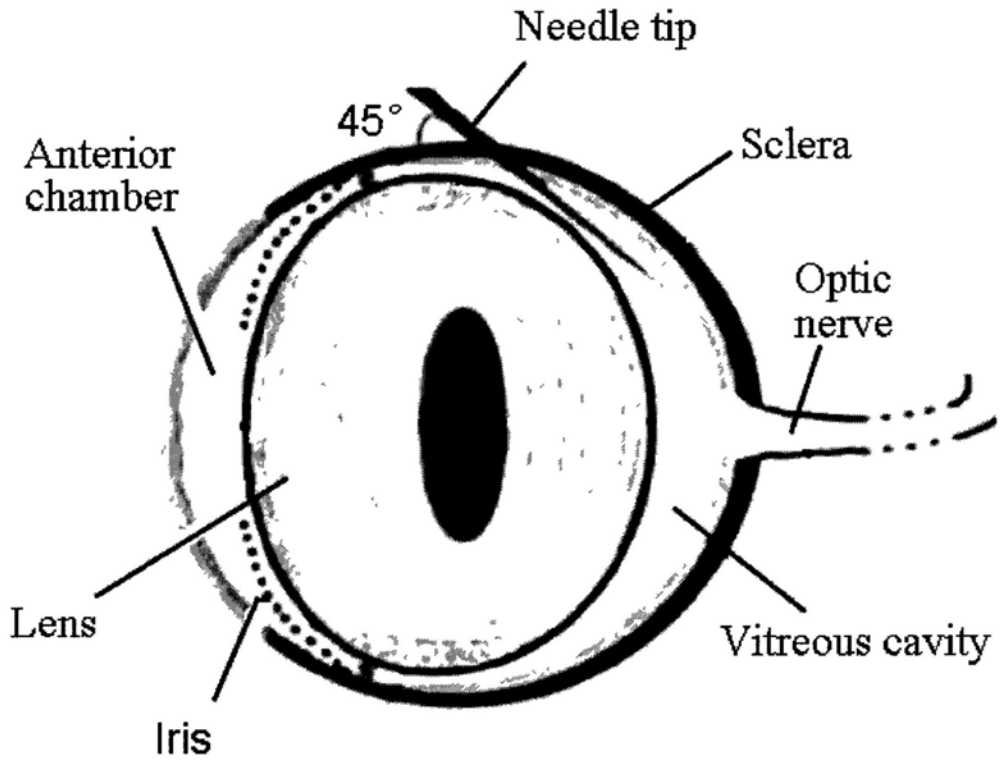


Figure 5.3 Schematic presentation of the intravitreous injection. Two μl of different solutions were injected into the vitreous of the right eye immediately after cauterization of the vortex veins. The needle tip was inserted into the superior temporal region of the eye at about 45° angle through the sclera into the vitreous cavity without touching the iris and the len.

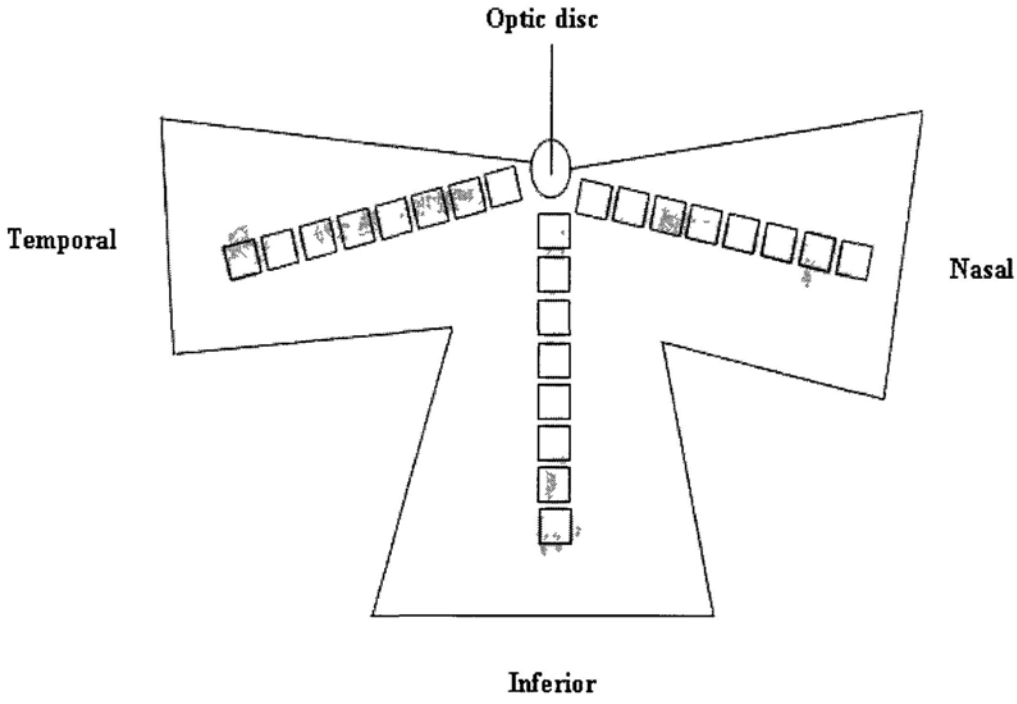


Figure 5.4 Counting of RGCs on the inferior half of the flat-mounted retina. The number of RGCs was quantified under an eyepiece grid of 200×200 μm^2 along the midline of each quadrant, from the optic disc to the border at 500 μm intervals. Eight microscopic fields for each quadrant were counted.

5.3 Results

5.3.1 Chrysin is not toxic to RGCs in normal rats

While flavonoids have long been considered to be beneficial to human health, they have also been reported that certain flavonoids may exert toxicity. Therefore, whether there was any toxicity from chrysin should be, at first, evaluated. Chrysin at the concentrations of 0.1, 1 or 10 μM were injected into the vitreous of normal rat eyes. Seven days later, surviving RGCs in the flat-mounted retina were counted by retrograde-labeled RGCs using FG. The percentage loss of RGCs was expressed by comparing the difference with the normal control group. Figure. 5.5 shows the percentage of RGC loss after intravitreous administration of chrysin at different concentrations. The loss of RGCs in chrysin groups at 0.1, 1 and 10 μM was 0.6 ± 0.3%, 1.1 ± 0.7% and 1 ± 0.2%, respectively. There was no significant difference in RGC loss among the different concentrations of chrysin-injected groups and the normal group. This result indicated that intravitreous application of chrysin at the concentrations up to 10 μM was not toxic to RGCs.

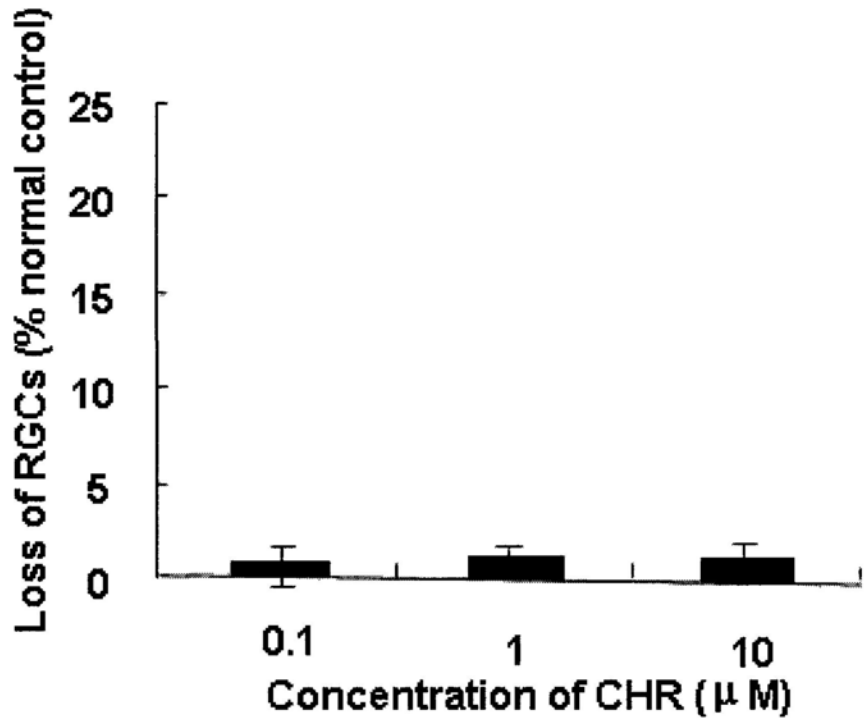
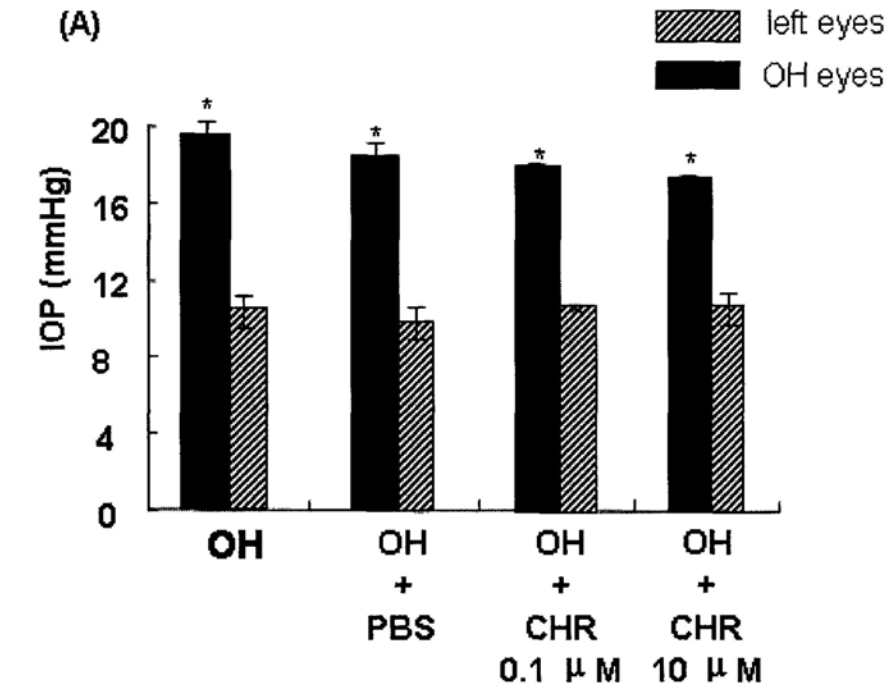


Figure 5.5 Examination of RGC loss in retinas receiving intravitreous injection of chrysin in normal rat eyes. There was no significant difference in RGC loss among normal and chrysin-injected retinas. N = 6 in each experimental group.

5.3.2 Chrysin protected RGCs challenged by OH after 2 weeks

At 2 weeks after induction of OH, there was a 22 ± 0.5% loss of RGCs. Intravitreous injection of PBS (the vehicle used to dissolve chrysin) right after the induction of OH resulted in 22 ± 0.7% loss of RGCs in the OH eyes, indicating PBS did not affect the survival of RGCs under OH. With chrysin treatment, there were 7 ± 1% and 13 ± 0.3% loss of RGCs in the 0.1 and 10 μM groups, respectively. There was statistically significant difference when compared chrysin 0.1 μM with either OH only or the OH with PBS injection control (#, $p < 0.001$ vs model and PBS control). It is interesting to note that increasing the chrysin concentrations to 10 μM did not further increase the neuroprotective effect (Figure 5.6B). As we have shown in Figure 5.5, intravitreous injection of chrysin did not affect RGC survival in the normal tension eyes. We also evaluated whether this neuroprotective effect of chrysin was accomplished by reduction of IOP. In normal eyes the average of IOP was 11 ± 0.6 mmHg. By using cauterization of the veins to induce OH, IOP was elevated from around 11 ± 0.2 mmHg (before) to 18 ± 0.4 mmHg (after). There was around 1.8-time increase of IOP at 2 weeks after cauterization. IOP of the cauterized right eye in all groups was significantly increased compared to the contralateral left eye ($p < 0.05$). Intravitreous injection of either PBS (18 ± 0.2 mmHg), chrysin at 0.1 (18 ± 0.1 mmHg) or 10 μM (17 ± 0.5 mmHg) did not affect the cauterization-induced IOP increase (Figure 5.6A). Therefore, neuroprotective effect of chrysin on RGCs was not mediated by reducing IOP in the OH eyes.


(A)
left eyes
OH eyes
IOP (mmHg)
20
16
12
8
4
0
*
OH
OH + PBS
OH + CHR 0.1 μM
OH + CHR 10 μM

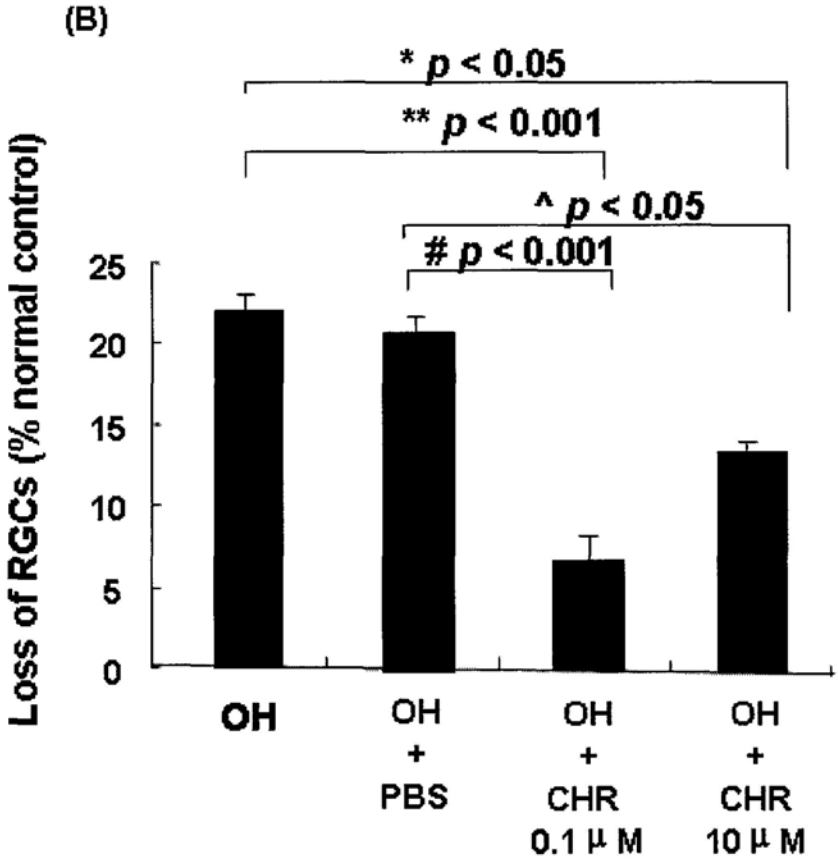

(B)
* p < 0.05
** p < 0.001
^ p < 0.05
# p < 0.001
Loss of RGCs (% normal control)
25
20
15
10
5
0
OH
OH + PBS
OH + CHR 0.1 μM
OH + CHR 10 μM

Figure 5.6 Effects of different doses of intravitreously administered-chrysin on the survival of RGCs in the OH eye at two weeks after the induction of OH. IOP in the right eyes was significantly increased compared with contralateral control left eyes (*, $p < 0.05$). IOP of right eyes in different groups did not show any significant difference (A). There was significant difference in the percentage of RGC loss when compared chrysin at 0.1 or 10 μM with the OH model or the PBS control groups (B). Results are expressed as means ± SEM. *, $p < 0.05$ and **, $p < 0.001$ vs model; ^, $p < 0.05$ and $^{\#}$, $p < 0.001$ vs control by one-way ANOVA for multiple comparisons and Student-Newman-Keuls test as post hoc test.

5.3.3 Chrysin protected RGCs challenged by OH after 4 weeks

Since glaucoma is a chronic disease, we also studied the long-term protective effect of chrysin on the survival of RGCs in OH eyes. At 4 weeks after cauterization, IOP was 16 ± 0.6 mmHg, 14 ± 0.3 mmHg and 15 ± 0.2 mmHg in the OH model, PBS control and chrysin 0.1 μM groups, respectively. IOP in the experimental eyes remained significantly high at 4 weeks after the cauterization (*, $p < 0.05$, Figure 5.7A).

The percentage loss of RGCs in the OH model and PBS-treated eyes was 24 ± 0.8% and 25 ± 0.6%, respectively, which was higher than that of 2 weeks. Intravitreous injection of 0.1 μM chrysin had significantly reduced the loss of RGCs to 9 ± 0.6% (#, $p < 0.001$, Figure 5.7B). One intravitreous injection of chrysin at the time of induction of OH was able to offer protection to RGCs under the challenge of 4-week high IOP.

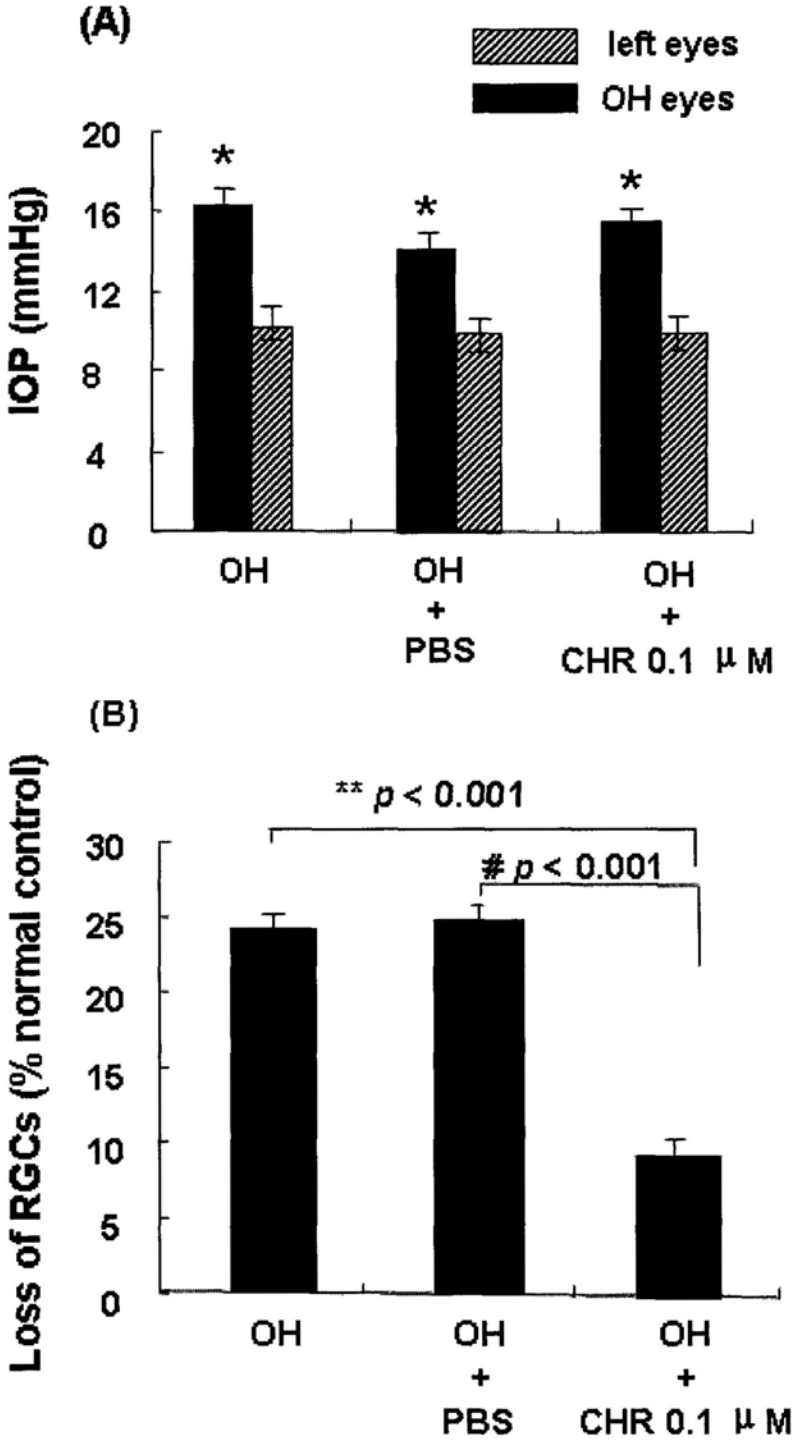

(A)
left eyes
OH eyes
IOP (mmHg)
20
16
12
8
4
0
*
*
*
OH
OH + PBS
OH + CHR 0.1 μM
(B)
** p < 0.001
# p < 0.001
Loss of RGCs (% normal control)
30
25
20
15
10
5
0
OH
OH + PBS
OH + CHR 0.1 μM

Figure 5.7 Effects of 0.1 μM of chrysin administered intravitreously in the OH eye at four weeks after the induction of OH. IOP of the right eyes in OH model, PBS control and chrysin group did not show any significant difference (A). At four weeks after OH induction, intravitreous injection of 0.1 μM chrysin significantly attenuated the loss of RGCs when compared with the OH model or the PBS control group (B). Results are expressed as means ± SEM. *, $p < 0.05$ and **, $p < 0.001$ vs model; ^, $p < 0.05$ and $^{\#}$, $p < 0.001$ vs control by one-way ANOVA for multiplecomparisons and Student-Newman-Keuls test as post hoc test.

5.4 Discussion

In this part of the study, we have shown the neuroprotective effect of chrysin against the loss of RGCs in ocular hypertension *in vivo*. To the best of our knowledge, this is the first study examining the possible neuroprotective effect of chrysin on RGCs in the retina against chronic ocular hypertension. Our *in vivo* experiments demonstrated that a single intravitreous injection of chrysin was able to rescue RGCs and the effects could last for 4 weeks.

Chrysin is a plant-derived flavonoid. If flavonoids access the brain, they must first cross the blood-brain-barrier (BBB), which controls the entry of xenobiotics into the brain (Abbott 2002). Studies have demonstrated that flavonoids are able to cross the BBB from periphery after oral administration, such as hesperentin, naringenin and their *in vivo* metabolites (Youdim *et al.* 2003a; Vauzour *et al.* 2008). The lipophilicity of flavonoids is considered to facilitate their passage across the BBB, as less polar O-methylated metabolites may be capable to greater brain uptake than the more polar flavomod glucuronides (Youdim *et al.* 2003b) (Mercer *et al.* 2005). Meanwhile, certain drug glucuronides may also cross the BBB to elicit pharmacological effects with special uptake mechanism for glucuronides *in vivo* (Kroemer & Klotz 1992; Aasmundstad *et al.* 1995; Sperker *et al.* 1997). One good example of flavonoid is epicatechin, which commonly presents in green tea, red wine and various fruit. Epicatechin shares the same polyphenolic structure with chrysin, and it can pass through the BBB (Abd El Mohsen *et al.* 2002). Since chrysin has high lipophilicity with small molecular weight, it is very likely that this flavonoid has this property.

Intravitreous injection has been used in the treatments of human ocular diseases for nearly a century. It has several advantages over other routes of drug administration especially for disorders affecting the posterior segment of the eye, most notably the ability to maximize intraocular concentrations while limiting systemic exposure (Jager *et al.* 2004). The drug concentration in the vitreous are determined not only by the amount of drug given, but also by the distribution and clearance of such compounds (Wingard *et al.* 1989; Coco *et al.* 1998). As with other routes of drug administration, pharmacokinetic characteristics are dependent on both the anatomical and physiologic features at the site of administration (Schindler *et al.* 1982; Wingard *et al.* 1989; Coco *et al.* 1998) and physicochemical properties of the agent administered (Stay *et al.* 2003). The most important advantage of intravitreous injection, compared to the other routes of drug administration, is that the drug is introduced precisely to the target tissue and therapeutic intraocular concentrations are achieved soon after the injection (Aiello *et al.* 2004) (Gragoudas *et al.* 2004). Because of this, we must ensure that our drug do not have any toxic effect on RGCs. On the other hand, as we mentioned before, flavonoids may exert toxicity, it was necessary for us to test whether chrysin at different concentrations was toxic to RGCs before further experiments were planned. As shown in Figure. 4.6, intravitreous application of chrysin at 0.1, 1 and 10 μM was not toxic to RGCs.

In our study, intravitreous injection of 0.1 and 10 μM could attenuate the RGC loss for 2 weeks in the eye with OH, and 0.1 μM is more effective. Furthermore, a

single intravitreous injection of 2 μl of 0.1 μM chrysin significantly reduces RGC loss for 4 weeks, suggesting that chrysin could directly protect RGCs against the insult of chronic ocular hypertension. In our *in vitro* experiment, chrysin at 0.1 μM was the most effective dose in attenuating H_2O_2-induced caspase-3 activity as shown in Figure 4.7. Similarly, in the post-treatment experiments, only 0.1 μM chrysin was able to attenuate glutamate-induced caspase-3 activity (Figure. 4.8). We postulate that chrysin can elicit neuroprotection at low concentration via a mechanism independent of its anti-oxidative effects. As glutamate is an important physiological factor involves in glaucoma. We believe that the protective effect of chrysin on RGC loss is also via a mechanism independent of its anti-oxidative effects. Further studies should be conducted both *in vivo* and *in vitro* to reveal the underlying mechanism.

Taken together, we have demonstrated that chrysin was able to exert neuroprotection against RGC loss in OH glaucoma rats and glutamate-induced damage in rat cortical neurons. In view of its effective neuroprotection, chrysin may have high potential to be further developed into pharmaceutical agent against aging-associated neurodegenerative diseases in the future.

Chapter 6

Discussion and Conclusions

In the present PhD study, we have successfully identified a neuroprotective compound, chrysin, from Fructus Alpinae oxyphyllae. We have evaluated the neuroprotective effect of chrysin on glutamate-induced neuronal death *in vitro,* and have further shown a possible neuroprotective effect of chrysin on RGCs against ocular hypertension *in vivo*. In addition, chrysin was found to protect neurons from glutamate-induced excitotoxicity and it attenuated the release of LDH and caspase-3-like activity. Moreover, it was able to suppress H_2O_2-triggered LDH release and caspase-3-like activity, as well as attenuate the intracellular ROS at high concentrations. Furthermore, our *in vivo* experiments demonstrated that a single intravitreous injection of chrysin was effective in rescuing RGCs and the effects could last for 4 weeks. While chrysin has been considered to exert neuroprotection by its anti-oxidative effects, low concentration of chrysin (i.e. 0.1 μM) appeared to elicit neuroprotection via an anti-oxidant-independent process. Our results suggest that chrysin, a plant-derived flavonoid, has good potential to be developed as neuroprotective agent against aging-associated neurodegenerative diseases.

Neurodegenerative disease is the result of the deterioration of neurons. Glaucoma is the second most common leading cause of vision loss worldwide. It is classified as a kind of conditions characterized by progressive damage to the optic nerve, deterioration of RGCs and ultimately visual field loss. Studies have shown that treatments intended only to lower IOP, the most common risk factor for glaucoma progression, are not always effective in preventing visual field loss. With regards to glaucoma, neuroprotection refers to any intervention aimed at preventing optic nerve damage or cell death (Sena *et al.* 2010). The rationale for treatment is

that by acting as pharmacological antagonists, neuroprotective agents will prevent optic nerve damage and cell death, thus preserving vision in patients with glaucoma. Pharmacological strategy that focuses directly on preventing the death of RGCs should play a more important role in the future of glaucoma treatment (Vass *et al.* 2007).

FAO is a perennial plant which grows in the oriental countries and its pharmacological effects include diuretic, anti-salivation and anti-diarrhea. Despite a number of reports on the effective neuroprotective compound isolated from FAO, there is still a lack of systematic investigations of the active ingredients in the herbal extracts. Using glutamate-induced neuronal death as an *in vitro* model, we successfully evaluated the neuroprotective effect of this plant. In addition, the bioassay-guided isolation method was used to identify the active ingredients. On the basis of experimental data, chrysin has been identified as the most active component on attenuating the glutamate-induced LDH toxicity on neurons.

Chrysin is classified as a flavonoid with a polyphenolic structure. A number of studies have demonstrated that dietary polyphenols are able to prevent oxidative damage and they can directly scavenge free radicals (Jiang & Dusting 2003; van Meeteren *et al.* 2004). Since chrysin has a number of beneficial effects, it is our goal to further explore its potential as a neuroprotective agent for aging-associated neurodegenerative diseases. Flavonoids are small molecules and studies have demonstrated that they are able to cross the blood-brain-barrier from periphery after oral administration (Vauzour *et al.* 2008). The lipophilicity of flavonoids also facilitate their passage across the BBB (Youdim *et al.* 2003a; Mercer *et al.* 2005).

The high lipophilicity with small molecular weight of chrysin makes it possible to cross BBB.

In our *in vivo* study, it is the first study examining the possible neuroprotective effects of chrysin on RGCs in the retina against chronic ocular hypertension. Intravitreous injection has several advantages over other routes of drug administration especially for disorders affecting the posterior segment of the eye (Jager *et al.* 2008). The most important is that the drug is introduced precisely to the target tissue and therapeutic intraocular concentrations are achieved soon after the injection (Aiello *et al.* 2004; Gragoudas *et al.* 2004). In our study, a single intravitreous injection of 2 μl 0.1 μM chrysin significantly reduced RGC loss for 4 weeks, suggesting that chrysin can directly protect RGCs against damage induced by chronic ocular hypertension.

Increasing lines of evidence support the idea that oxidative stress plays an important role in the pathogenesis of RGC loss in glaucoma (Tezel & Wax 2000). Free radicals can damage the trabecular meshwork leading to alteration of the aqueous humor outflow pathway (Tamm *et al.* 1996; Sacca *et al.* 2005; Sacca & Izzotti 2008). Consequently, all these events will increase IOP and damage RGCs (Izzotti *et al.* 2003). Apart from free radicals, other pathological factors such as glutamate excitotoxicity are also involved in glaucoma (Komara *et al.* 1986; Lazzarino *et al.* 1992). Therefore, it is essential to find neuroprotective agent to

prevent the damage from the above pathological factors (Simonian & Coyle 1996; Ritch 2000; Mozaffarieh & Flammer 2007).

In our study, we found that chrysin was able to protect neurons against glutamate neurotoxicity. Exposure of chrysin at 1 or 10 μM significantly attenuated glutamate-induced LDH release and apoptosis in a dose-dependent manner. Since oxidative stress is one of the key mediators in glutamate neurotoxicity. We also performed NBT assay to access the effects of chrysin on glutamate-induced ROS. Our data were congruent with previous reports showing that chrysin can elicit anti-oxidative effects (Mercer *et al.* 2005; Weng *et al.* 2005). Therefore, in our *in vitro* and *in vivo* models, the results demonstrated that the anti-oxidative property of chrysin could not completely account for its neuroprotective effects. Chrysin at 0.1 μM was more effective in rescuing RGC loss than 10 μM 2 weeks after induction of OH model. Moreover, chrysin at 0.1 μM was the most effective dose in attenuating H_2O_2-induced caspase-3 activity. Similarly, in the post-treatment experiments, only 0.1 μM chrysin was able to attenuate glutamate-induced caspase-3 activity. Furthermore, only this concentration can safeguard neurons from glutamate-induced caspase-3 activity but not LDH release. All these results may suggest that anti-oxidant activity cannot fully explain the protective effects of chrysin.

In addition to anti-oxidative effects, chrysin may elicit neuroprotection by other possible mechanisms. Our results demonstrated that chrysin was able to attenuate

glutamate-induced neurotoxicity in the pretreatment experiment and also decreased the caspase-3 activity in the post-treatment experiments. These findings suggest that chrysin may act as ligand for glutamate receptor to interfere with their activities; or it could affect glutamate receptor activity resulting in the neuroprotective effects. Further examination is required to clarify the exact neuroprotective mechanism(s).

Another possibility is that chrysin is able to modulate intracellular signaling pathways. By using an endoplasmic reticulum (ER)-stress inducer, tunicamycin, chrysin elicites neuroprotection by concentration-dependently inhibit tunicamycin-induced SH-SY5Y cell death (Izuta *et al.* 2008). There are two main pathways through which ER-stress can trigger apoptosis. One is mitochondria-dependent (Csordas *et al.* 2006), and another is impairment of ER functions (Breckenridge *et al.* 2003). Chrysin has been found to protect neuroblastoma SH-SY5Y cells from ER-stress responses by a mitochondria-dependent pathway, inhibition of $\Delta\Box_m$ depolarization and cytochrome *c* release (Izuta *et al.* 2008). Therefore, chrysin may affect a wide array of signaling pathways.

In summary, we have successfully identified an active neuroprotective compound, chrysin, from FAO. Furthermore, we have demonstrated that chrysin is able to exert neuroprotection against RGCs loss in OH glaucoma rats and glutamate-induced damage in rat cortical neurons. The mechanisms of neuroprotection may be partly

related to its anti-oxidative activity when chrysin is at high concentrations. Chrysin can also elicit neuroprotection at low concentration via a mechanism independent of its anti-oxidative effects. In view of its effective neuroprotection, chrysin may have good potential to be further developed into pharmaceutical agent for the treatment of aging-associated neurodegenerative diseases in future.

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