ผลของเอเชียติโคซัยด์ต่อการตายของเซลล์เนื่องจากสภาวะโพแทสเซียมต่ำ ในเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาว

นางสาวศรีรัตน์ ศุภวรรธนะกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECTS OF ASIATICOSIDE ON LOW POTASSIUM MEDIUM INDUCED CELL DEATH IN CULTURED RAT CEREBELLAR GRANULE NEURONS

Miss Srirat Supawantanakul

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for the Degree of Master of Science in Pharmacology

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ศรีรัตน์ ศุภวรรธนะกุล : ผลของเอเชียติโคซัยด์ต่อการตายของเซลล์เนื่องจากสภาวะโพแทสเซียมต่ำในเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาว (EFFECTS OF ASIATICOSIDE ON LOW POTASSIUM MEDIUM INDUCED CELL DEATH IN CULTURED RAT CEREBELLAR GRANULE NEURONS) อ. ที่ปรึกษา : ผศ. ดร. สุรชัย อัญเชิญ 74 หน้า. ISBN 974-17-5365-9

แพทย์แผนโบราณในหลายประเทศของทวีปเอเชียใช้ บัวบก (*Centella asiatica*) ในการรักษาโรคมานาน เอเชียติโคซัยด์เป็นส่วนประกอบสำคัญหนึ่งในบัวบกซึ่งมีศักยภาพในการใช้รักษา การศึกษานี้ออกแบบเพื่อทดสอบผลของเอเชียติโคซัยด์ต่อการตายของเซลล์ประสาทแกรนูลเพาะเลี้ยงอันเกิดจากสภาวะโพแทสเซียมต่ำ โดยใช้การดำรงชีวิตของเซลล์ (วัดโดย MTT reduction และ LDH release), lipid peroxidation และปริมาณ glutathione เป็นตัวชี้วัด และการทดลองทุกแบบใช้เซลล์ประสาทแกรนูลเพาะเลี้ยงอายุ 8 วัน เมื่อเซลล์ประสาทเพาะเลี้ยงสัมผัสกับเอเชียติโคซัยด์ความเข้มข้นสูง (200-400 μM) ด้วยระยะเวลาต่างๆ จนถึง 72 ชั่วโมง ปรากฏว่าสมรรถนะเมตาบอลิสม์ของไมโตคอนเดรียลดลง ในขณะที่เมื่อสัมผัสกับความเข้มข้นต่ำ (0.05-100 μM) สมรรถนะเมตาบอลิสม์ของไมโตคอนเดรียจะเพิ่มขึ้นจนถึง 48 ชั่วโมง และต่อมาลดลงเมื่อถึง 72 ชั่วโมง

หลังจากสลับเซลล์ประสาทแกรนูลเพาะเลี้ยงจาก medium ซึ่งผสมซีรัมและมีความเข้มข้น K^+ 25 mM (K^+ สูง) มายัง medium ซึ่งปราศจากซีรัมและมีความเข้มข้น K^+ 5 mM (K^+ ต่ำ) เป็นเวลา 24 ชั่วโมง ปรากฏว่าเซลล์ประสาทประมาณ 50% ไม่สามารถดำรงชีวิตอยู่ได้ นอกจากนั้นระดับ lipid peroxidation ในเซลล์เพิ่มขึ้นเป็นประมาณ 130% และปริมาณ glutathione ทั้งหมดลดลงเหลือเพียงประมาณ 65% ของเซลล์เพาะเลี้ยงกลุ่มควบคุม การสัมผัสกับเอเชียติโคซัยด์ (1-100 μM) พร้อมกับสภาวะ K^+ ต่ำ (5 mM) เป็นเวลา 24 ชั่วโมง ไม่มีผลต่อการตายของเซลล์ประสาทแกรนูลที่เกิดจากสภาวะ K^+ ต่ำ การสัมผัสล่วงหน้ากับเอเชียติโคซัยด์ (1-100 μM) เป็นเวลา 24 ชั่วโมง ก่อนสลับมาสัมผัสกับสภาวะ K^+ ต่ำ 24 ชั่วโมง ปรากฏว่ามีเพียงเอเชียติโคซัยด์ความเข้มข้น 1 μM เท่านั้นที่แสดงผลปกป้องเซลล์ประสาทอย่างมีนัยสำคัญ ในขณะที่การสัมผัสล่วงหน้ากับเอเชียติโคซัยด์ทุกความเข้มข้นดังกล่าวเป็นเวลา 48 ชั่วโมง ไม่แสดงผลปกป้องเซลล์ประสาทจากการตายด้วยสภาวะ K^+ ต่ำ การสัมผัสล่วงหน้ากับเอเชียติโคซัยด์ 1 μM เป็นเวลา 24 ชั่วโมง ก่อนสลับมาสัมผัสกับสภาวะ K^+ ต่ำ 24 ชั่วโมง ไม่ลดระดับ lipid peroxidation พื้นฐานของเซลล์ แต่สามารถป้องกันการเพิ่มขึ้นของ lipid peroxidation ที่เกิดจากสภาวะ K^+ ต่ำได้อย่างมีประสิทธิภาพ นอกจากนั้นการสัมผัสล่วงหน้ากับเอเชียติโคซัยด์ดังกล่าวไม่มีผลต่อปริมาณ glutathione พื้นฐาน และแสดงผลป้องกันการลดปริมาณ glutathione ที่เกิดจากสภาวะ K^+ ต่ำได้อย่างจำกัด

โดยสรุป เอเชียติโคซัยด์มีทั้งผลป้องกันเซลล์ประสาทและผลพิษต่อเซลล์ประสาทเมื่อทดสอบกับเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัม โดยขึ้นอยู่กับความเข้มข้นของเอเชียติโคซัยด์และระยะเวลาสัมผัส ผลป้องกันเซลล์ประสาทจะปรากฏชัดภายใต้สภาวะการสัมผัสที่จำเพาะเจาะจงเท่านั้น ได้แก่ การสัมผัสล่วงหน้ากับเอเชียติโคซัยด์ 1 μM เป็นเวลา 24 ชั่วโมง การสัมผัสอย่างจำเพาะดังกล่าวสามารถป้องกันการตายจากสภาวะ K^+ ต่ำที่เกิดกับเซลล์ประสาทแกรนูลเพาะเลี้ยง นอกจากนั้นยังมีผลกระตุ้นการทำงานของไมโตคอนเดรีย ลดระดับ lipid peroxidation ของเซลล์ และอาจเสริมสร้างปริมาณ glutathione ที่ลดลงได้บ้าง ในขณะที่เซลล์ประสาทสลับมาอยู่ในสภาวะ K^+ ต่ำ อย่างไรก็ตามกลไกการออกฤทธิ์ของผลทั้งสองแบบที่เกิดจากเอเชียติโคซัยด์ซึ่งมีต่อเซลล์ประสาทเพาะเลี้ยงยังไม่ทราบชัดในปัจจุบัน

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEYWORDS: ASIATICOSIDE / LOW POTASSIUM / CEREBELLAR GRANULE NEURONS / CELL DEATH

SRIRAT SUPAWANTANAKUL: EFFECTS OF ASIATICOSIDE ON LOW POTASSIUM MEDIUM-INDUCED CELL DEATH IN CULTURED RAT CEREBELLAR GRANULE NEURONS. THESIS ADVISOR: ASST. PROF. SURACHAI UNCHERN, Ph.D., 74 pp. ISBN 974-17-5365-9

Centella asiatica has been used in folk medicine in many Asian countries. Asiaticoside is one of major components found in this herbal plant with potential therapeutic uses. This study was designed to investigate effects of asiaticoside on low potassium medium-induced cell death in cultured rat cerebellar granule neurons by using cell viability (assessed by MTT reduction and LDH release), lipid peroxidation and content of glutathione, as the measuring endpoints. Eight-day cultured cerebellar granule neurons were used in all experiments. When cultured neurons were exposed to higher concentrations of asiaticoside (200-400 μM) for different time intervals up to 72 hr, mitochondrial metabolic activity was suppressed, but when exposed to lower concentrations (0.05-100 μM), mitochondrial metabolic activity was increased up to 48 hr and thereafter decreased at 72 hr.

After switching cultured cerebellar granule neurons from a serum-supplemented medium containing 25 mM K^+ (high K^+) to a serum-free medium containing 5 mM low K^+ (low K^+) for 24 hr, approximately 50% of neurons had loss their viability. In addition, cellular lipid peroxidation levels increased to approximately 130% and total glutathione content decreased to approximately 65% of control cultures. Coexposure of cerebellar granule neurons with asiaticoside (1-100 μM) and low potassium (5 mM) medium for 24 hr had no effect on low K^+-induced cell death. Preexposure of cerebellar granule neurons with asiaticoside (1-100 μM) for 24 hr and then shifting to low potassium medium for 24 hr revealed a significant neuroprotective effect only at 1 μM of asiaticoside while similar preexposure with asiaticoside for 48 hr failed to protect cultured neurons from low K^+-induced cell death at all concentrations used. Preexposure of cultured neurons with 1 μM asiaticoside for 24 hr in high K^+ medium did not decrease basal cellular lipid peroxidation levels but effectively prevented low K^+-induced increase in lipid peroxidation. Moreover, this preexposure had no effect on basal total glutathione content and displayed a marginal preventive effect on low K^+-induced decrease in total glutathione content.

In conclusion, asiaticoside by itself displayed both neuroprotective and neurotoxic effects on cultured cerebellar granule neurons, depending on the concentration and duration of exposure. Neuroprotective effect against low K^+-induced cell death is clearly evident only under specific conditions of exposure, namely, preexposure to 1 μM asiaticoside for 24 hr. This particular preexposure can prevent low potassium medium induced cell death in cultured rat cerebellar granule neurons, in conjunction with increasing mitochondrial function, decreasing cellular lipid peroxidation, and marginal restoring of glutathione diminution. However, the mechanism of action underlying biphasic effects of asiaticoside on cultured neurons is still unclear at the present time.

Department Pharmacology Student's signature.....….

Field of study Pharmacology Advisor's signature.....…..

Academic year 2003

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LIST OF ABBREVIATIONS

κ	= kappa
γ	= gamma
β	= beta
μM	= micromolar
°C	= degree celcius
μg	= microgram
μl	= microliter
BAD	= Bcl-2 antagonist of cell death
Bcl	= B-cell lymphoma
Ca^{2+}	= calcium ion
$CaCl_2$	= calcium chloride
CGC	= cerebellar granule cell
CNS	= central nervous system
DMEM	= Dulbecco's Modified Eagle's Medium
DMSO	= dimethyl sulfoxide
DNase	= deoxyribonuclease
DBPS	= Dulbecco's Phosphate Buffered Saline
DNA	= deoxyribonucleic acid
DTNB	= 5,5'-dithiobis (2-nitrobenzoic acid)
et al.	= et alii (and other)
FBS	= fetal bovine serum
GABA	= gamma aminobutyric acid
GSH	= glutathione
GSSG	= glutathione disulfide
HCl	= hydrochloric acid
HK	= high potassium
ICE	= interleukin 1β-converting enzyme

LIST OF ABBREVIATIONS (CONTINUED)

K^+	= potassium ion
KCl	= potassium chloride
L	= liter
LDH	= lactate dehydrogenase
LK	= low potassium
nm	= nanometer
MDA	= malondialdehyde
mg	= milligram
mM	= millimolar
MTT	= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADP	= nicotinamide adenine dinucleotide phosphate
NADPH	= nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	= sodium hydroxide
nmole	= nanomole
NMDA	= N-methyl-D-aspartate
PBS	= phosphate buffered saline
PI-3	= phosphatidylinositol-3
PNS	= peripheral nervous system
PTZ	= pentylenetetrazole
RNA	= ribonucleic acid
ROS	= reactive oxygen species
SEM	= standard error of mean
SOD	= superoxide dismutase
TBA	= thiobarbituric acid
TBARS	= thiobarbituric acid reactive substance
TECA	= titrated extract of *Centella asiatica*
TNB	= 5-thio-2-nitrobenzoate

CHAPTER I

INTRODUCTION

During the development of the vertebrate nervous system, about half of embryonic neurons that are produced ultimately die by process of naturally occurring or programmed cell death (Johnson, and Deckworth, 1988; Oppenheim, 1991). Programmed cell death is an essential regulator of normal development and homeostasis of the central nervous system (Steller, 1995; Jacobson et al., 1997). The time of cell death (apoptosis) is limited to the period of formation of synapses with the target cells, and the neurons which fail to obtain sufficient amounts of trophic factors released from the target cells are eliminated. This selection system is considered to be a mechanism to ensure formation of a physiologically relevant neuronal network. Mature neurons which correctly execute their functions, however, undergo apoptosis in response to exogenous toxic stimuli. Such stimuli may be responsible for neurodegenerative diseases (Shimoke et al., 1999).

Among the most common neurological diseases are neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). As the elderly population increases, the prevalence of these age-related diseases is likely to increase. Thus, among few risk factors that have been identified for these diseases, increased age is the only one that is common to AD, PD, and ALS. Inappropriate apoptosis has been suggested to be involved in neuronal death which occurs in various human neurodegenerative disorders, including AD, PD, ALS, and Huntington's disease (Nijhawan et al., 2000; Sastry and Rao, 2000).

The mammalian nervous system is critically dependent on trophic supports for proper development and survival (Levi-Montalcini, 1987; Barde, 1989). Among the molecules shown to influence neuronal differentiation and survival are the neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 and 4 (NT3 and NT4), fibroblast growth factors (FGFs), ciliary neurotrophic factor (CNTF), insulin, and insulin-like growth factors (IGFs) (Knusel et

al., 1990; Maisonpierre et al., 1990). In culture, however, neuronal survival can also be supported by variety of agents in the absence of any neurotrophic factors. One of these agents is K^+. High levels of K^+ promote survival of several types of cultured neurons (Gallo et al., 1990; Kaike et al., 1989; Franklin and Johnson, 1992). Besides supporting neuronal survival, high K^+ has also been suggested to influence neuronal development and phenotypic characteristics (Chalazonitis and Fishbach, 1980; Ishida and Deguchi, 1983; Resink et al., 1992). Although the ability of high K^+ to promote neuronal survival is well established, the mechanism by which it acts is unclear.

Among various cell types known to be dependent on high K^+ for survival *in vitro* are cerebellar granule neurons (Gallo et al., 1990; Lasher and Zaigon, 1972; Levi et al., 1984). Primary cultures of cerebellar granule neurons have been proposed as a suitable *in vitro* model for studying the mechanisms of neuronal apoptosis (D'Mello et al., 1993; Galli et al., 1995). This neuronal population, cultured in 25 mM KCl-containing medium, develops typical *in vitro* features of mature CGCs *in vivo*, such as an extensive neuritic network, expression of excitatory amino acid receptors, and production and release of glutamate (Burgone et al., 1993). When deprived of depolarizing levels (25 mM) extracellular K^+, CGCs undergo apoptosis characterized by chromatin condensation, pyknosis, and internucleosomal DNA fragmentation (D'Mello et al.,1993; Galli et al., 1995). Production of reactive oxygen species (ROS) is thought to represent a relevant mechanism in the series of biochemical events ultimately leading to apoptosis (Hockenbery et al., 1993; Greenlund et al., 1995; Schulz et al., 1996; Alavez et al., 2003). Consistent with this hypothesis is the finding that various antioxidant compounds block apoptosis induced by low K^+ concentrations (5 mM) and glutamate in cultured cerebellar granule cells (Schulz et al., 1996; Atabay et al., 1996; Ciani et al., 2000; Bisaglia et al., 2000; Valencia and Moran, 2001; Amodio et al., 2003). Therefore, elucidation of the mechanism underlying the anti-apoptotic activity of antioxidants may lead to the development of neuroprotective treatment.

Antioxidant properties of natural compounds extracted from dietary or herbal plants have aroused much attention. Asiaticoside is a triterpene ester glycoside isolated from *Centella asiatica* (Linn.) Urban, family Apiaceae, a herbal plant that has been known as Gotu kola and pennywort. In the Indian system of traditional medicine, Ayurveda, *Centella asiatica* (CA) has been used in various parts of India for different ailments like headache, body aches, insomnia, asthma, leprosy, ulcers, eczema and wound healing (Chatterjee et al., 1992; Shukla et al., 1999; Suguna et al., 1996). Its wound healing effects may be due to its up regulation of human collagen I expression (Bonte et al., 1997) and an increase in tensile strength of the wounds (Suguna et al., 1996). Methanol extract of CA showed an inhibitory effect on lipid peroxidation in biological membranes (Kumar and Muller, 1999). Asiaticoside has been reported to increase enzyme and non-enzymatic antioxidant (superoxide dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid in newly synthesized tissue and decrease lipid peroxide content (Shukla et al., 1999). In the course of pharmacological studies, the plant showed CNS depressant activity (Sakina and Dandiya, 1990), antitumor activity (Babu et al., 1995) and an inhibitory effect on the biosynthetic activity of fibroblast cells (Veechai et al., 1984). The whole plant of CA has been shown to be beneficial in improving memory (Mukerji, 1953; Vaidyaratnam, 1994) and is also reported to improve the general mental ability of mentally retarded children (Appa rao et al., 1973; Kakkar, 1990). In addition, fresh leaf juice of CA has been showed to improve passive avoidance task in rats (Nalini, 1992). An earlier study demonstrated that the aqueous extract of CA has cognition-enhancing properties with an associated decrease in the brain oxidative stress parameters of the normal rats (Veerendra et al., 2002). One supporting study has been reported that CA significantly prevented the cognitive impairment and attenuated the oxidative stress induced by PTZ kindling. CA induced a decrease in MDA levels and an increase in glutathione levels that may be due to its antioxidant property (Gupta et al., 2003).

From the reported evidence for potential protective activities of asiaticoside, it is quite considerable that it may be useful in preventing neuronal damages in various neurodegenerative disorders. Therefore, the main purpose of this study was to investigate beneficial effects of asiaticoside on low potassium-induced cell death in

primary cultured rat cerebellar granule neurons, a well established *in vitro* model for neurodegeneration, by using biochemical analyses as follows:

1. MTT reduction assay – an indication of cell metabolic activity, especially mitochondrial dehydrogenase activity.

2. Lactate dehydrogenase (LDH) release assays – an indication of cell membrane damage and cell death.

3. Thiobarbituric acid reactive substance (TBARS) assay – an indication of lipid peroxidation-induced oxidative stress.

4. Reduced plus oxidized glutathione (total glutathione) assay – an indication of cellular antioxidant capacity.

CHAPTER II

LITERATURE REVIEW

The cerebellum

Cerebellum is located at the lower back of the brain, beneath occipital lobes, lies behind the pons and connects to the brainstem by fiber tracts called “peduncles”(Figure 1). Cerebellum is concerned with balance and receives a profuse input from the vestibules. Cerebellum is also very important in coordinating the movements, particularly, of fingers, where some of the finest of all movements in the body are executed. Cerebellum influences motor systems by evaluating disparities between intention and action and by adjusting the operation of motor centers in the cortex and brain stem while a movement is in progress as well as during repetitions of the same movement (Kandel et al., 2000). It has been suggested that cerebellum has the function of calculating the difference between position of the limb and the target, and provides a signal related to this ‘error’, or the amount of movements still needed. Cerebellum sends this information up to the movement generators in the motor region of cerebral cortex for limb movement, to the vestibular nuclei for eye movements, etc., and changes their gain appropriately. Cerebellum is thus acting as a calibrating center and also involved in learning of skilled movements such as writing and playing the violin (Holmes, 1993)

Removal of cerebellum does not alter sensory thresholds or the strength of muscle contraction. Thus cerebellum is not necessary to basic elements of perception or movement. Rather, damage to cerebellum disrupts the spatial accuracy and temporal coordination of movements. It impairs balance and reduces muscle tone. It also markedly impairs motor learning and certain cognitive functions.

Cerebellum constitutes only 10% of total volume of the brain but contains more than half of all its neurons. These neurons are arranged in a highly regular manner as repeating units, each of which is a basic circuit module (Kandel et al., 2000).

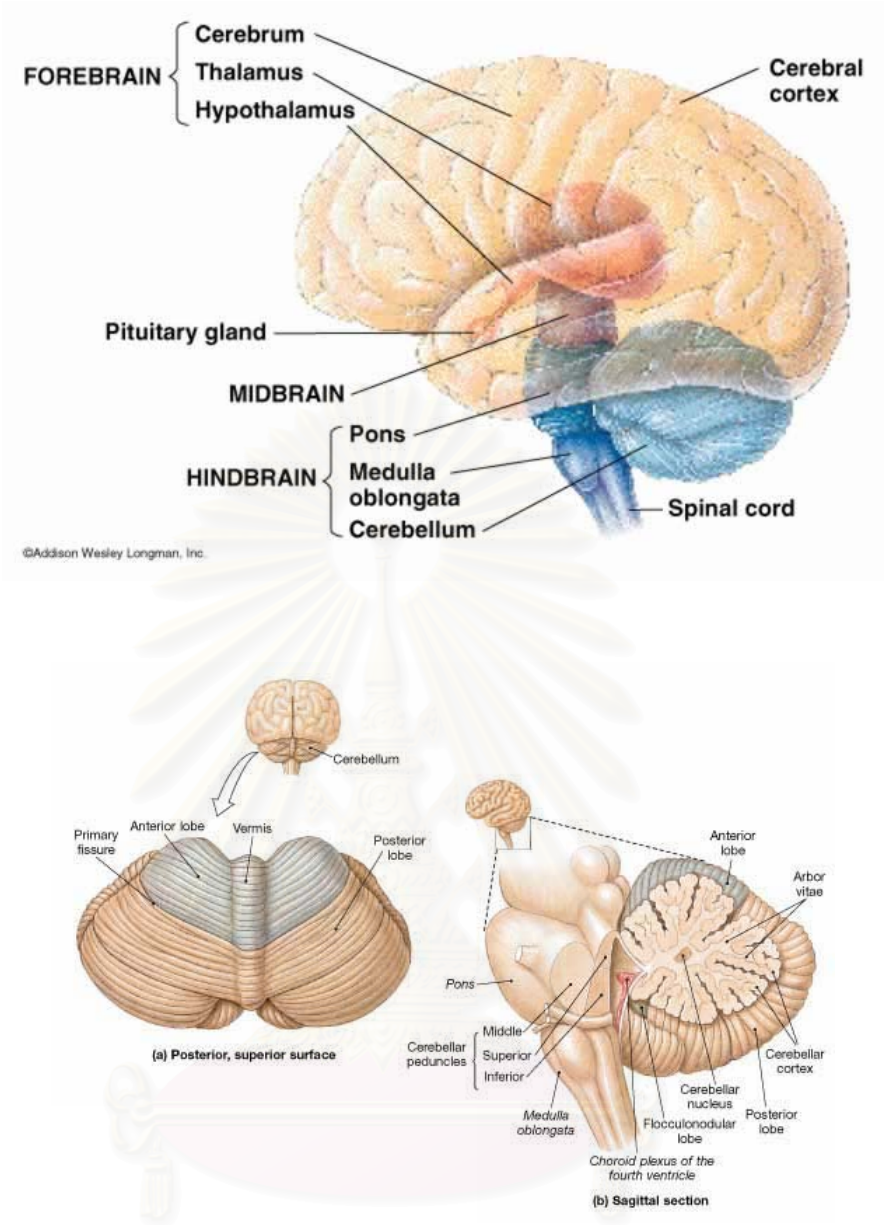

Figure 1 The cerebellum (Human anatomy and Physiology, n.d.)

Neurons in the cerebellar cortex

Neurons in cerebellar cortex are organized into three layers, the outermost or molecular layer of cerebellar cortex contains cell bodies of two types of inhibitory interneurons, the stellate and basket cells, dispersed among excitatory axons of granule cells and dendrites of inhibitory Purkinje cells, whose cell bodies lie in deeper layer (Figure 2). The axons of granule cells in this layer run parallel to the long axis of folia and therefore are called ‘parallel fibers’. Dendrites of Purkinje neurons are oriented perpendicular to these axons.

Beneath the molecular layer is the Purkinje cell layer, consisting of a single layer of Purkinje cell bodies. Purkinje neurons have large cell bodies (50-80 μM) and fan-like dendritic arborizations that extend upward into the molecular layer. Their axons project into the underlying white matter to the deep cerebellar or vestibular nuclei and provide the output of cerebellar cortex. This output is entirely inhibitory and mediated by a neurotransmitter γ-aminobutyric acid (GABA)

The innermost or granular layer contains a vast number of granule cells and few larger Golgi interneurons. Mossy fibers, the major source of afferent input to cerebellum, terminate in this layer. The bulbous terminals of mossy fibers contact granule cells and Golgi neurons in synaptic complexes called cerebellar glomeruli (Figure 2)

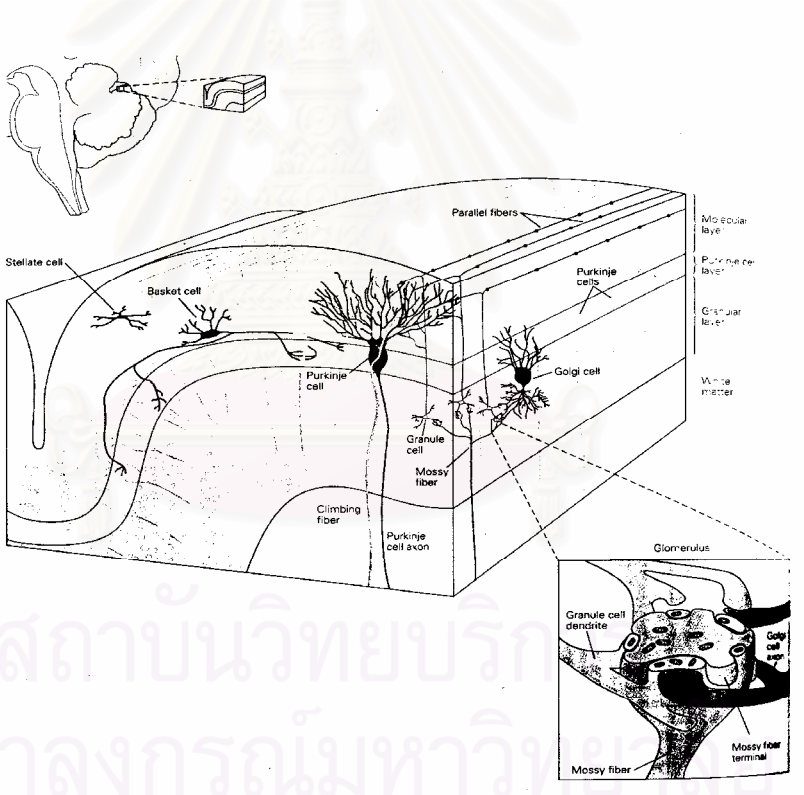

Figure 2 Cerebellar cortex is organized into three layers and contains five types of neurons. A vertical section of a single cerebellar folium, in both longitudinal and transverse planes, illustrates the general organization of cerebellar cortex. The detail of a cerebellar glomerulus in the granule layer is also shown. Glomerulus is a clear space where the bulbous terminal of a mossy fiber makes synaptic contact with Golgi and granule cells (Kandel et al., 2000).

The basic circuit of cerebellum is illustrated in Figure 3, which shows the excitatory and inhibitory connections between different cell types.

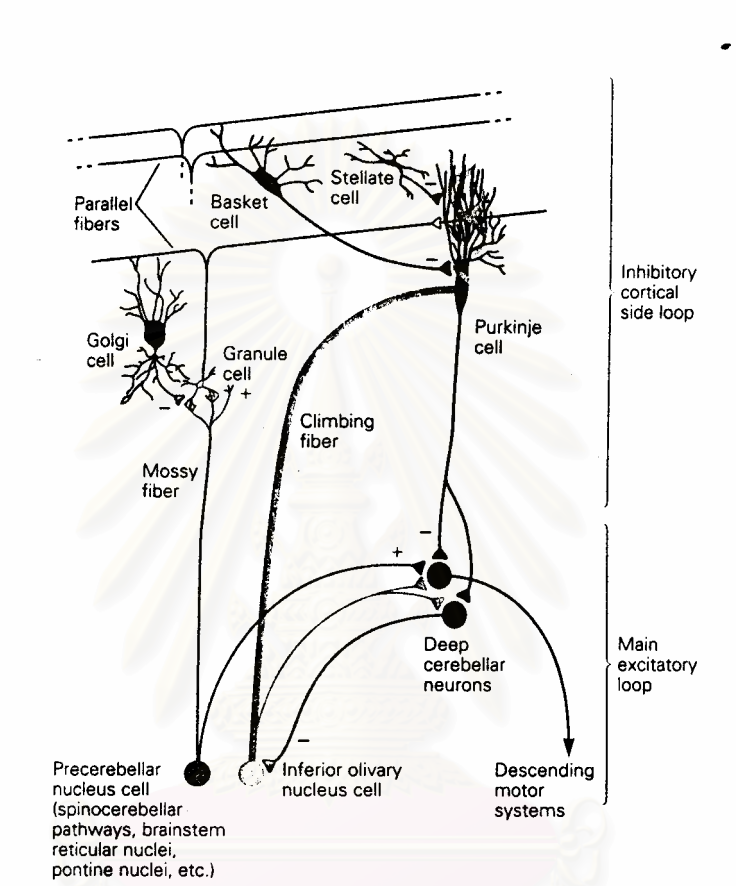

Figure 3 Synaptic organization of the basic cerebellar circuit module. Mossy and climbing fibers convey output from cerebellum via a main excitatory loop through the deep nuclei. This loop is modulated by an inhibitory side-loop passing through the cerebellar cortex. This figure shows the excitatory (+) and inhibitory (-) connections among the cell types (Kandel et al., 2000).

Granule cells

These cells are very small in comparison to Purkinje cells, but far more numerous. It is estimated that about 2×10^9 granule cells (so called because they appear as small and densely packed darkly stained nuclei in histological sections) exist within cerebellum, located in the deepest cortical layer of cerebellum called the

granular layer. Granule cells have only three to seven dendrites, receiving signals from the brain stem and cerebral cortex. Axons of granule cells course vertically through the Purkinje layer and arbor many branches thus establishing contact with 200 to 500 Purkinje cells. Each Purkinje cell receives approximately 150,000 synaptic inputs from the granule layer.

Centella asiatica

Centella asiatica (Gotu kola, pennywort) is a perennial herbaceous creeper of the *Apiaceae* family. The stem is glabrous, pink and striated, rooting at the nodes. The leaves are fleshy, orbicular-reniform, crenate, base cordate and often lobed and long-petioled. The flowers are red, pink or white, in fascicled umbels. The fruits are oblong, dull brown, laterally compressed; the pericarp hard, thickened, woody and white. (Figure 4)

Figure 4 *Centella asiatica* (http://www.fitomedicina.it/images/centella.GIF)

Centella is an important herb in Ayurvedic medicine. Traditionally, it is widely used as a blood purifier as well as for treating a variety of other illnesses. It was believed that the leaves promoted longevity. In Ayurveda, Gotu kola is one of the chief herbs for revitalizing the nerves and brain cells. The use of *Centella asiatica* in the treatment of dermatological conditions has a long tradition in its native areas, such as India and Sri Lanka, where it is used for quicker healing of small wounds, chap and scratches, as a treatment for surgical wounds. In India and Madagascar, this plant was used to treat leprosy, while the Chinese prescribed leaves in curing leukorrhea and

toxic fever. In Malaysia, it is also said to have beneficial effects in improving memory and in treating mental fatigue, anxiety, and eczema (Abdul Hamid et al., 2002).

The major principles in *Centella asiatica* are triterpene saponins, asiaticoside and madecassoside, and their aglycones, asiatic acid and madecassic acid, respectively. It also contains essential oils and other volatile constituents. The plant contains other saponins and triterpene acids, namely, brahmoside, brahminoside, brahmic acid, isobrahmic acid, betulic acid, centelloside, cetillic acid, etc. (Asakawa et al., 1982; Singh et al., 1969)

Asiaticoside is the most essential ingredient and founded to be the most content extracted from *Centella asiatica* (the structure showed in Figure 5).

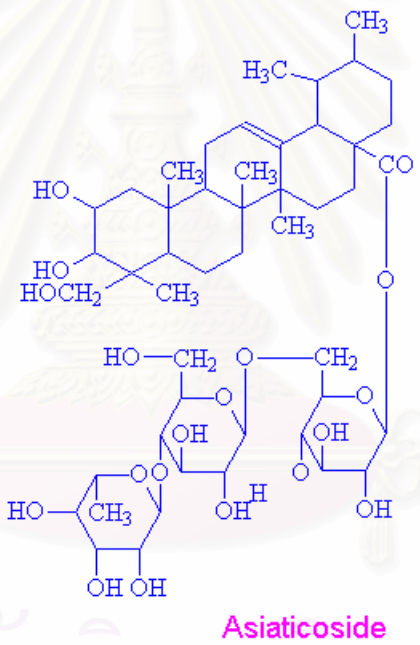

Figure 5 The structure of asiaticoside (Minsun et al.,1999)

Asiaticoside

Molecular Formula	$C_{48}H_{78}O_{19}$
Molecular Weight	959.13
Melting point	235-237^{o}C

Pharmacological activities of *Centella asiatica* and asiaticoside

Anti-inflammatory

Asiaticoside was reported to reduce acute skin reactions in radiotherapy treated rat (Chen et al., 1999).

Antimicrobial

Asiaticoside is reported to be active against *Mycobacterium tuberculosis*, *Bacillus leprae* and *Entamoeba histolytica* (Oliver-Bever, 1986).

Antioxidant

Topical administration of asiaticoside (0.2% solution) twice daily for 7 days to skin wounds in rats induced significant increases in both enzymatic and non-enzymatic antioxidants, namely superoxide dismutase (35%), catalase (67%), glutathione peroxidase (49%), vitamin E (77%) and ascorbic acid (36%) in newly formed tissue. It also resulted in a several fold decrease in lipid peroxide level (69%) as measured in terms of thiobarbituric acid reactive substance (TBARS). The level of antioxidant activity was highest during the initial stage of treatment (Shukla et al., 1999).

Antitumor

Researchers at Amala Cancer Research Center in Kerala, India, tested both a crude extract of *Centella asiatica* (CE) and its partially purified fraction (AF) for their antitumor activity. AF significantly inhibited the proliferation of transformed cell lines in Ehrlich's ascites tumor cells and Dalton's lymphoma ascites tumor cells with no toxic effects on normal human lymphocytes. AF was also found to inhibit the development of solid and ascites tumors, and increased the life span of tumor bearing mice. "Tritriated" thymidine, uridine and leucine incorporation assay suggested that the fraction acts directly on DNA synthesis". (Babu et al., 1995).

Antiulcer

An extract of *Centella asiatica* significantly inhibited gastric ulceration induced by cold and restraint stress in Charles-Foster rats (Chatterjee et al., 1992). The antiulcer activity was compared to famotidine (H_2-receptor antagonist) and sodium valproate (antiepileptic). Both drugs and plant extract showed a dose dependent reduction of gastric ulceration, which, except for the antiulcer effect of famotidine, could be reversed with bicucullin methiodide (specific $GABA_A$ receptor antagonist). It seems that the plant extract, which increased GABA levels in the brain, protected the rats against the cold restraint ulceration.

Autoimmune

The usage of asiaticoside in tablet, ointment and powdered form, was found to be efficacious in the treatment of chronic or subchronic system scleroderma with limited skin involvement, and in progressive and/or advanced focal scleroderma (Guseva et al.,1998)

Cardiovascular

A three-week treatment of a triterpene fraction of *Centella asiatica* in clients with postphlebitic syndrome significantly reduced the number of circulating endothelial cells, as compared to normal subjects (Montecchio et al., 1991). The use of an oral extract of *Centalla asiatica* in a randomized controlled trial of 87 clients with chronic venous hypertensive microangiopathy was found efficacious, without side effects (Cesarone et al., 1994). A clinical trial of an extract of *Centella asiatica* found that it was efficacious in the treatment of venous insufficiency, reducing ankle edema and foot swelling, and improving capillary filtration rate and microcirculatory parameters (Cesarone et al., 1992).

Central Nervous system

Asiaticoside derivatives were found to inhibit or reduce H_2O_2-induced cell death and lower intracellular free radical concentrations, protecting against effects of beta-amyloid neurotoxicity (Mook-Jung et al., 1999). A two compartment passive avoidance task test (with rats) showed an improvement in 24 hr retention. Assessment of the turnover of biogenic amines (norepinephrine, dopamine, and serotonin) showed

significant reductions of these amines and their metabolites in the brain following oral administration of a fresh juice (1 ml = 0.38 g fresh leaves), at a dose of 0.18 g/kg for 15 days. The decrease of amine levels was correlated to improved learning and memory in rats. An earlier study demonstrated that the aqueous extract of CA has cognition-enhancing properties with an associated decrease in the brain oxidative stress parameters (MDA) with simultaneous significant increase in levels of glutathione of the normal rats (Veerendra et al., 2002). A water-soluble fraction of *Centella asiatica* was found to have an anxiolytic effect in animals comparable to diazepam (Chatterjee et al, 1992). In a study upon effects of *Centella asiatica* on mentally-challenged children, half were given 500 mg tablets of dried *Centella asiatica* (whole plant), and half placebo. Intelligence quotient tests were conducted at the outset of the study and again after three month. Results indicated that children who took *Centella asiatica* tablets showed significant improvements in co-operation, memory, concentration, attention, vocabulary and social adjustment (Appa-Rao, 1973). Intraperitoneal injections of brahmoside and brahminoside were found to have CNS-depressant effect in mice and rats (Ramaswamy et al, 1970). Six-week treatment in patients of anxiety neurosis reduced anxiety levels and showed improvement in the mental fatigue rate and immediate memory span.

Hepatitis

Researchers found improvement in 5 of 12 clients with chronic hepatic disorders, treated with a titrated extract of *Centella asiatica* (Darnis et al, 1979).

Wound-healing

Titrated extract of *Centella asiatica* (TECA), consisting of a mixture of three triterpenes (asiatic acid, madecassic acid and asiaticoside) stimulated collagen and glycosaminoglycan synthesis in rats surgically inserted with stainless-steel wound chambers (Maquart et al, 1999). The administration of a topical solution of asiaticoside (0.2%) in guinea-pig punch wounds produced a 56% increase in hydroxyproline, a 57% increase in tensile strength, increased collagen content and better epithelialization than controls. A similar increase was also found with the oral administration of asiaticoside (91 mg/kg) in guinea pigs with punch wounds. The topical administration of a 0.4% solution of asiaticoside in punch wounds of

streptozocin diabetic rats, in which healing is delayed, increased hydroxyproline content, tensile strength, collagen content and epithelialization (Shukla et al, 1999). An alcoholic extract of *Centella asiatica* increased cellular proliferation and collagen synthesis in rat dermal wounds, with better cross-linking of collagen and faster epithelialization, as compared to control wounds (Suguna et al., 1996). In the treatment of keloids, asiaticoside compared favorably with compression bandaging and provided more lasting results than intralesional cortisone or radiation therapy (Bosse et al., 1979).

Antiepilepsy

The administration of aqueous extract of *Centella asiatica* (300 mg/kg orally) decreased the PTZ-kindled seizures and showed improvement of behavior due to its antioxidant property which decreased MDA levels and increased glutathione levels (Gupta et al., 2003).

Potassium deprivation-induced cell death

In culture, survival of several neuronal types can be maintained in the absence of neurotrophic factor, if depolarizing amounts of extracellular potassium are provided. The mechanism by which elevated K^+ supports neuronal survival is believed to similar to those activated by neuronal activity during embryogenesis (Collins and Lile, 1989; Collins et al., 1991; Gallo et al., 1987). Chronic depolarization of neuronal cultures by elevated K^+ therefore serves as a convenient model system to investigate the mechanism by which electrical activity maintains survival *in vivo*. Electrophysiological experiments have shown that membrane depolarization by elevated K^+ results in a sustained rise in the level of intracellular free calcium, $[Ca^{2+}]_i$ due to an efflux through dihydropyridine-sensitive type Ca^{2+} channels which is necessary for high K^+ (HK)-mediated survival (Collins and Lile, 1989; Collins et al., 1991; Gallo et al., 1987).

When cerebellar granule neurons obtained from postnatal rats are cultured in medium containing serum and depolarizing level of K^+ (25 mM), they can survive and mature *in vitro*. After culture and maturation in HK medium, lowering the K^+ concentration (5 mM) (LK) in the medium without serum induced death of cultured

granule neurons. This LK-induced cell death occurs via apoptosis, as demonstrated by the typical hallmarks of apoptosis such as membrane blebbing, nuclear and cytoplasmic condensation, and DNA fragmentation (D'Mello et al., 1993; Galli et al., 1995; Schulz et al., 1996). An alteration of reactive oxygen species (ROS) metabolism and cellular redox state have also been implicated as mediators of CGC apoptotic death (Simonian and Coyle, 1996; Atlante et al., 1998). A sequential requirement for new mRNA formation, protein synthesis, caspase activation, and finally ROS overproduction during CGC apoptosis has been demonstrated (Schulz et al., 1996). Free radicals can act either as effectors of DNA, lipid, and protein damage or as signaling molecules via redox-sensitive cellular factors, such as nuclear factor-κB (NF-κB) (Bredesen, 1995). Other events during potassium deprivation-induced apoptosis include synthesis and phosphorylation of c-Jun (Watson et al., 1998), inactivation of phosphatidylinositol-3 (PI-3) and Akt kinase (D'Mello et al., 1997; Shimoke et al., 1997; Skaper et al., 1998) and BAD dephosphorylation (Gleichmann et al., 2000). The pathways that potassium withdrawal induced cell death may be summarized as shown in Figure 6.

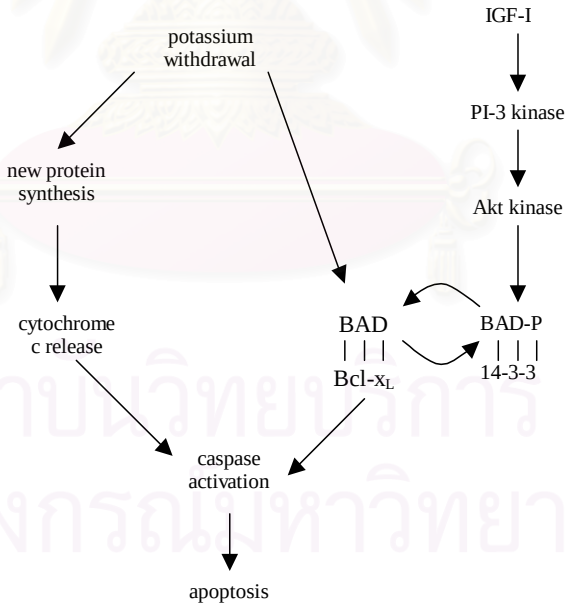

Figure 6 Pathways leading to apoptosis of cerebellar granule neurons after potassium withdrawal. Interfering with either the protein synthesis dependent- (left side) or the posttranslational, PI-3 kinase-dependent pathway (right side) blocks apoptosis (Gleichmann et al., 2000)

CHAPTER III

MATERIALS AND METHOD

Materials

1. Experimental Animals

Postnatal day 8, Wistar rat pups (both sexes), were used in this study. They were obtained from time-pregnant mother rats of embryonic day 18 purchased from the National Laboratory Animal Center, Nakornpathom, Thailand. All pregnant rats were housed in stainless-steel cages at Faculty of Pharmaceutical Sciences, Chulalongkorn University, until delivery and then rat pups were kept with their mother until 8-day old. They were freely accessed to food pellets (C.P. rat fed, Pokaphand Animal Fed Co. Ltd., Bangkok, Thailand) and tap water *ad libitum*.

2. Chemicals

The following chemicals were used in the experiments:

Asiaticoside (kindly provided by Ms. Suvipha Sermboonsang, International Graduate Program in Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

Butylated hydroxytoluene [Sigma]

Cytosine arabinoside (Ara-C) [Sigma]

Cytotoxicity Assay Kit [Sigma]

Dimethyl Sulfoxide (DMSO) [BDH]

5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [Sigma]

DNase I [Sigma]

Dulbecco's Modified Eagle's Medium (DMEM) [Sigma]

Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM [Sigma]

Dulbecco's Modified Eagle's Medium (DMEM) without phenol red [Sigma]

Dulbecco's Phosphate Buffered Saline (DPBS) without $CaCl_2$ [Sigma]

Equin Serum [Hyclone]

Ethanol [Merck]

Fetal Bovine Serum [Hyclone]

D-glucose, monohydrate [Unilab]

Glutathione, reduced form [Sigma]

Glutathione reductase [Sigma]

HEPES [Sigma]

Hydrochloric acid [Merck]

Insulin [Sigma]

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) [Sigma]

β-Nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) [Sigma]

Penicillin G [Sigma]

Phosphotungstic acid [Sigma]

Potassium chloride [Ajax chemicals]

Poly-D-Lysine Hydrobromide [Sigma]

Progesterone [Sigma]

Putrescine [Sigma]

Pyruvic acid sodium salt [Fluka]

Sodium dodecyl sulfate [Sigma]

Sodium hydrogencarbonate [Ajax chemicals]

Sodium hydroxide [Sigma]

Sodium phosphate, dibasic anhydrous [Sigma]

Sodium phosphate, dibasic heptahydrate [Sigma]

Sodium phosphate, monobasic anhydrous [Sigma]

Sodium selenite [Sigma]

Streptomycin [Sigma]

Sulfosalicylic acid [Sigma]

Sulfuric acid [BDH]

Thiobarbituric acid [BDH]

Transferrin [Sigma]

0.04% Trypan Blue [Sigma]

3. Instruments

The following instruments were used in the experiments:

Adjustable pipette: 10-100 μl [Nichiyo]

Adjustable pipette: 200-1000 μl, 1-5 ,ml [Lab systems]

Aluminum Foil [Tops]

Bunsen burner

Carbon dioxide incubator [Forma Scientific]

Cell culture dish: diameter 35 mm, diameter 100 mm [Nunc]

24-well cell culture plate [Nunc]

96-well cell culture plate [Nunc]

Cell strainer: 40 μm Nylon [Becton Dickinson]

Centrifuge [Kokusan]

Conical tube: 15 ml, 50 ml [Nunc]

Disposable glass Pasteur pipettes: 230 mm [Volac]

Fluorescence spectrophotometer FS 777 [Jasco]

Glass bottle: 100 ml, 500 ml, 1000 ml [Schott Duran]

Hemocytometer (Depth 0.100 mm) [Improved Neubauer]

Inverted microscope, Axiovent 135 [Zeiss]

Latex Free Syringe: 10 ml [Becton Dickinson]

Laminar air flow hood [Hepaco]

96-well microtiter plate [Nunc]

Microliter pipette: 10-100 μl, 200-1000 μl, 1-5 ml [Labsystems]

Microplate reader [Biorad model 3550]

pH meter [Beckman Instruments]

Pipettes tip: 1-200 μl, 200-1000 μl, 1-5 ml [Labsystems]

Sonicator [Elma]

Sterile Millex-GV (0.22 μm filter unit) [Millipore]

Sterivex-GS (0.22 μm filter unit with filling bell) [Millipore]

Surgical equipments

Syringe filter holder: 13 mm [Satorius]

Vortex mixer [Clay adams]

Water distiller

Water bath

Experimental methods

1. Preparation of culture media, buffers, and solutions

All culture media were obtained from commercial sources in powder form. They were prepared by dissolving ingredients in non-pyrogenic distilled water followed by filter sterilization through a 0.22 μm membrane filter. They were kept at 4 °C until used.

Dulbecco's modified Eagle's medium (DMEM, high glucose) was used in culturing cerebellar granule neurons. The medium was supplemented with 99 μg/ml sodium pyruvate, 3.7 mg/ml sodium bicarbonate, 54 μg/ml penicillin G sodium and 90 μg/ml streptomycin sulfate. Where indicated, 10 % (v/v) fetal bovine serum (FBS) was added. Serum-free DMEM was composed of normal DMEM without phenol red supplemented with N-2 supplement. The supplement was used for the survival and expression of post-mitotic neurons in primary cultures from both the peripheral

nervous system (PNS) and the central nervous system (CNS). It contained 30 μg/ml transferrin, 5 μg/ml insulin, 100 μM putrescin, 20 nM progesterone and 30 nM sodium selenite. The media was adjusted to pH 7.3 using 1 N NaOH and 1 N HCl.

Dulbecco's phosphate buffered saline (without $CaCl_2$) (DPBS) was purchased from Sigma. It was used for the preparation of cerebellar granule cell culture and 0.5% Triton-X 100 solution. For 1 liter preparation, it consisted of $MgCl.6H_2O$ 0.10 g, KCl 0.20 g, KH_2PO_4 0.20 g, NaCl 8.00 g and Na_2HPO_4 1.150 g. The solution was adjusted to pH 7.4 using 1 N NaOH and 1 N HCl.

Hank's balanced salt solution was used for the preparation of 0.25% trypsin and 0.01% Dnase I. For 1 liter preparation, it contained KCl 0.40 g, KH_2PO_4 0.06 g, $MgCl_2.H_2O$ 0.10 g, $MgSO_4.7H_2O$ 0.10 g NaCl 8.00 g, $NaHCO_3$ 0.35 g and $Na_2HPO_4.7H_2O$ 0.09 g, adjusted to pH 7.4 using 1 N NaOH and 1 N HCl.

0.1 M sodium phosphate buffer was used for dissolving 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB). It contained 94.7 ml of 0.2 M sodium phosphate dibasic anhydrous and 5.3 ml of 0.2 M sodium phosphate monobasic anhydrous. The solution was adjusted to 200 ml by distilled water and adjusted to pH 7.4 using 1 N NaOH and 1 N HCl.

2. Cerebellar granule neurons cultures (Figure 7)

Cultures of cerebellar granule cells were prepared from pooled cerebella of 8-day old Wistar rat pups, a time when many of granule neurons were still at an early post-mitotic stage of differentiation. Postnatal rats were decapitated under ether anesthesia by a pair of scissors. Cerebellar were removed aseptically from the brain. After removal of the meninges, the tissue was cut into cubes of about 0.4 mm side dimension, and incubated for 20 min at 37 °C with 0.25% trypsin and 0.01% Dnase I in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution. The incubation was terminated by the addition of horse serum and tissue fragments were centrifuged at 3,000 rpm for 10 min. The tissue pellet was gently rinsed and resuspended by gentle passing the suspension through a 10-ml plastic pipette tip and then a 5-ml plastic pipette tip. The cell suspension was filtered through two sheets of nylon net (50 μm-mesh) to remove cell lumps, and was further diluted as appropriate with high K^+ (25 mM KCl) DMEM

containing 10% FBS and plated on poly-D-lysine (100 μg/ml) coated plates at density of 1×10^6 cells/cm^2 in 24-well culture plates. The cultures were grown in a humidified 5% CO_2-95% air atmosphere at 37 °C. At 18-24 hr after plating, cytosine arabinoside was added to the medium to a final concentration of 10 μM; this curtails the number of astrocytes that develop in the cultures. Two-third volume of medium in each well was replaced with fresh medium on day 4 *in vitro*. Cultures were used on day 8 at which the medium was changed to serum free high K^+ DMEM without phenol red containing N-2 supplement plus desired concentrations of asiaticoside or serum free low K^+ DMEM plus desired concentrations of asiaticoside.

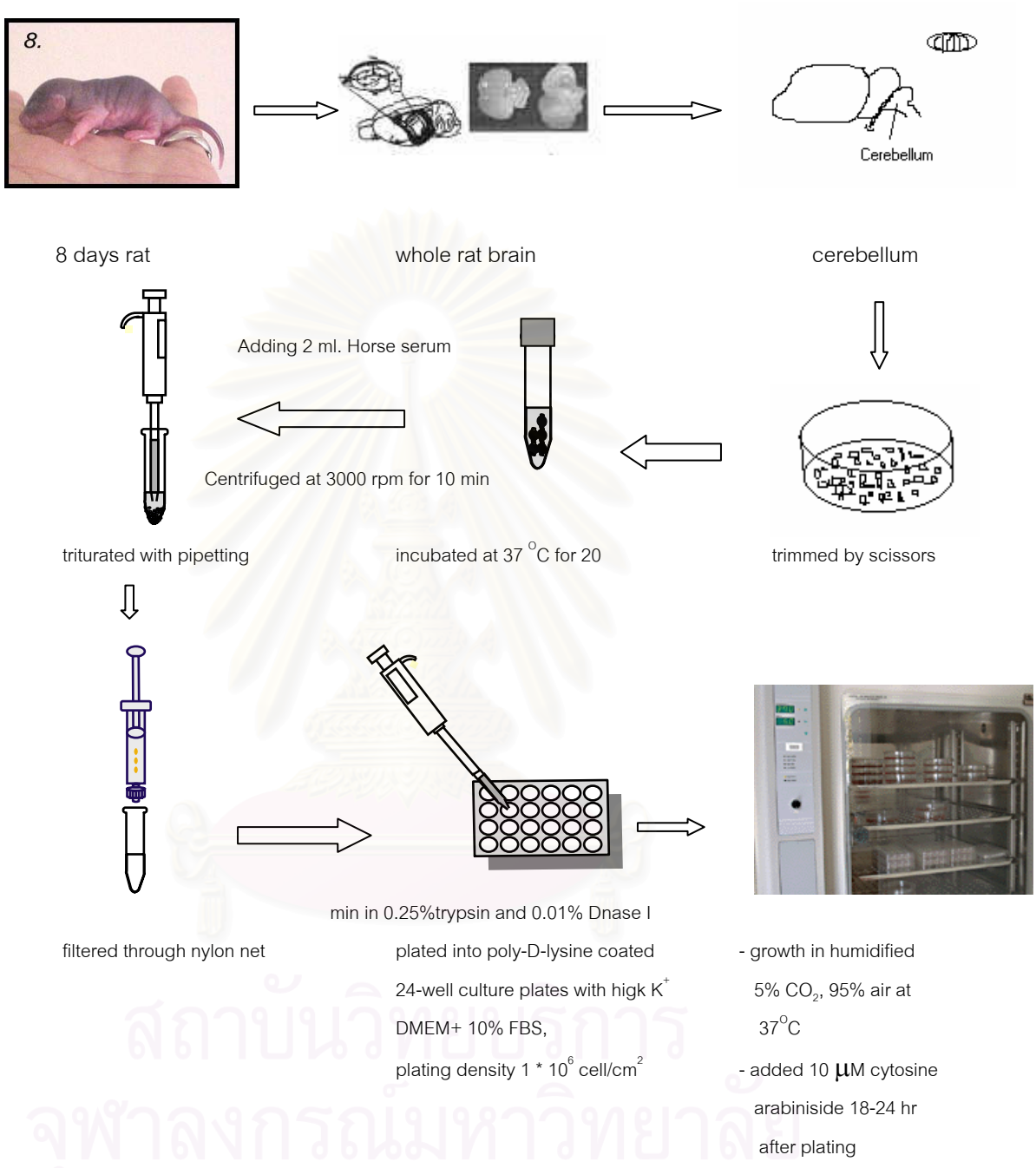

Figure 7 Preparation of cerebellar granule neuron cultures.

3. MTT reduction assay

The MTT assay is widely used in cell proliferation and cytotoxicity assays. The colorimetric MTT assay based on the reduction of yellow-colored 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into a purple insoluble formazan reduction products in living cells but not in dead cells or their lytic debris (Figure 8) (Mosmann, 1983). The MTT assay is though to measure the ability of MTT to be reduced by electrons flowing through the mitochondrial electron transport chain and therefore reflects early redox changes within the cell. MTT is also reduced by xanthine oxidase (Behl et al., 1994). The intracellular purple formazan, solubilized in dimethyl sulfoxide, is easily measured with microplate reader (Skaper et al., 1998).

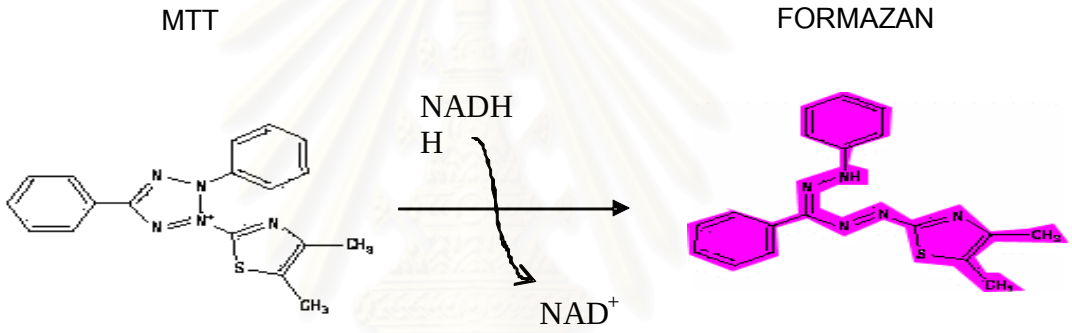

Figure 8 Molecular structure of MTT and its corresponding reaction product. (Methods for studying cell proliferation and viability in cell populations: an assay that measures metabolic activity)

To evaluate cell survival, the MTT assay was performed with modification (Hansen et al., 1989, Ishikawa et al., 2000) of the original procedure (Mosmann, 1983). MTT reduction was analyzed by adding 10 μl of MTT stock solution, 5 mg/ml in phosphate-buffered saline (PBS), to medium in each well (final concentration was 100 μg/ml). Cultures were incubated in a CO_2 incubator for 1 hr at 37 °C and the medium in each well was aspirated off without disturbing the formazan precipitate. Then 400 μl of DMSO was added to each well to solubilize the formazan crystals. Following through formazan solubilization, 200 μl aliquots of soluble formazan were transferred to 96-well microtiter plate. Absorbance (600 nm) in each well was

quantified using a microplate reader (Biorad, model 3550). The cellular reduction of MTT, which reflects metabolic activity and viability, was expressed in term of the percentage of absorbance as compared to that in control cultures.

4. Lactate dehydrogenase (LDH) release assay

The lactate dehydrogenase release assay is a means of measuring membrane integrity as a function of the amount of cytoplasmic lactate dehydrogenase (LDH) released into the medium. The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye (Figure 9).

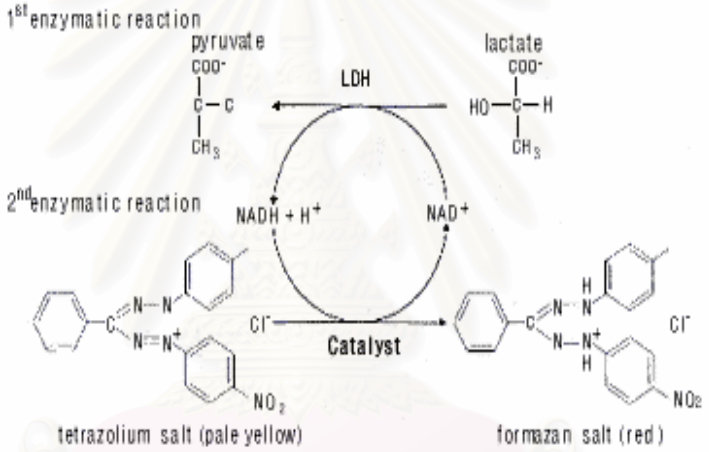

Figure 9 The reaction in lactate dehydrogenase (LDH) assay. (Cytotoxicity detection kit (LDH): Instruction manual version 5, 1999)

Cell viability was determined by assaying the medium from each well for LDH activity using a cytotoxic test kit with a procedure according to the manufacture's instructions (Sigma). Medium LDH was assayed by pipetting 100 μl of culture medium from each well into a 96-well microtiter plate. Cellular LDH in cultures was measured by careful removing culture medium from adherent cells and solubilized cells with 1 ml of 0.5% Triton X-100 in PBS after which 100 μl aliquots were pipetted into a 96-well microtiter plate. The reaction was started by adding 50 μl of assay mixture into each well. The reaction mixtures were left at room temperature for 30 min after which 50 μl of 0.5 N HCl was added into each well. During this incubation

period, the reaction should be protected from light. The light absorbance in each well was measured at 510 nm with reference wavelength at 610 nm with a Biorad Model 550 dual wavelength microplate reader. The LDH release which reflects cell death was presented as percentage of total LDH activity by the following formula:

$$\% \text{ LDH release} = \frac{\text{LDH activity in medium} \times 100}{\text{LDH activity in medium + LDH activity in cells}}$$

In most cases, comparative LDH release in test conditions was expressed as the percentage of that in control conditions.

5. Thiobarbituric acid reactive substances (TBARS) assay

The TBARS assay measures the amount of malondialdehyde (MDA), an end product of peroxidative decomposition of polyeonic fatty acids, and is widely used as a screening assay to quantify the extent of lipid peroxidation *in vitro* (Holly and Cheeseman, cited in Ljybucid et al., 1996). One molecule of MDA can react with two molecules of thiobarbituric acid (TBA) to generate the production of pink pigment which can be determined by spectrofluorometric method (Halliwell and Gutteridge, 1989; Esterbauer and Cheeseman, 1990; Sattler et al., 1998). The principle of reaction was described below (Figure 10).

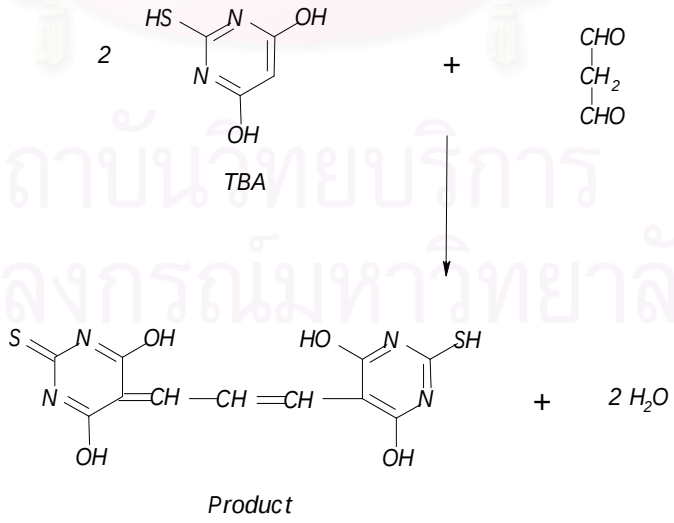

Figure 10 The reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA). (Halliwell and Gutteridge, 1989)

TBARS was measured using a technique modified from Ohkawa et al. (1979) and Storch et al. (2000). In this study, granule neuron cultures grown in 24-well plate were lysed with 160 μl of 2% sodium dodecyl sulfate for 30 min. The lysates from 6 culture wells were pooled and adjusted with 2% sodium dodecyl sulfate to 1 ml. The lysates were added serially with 50 μl of butylated hydroxytoluene (4% in ethanol), 1 ml of phosphotungstic acid (10% in 0.5 M sulfuric acid) and 1.5 ml of thiobarbituric acid (0.7%). The mixtures were incubated at 95 °C for 60 min, cooled by tap water, and extracted with n-butanol. After centrifugation at 3,500 rpm for 10 min, the fluorescence of the n-butanol layer was measured at 515 nm excitation and 553 nm emission wavelengths. Measurements were expressed in term of the percentage to that in control cultures. Fluorescence was measured with Jasco FS 777 spectrofluorometer.

6. Reduced plus oxidized glutathione (total glutathione) assay

The total glutathione, comprising reduced glutathione (GSH) and glutathione disulfide (GSSG), content of biological samples is conveniently determined with an enzymatic recycling assay based on glutathione reductase (Tietze, 1969; Xu and Thornalley, 2001). The sum of reduced and oxidized forms of glutathione can be determined using a kinetic assay in which catalytic amounts of GSH or GSSG and glutathione reductase bring about the continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) by NADPH according to the following reactions.

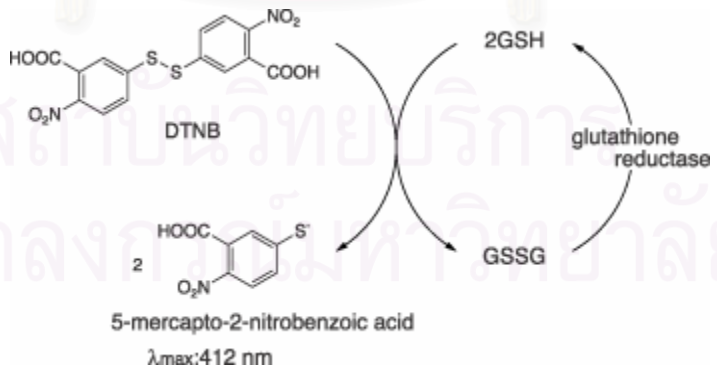

Figure 11 The reaction of GSH, GSSG and GSH reductase. (Total Glutathione Quantification Kit : Technical manual, 2003)

In this study, cultured granule neurons were washed twice in PBS, dissolved in 150 μl of 1% sulfosalicylic acid and left on ice for 10 min. Then, two wells of samples were pooled and centrifuged at 6,000 × g for 5 min at 4 °C. The supernatant was analyzed for total glutathione. A 20 μl aliquot of cell extract was added to 80 μl of PBS in the well of 96-well microtiter plate. The assay was initiated by adding 100 μl GSH reductase solution in 0.1 M sodium phosphate buffer. This reagent was prepared as follows: 25 μl of 0.15 mM 5,5'-dithiobis(2-nitrobenzoic acid), 25 μl of 0.2 mM NADPH and 50 μl of 1 U GSH reductase. The rate of formation of 5-thio-2-nitrobenzoate (TNB) was followed at 410 nm over the initial 3 min of the reaction time and the rate of increase in absorbance (dA/dt_0) was determined and expressed in term of the percentage to that of control.

Experimental protocols

The study was divided into 6 parts as follows:

1. Determining effects of asiaticoside on cultured cerebellar granule neurons.
2. Determining effects of low K^+ medium on cultured cerebellar granule neurons.
3. Determining effects of coexposure with asiaticoside on low K^+-induced cell death in cultured cerebellar granule neurons.
4. Determining effects of preexposure with asiaticoside on low K^+-induced cell death in cultured cerebellar granule neurons.
5. Determining effects of asiaticoside on low K^+-induced lipid peroxidation in cultured cerebellar granule neurons
6. Determining effects of asiaticoside on low K^+-induced glutathione diminution in cultured cerebellar granule neurons

1. Determining effects of asiaticoside on cultured cerebellar granule neurons

Effects of asiaticoside on cerebellar granule neurons were assay after 8 days of culture. Culture medium was removed. Cells were then replenished with serum-free high K^+ DMEM containing N-2 supplement in the presence of asiaticoside. Asiaticoside was dissolved in 0.2% dimethylsulfoxide (DMSO) and then diluted to final concentrations of 0-400 μM in culture medium. The cell was incubated at 37 °C in a humidified 5% CO_2 - 95% air atmosphere. Cell viability was measured 24, 48 and 72 hr later, by MTT cell viability assay and LDH release assay to determine the effects of time and concentration of asiaticoside exposure.

2. Determining effects of low K^+ medium on cultured cerebellar granule neurons

Cultured medium was removed on the experimental day. Cells were then replenished with serum-free low K^+ (5 mM KCl) DMEM containing N-2 supplement and incubated for 6, 12, 24, 48 and 72 hr at 37 °C in a humidified 5% CO_2 - 95% air atmosphere. Control cultures were maintained in serum-free medium containing 25 mM KCl. Cell viability was determined by MTT cell viability assay and LDH release assay to determine the effects of time of low K^+ medium on cultured cerebellar granule cells.

3. Determining effects of coexposure with asiaticoside on low K^+ medium induced cell death in cultured cerebellar granule neurons

On the day of experiment, the medium was removed. Cells were then replenished with serum-free low K^+ DMEM containing N-2 supplement with different concentrations of asiaticoside (0-100 μM). Control cultures were maintained in serum-free medium containing 25 mM KCl. They were maintained at 37 °C in a humidified 5% CO_2 - 95% air atmosphere. After the incubation period, MTT cell viability assay and LDH release assay were determined 24 hr later.

4. Determining effects of preexposure with asiaticoside on low K^+-induced cell death in cultured cerebellar granule neurons

On the day of the experiment, medium was removed. Cells were then incubated with serum-free high K^+ DMEM containing N-2 supplement in the presence of different concentration of asiaticoside (0-100 μM) for 24 and 48 hr at 37 °C in a humidified 5% CO_2 - 95% air atmosphere. After the incubation period, medium was removed and replaced with serum-free low K^+ DMEM containing N-2 supplement. Control cultures were maintained in serum-free medium containing 25 mM KCl. MTT cell viability assay and LDH release assay were determined 24 hr later.

5. Determining effects of asiaticoside on low K^+-induced lipid peroxidation in cultured cerebellar granule neurons

On the day of the experiment, medium was removed. Cells were then incubated with serum-free high K^+ DMEM containing N-2 supplement in the presence of asiaticoside at concentration and time that showed the most benefit on cell viability in prior experiment at 37 °C in a humidified 5% CO_2 - 95% air atmosphere. After the incubation period, medium was removed and replaced with serum-free low K^+ DMEM containing N-2 supplement. Control cultures were maintained in serum-free medium containing 25 mM KCl. TBARS assay were determined 24 hr later.

6. Determining effects of asiaticoside on low K^+-induced glutathione diminution in cultured cerebellar granule neurons

On the day of the experiment, medium was removed. Cells were then incubated with serum-free high K^+ DMEM containing N-2 supplement in the presence of asiaticoside at concentration and time that showed the most benefit on cell viability in prior experiment at 37 °C in a humidified 5% CO_2 - 95% air atmosphere. After the incubation period, medium was removed and replaced with serum-free low K^+ DMEM containing N-2 supplement. Control cultures were maintained in serum-free medium containing 25 mM KCl. Total GSH assay were determined 24 hr later.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) from groups of samples with $n \geq 8$ separate experiments. Differences between control and treatment groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Groups were considered to show statistically significant difference if the P-value was less than 0.05.

CHAPTER IV

RESULTS

1. Effect of asiaticoside on cell viability of cultured cerebellar granule neurons

Exposure of cultured cerebellar granule neurons for 24-72 hr to asiaticoside at different concentrations (0.05-400 μM) induced changes in cell metabolic activity (MTT reduction) and cell death (LDH release) in a concentration- and exposure time-dependent manner. With 24 hr exposure, MTT reduction tended to gradually increased dependent on asiaticoside concentrations with significant maximal increase at 100 μM of asiaticoside (127.13 % of control, Figure 12A). With 48 hr exposure, MTT reduction was significantly increased in the presence of 1-100 μM asiaticoside. With 72 hr exposure, in contrast, MTT reduction tended to decrease even at low concentrations of asiaticoside. It was apparent that cultured neurons exposed to high concentrations (200-400 μM) of asiaticoside showed a significant decrease of MTT reduction at all exposure times. The maximal reduction of mitochondrial metabolic activity (54.5% of control) was seen with 72 hr exposure to 400 μM of asiaticoside (Figure 12A).

In addition, the profile of LDH release following exposures to various concentrations of asiaticoside (.05-400 μM) for 24-72 hr seemed to be the mirrored pattern to the profile of MTT reduction. Asiaticoside induced a significant increase of LDH release at high concentrations (200-400 μM) after all exposure times. With 72 hr exposure, significant increase of LDH release was observed after nearly all concentrations of asiaticoside (Figure 12B). Based on these results, asiaticoside concentrations in a range of 1-100 μM were selected for further investigation on neuroprotective effects of asiaticoside.

2. Effect of potassium deprivation on cell viability of cultured cerebellar granule neurons

To investigate the effect of potassium deprivation, neurons were allowed to develop for 8 days in cultured medium with 10% FBS and 25 mM KCl. The mature neurons were then shifted to serum-free medium containing 5 mM KCl for various exposure times. Control cultures were maintained in serum-free medium with 25 mM KCl. At 6 hr after switching to 5 mM KCl, suppression of cell metabolic activity as measured by MTT reduction was apparent and less than 60% of activity was seen at 24 hr (Figure 13A). Metabolic activity continued to decrease and was less than 15% by 72 hr. In addition to cell metabolic activity, cell death was estimated by measuring release of LDH into the medium. LDH release was low until 12 hr of potassium deprivation but progressively increased to more than 150% of control by 24 hr of potassium deprivation (Figure 13B). LDH release continued to increase gradually to about 230% by 48 hr and nearly 300% by 72 hr. The results of these two assays indicate that suppression of neuronal mitochondrial activity started earlier than neuronal cell death after the beginning of potassium withdrawal (6 hr and 12 hr, respectively) and proceed rapidly within the next 48 hr. Exposure to 5 mM KCl for 24 hr in which cell metabolic activity was suppressed to approximate 50% of control had been selected as the insulting condition for further investigation on neuroprotective effects of asiaticoside.

3. Effect of coexposure with asiaticoside on potassium deprivation-induced cell death in cultured cerebellar granule neurons

Coexposure of cultured cerebellar granule neurons with asiaticoside (at different concentrations ranging from 1-100 μM) in serum-free medium containing 5 mM KCl for 24 hr failed to significantly prevent suppression of cell metabolic activity and cell death caused by potassium deprivation (Figure 14). In MTT reduction assay, asiaticoside had a small but not statistically significant effect on cell metabolic activity. In addition, results of LDH release assay were in accordance with those observed in MTT reduction assay. These results suggest that asiaticoside may be ineffective in protecting cultured cerebellar granule neurons against KCl withdrawal-induced neuronal injury and death.

4. Effects of preexposure with asiaticoside on potassium deprivation-induced cell death in cultured cerebellar granule neurons

Preexposure of cultured cerebellar granule neurons with asiaticoside (at various concentrations ranging from 1-100 μM) for 24 hr in serum-supplemented medium containing 25 mM KCl before switching to serum-free medium containing 5 mM KCl for 24 hr showed a trend to attenuate the suppression of cell metabolic activity as measured by MTT reduction assay, especially at lower concentration of asiaticoside. However, this effect was statistically significant only with exposure to 1 μM of asiaticoside (Figure 15A). In addition, asiaticoside showed the similar results in LDH release assay. Decrease in cell death was statistically significant only after preexposure to 1 μM asiaticoside (Figure 15B). With 48 hr preexposure in serum-supplemented medium containing 25 mM KCl before switching to serum-free medium containing 5 mM KCl for 24 hr, asiaticoside at all concentrations failed to exert any beneficial effects on cell metabolic activity and cell death similar to the results observed in coexposure experiment (Figure 16).

5. Effect of preexposure with asiaticoside on potassium deprivation-induced lipid peroxidation in cultured cerebellar granule neurons

Cellular lipid peroxidation was measured with a spectrofluorometric method (TBARS). When cultured neurons were switched from serum-supplemented medium containing 25 mM KCl to serum-free medium containing 5 mM KCl, an increase in lipid peroxidation was observed (130% of control cultures, Figure 17). Preexposure of cultured neurons with 1 μM asiaticoside for 24 hr in high K^+ medium had no effect on basal levels of lipid peroxidation. However, preexposure to asiaticoside effectively attenuate potassium deprivation-induced increment of cellular lipid peroxidation in cultured neurons to near control levels. These results suggest that asiaticoside preexposure may induce some cellular antioxidant mechanisms that are still functioning in its absence.

6. Effect of preexposure with asiaticoside on potassium deprivation-induced glutathione diminution in cultured cerebellar granule neurons

Potassium deprivation markedly decreased total glutathione contents to about 60% of control cultures (Figure 18). Preexposure of cultured neurons with 1 μM asiaticoside for 24 hr in high K^+ medium had no effect on basal levels of total glutathione content. However, preexposure to asiaticoside partially attenuated potassium deprivation-induced glutathione diminution. However, this beneficial effect was marginal and did not reach statistically significant level (Figure 18).

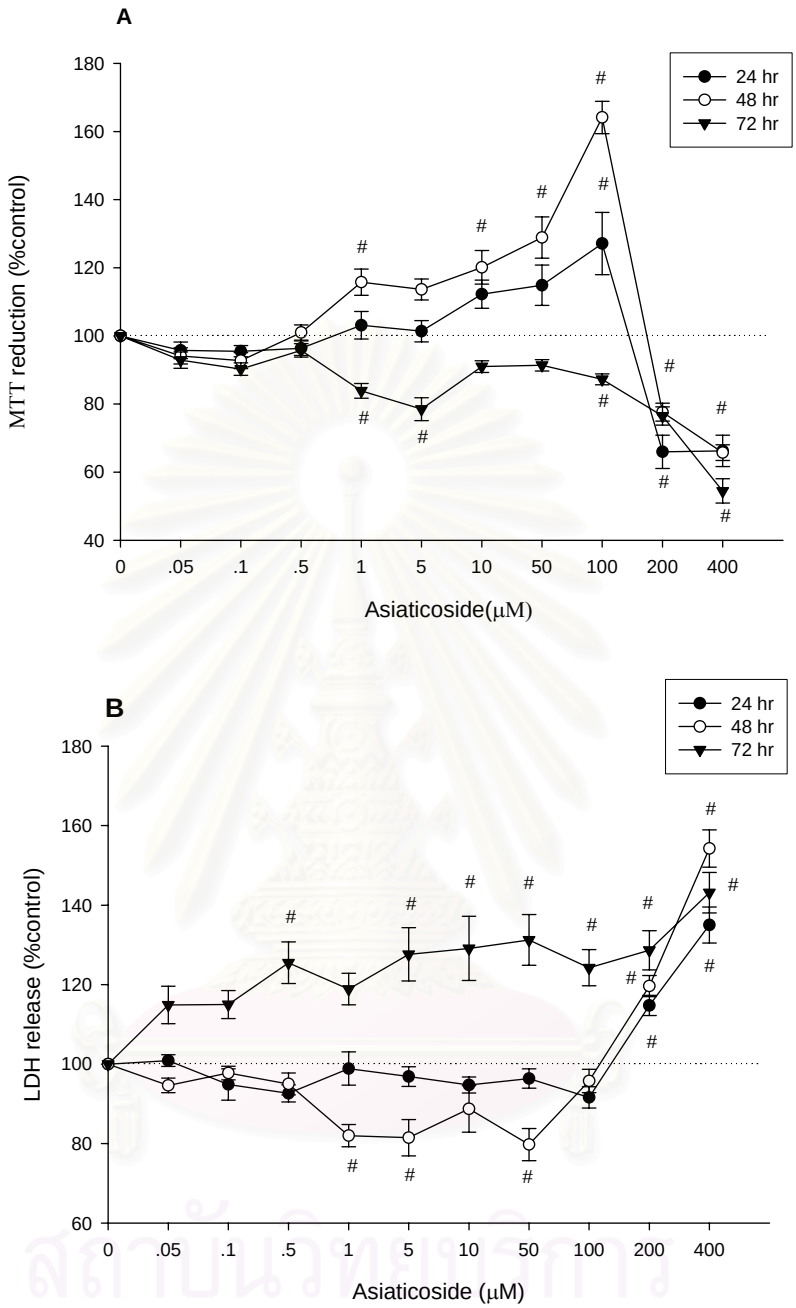

Figure 12 Effects of asiaticoside on viability of cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then treated with various concentrations of asiaticoside (0.05-400 μM) for 24, 48, and 72 hr. Cell viability was determined by MTT reduction assay (A) and cell death was determined by LDH release assay (B). Data are presented as mean ± SEM from 8 independent experiments with duplicate samples.

$^{\#}P < 0.05$ vs control cultures by one-way ANOVA followed by Tukey's test.

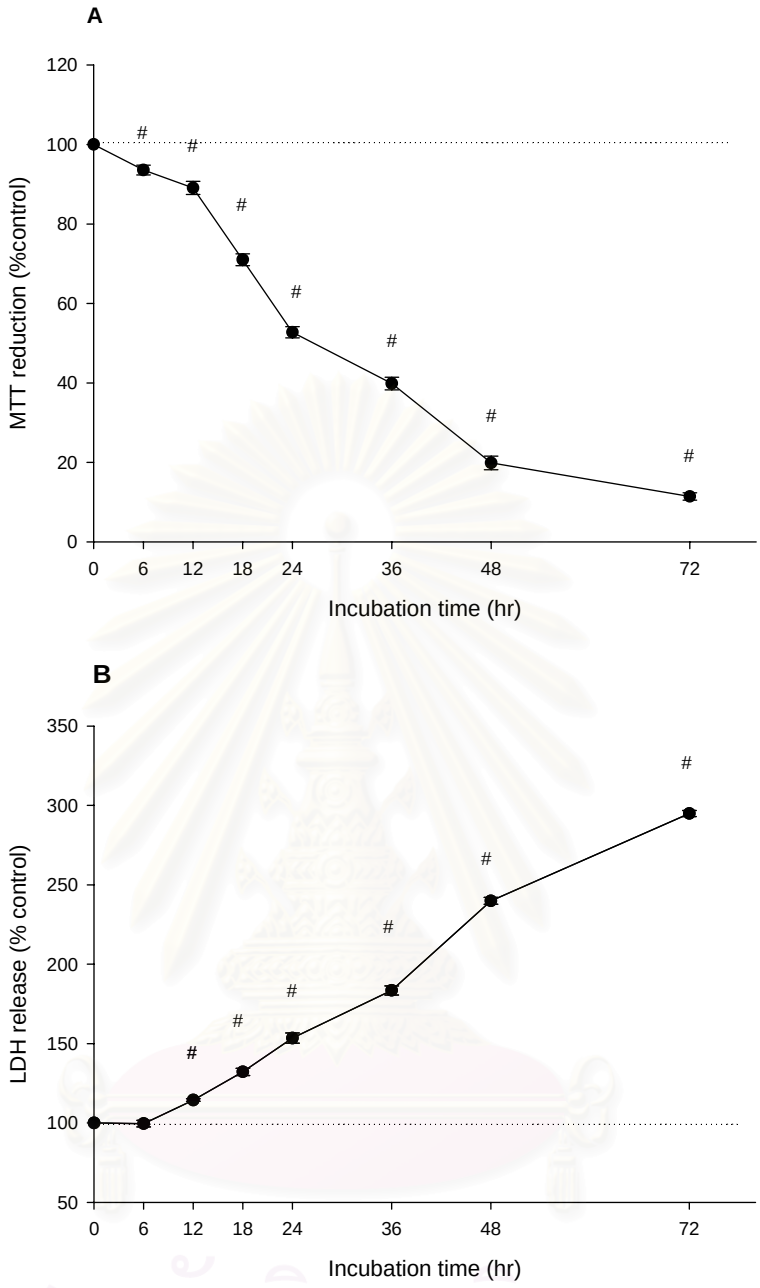

Figure 13 Effect of potassium deprivation on cell survival of cultured cerebellar granule neurons. Cerebellar granule neurons were cultured in serum-containing medium with 25 mM KCl for 8 days. The medium was then replaced with a serum-free medium with containing 5 mM KCl. At the indicated times after the change of medium, cell viability and death were determined by MTT reduction assay (A) and LDH activity in the extracellular medium (B), respectively. Control cells were switched to a serum-free medium containing 25 mM KCl. Results represent the mean ± SEM of eight independent experiments with duplicate samples.

$^{\#}P < 0.05$ vs control cultures by one-way ANOVA followed by Tukey's test.

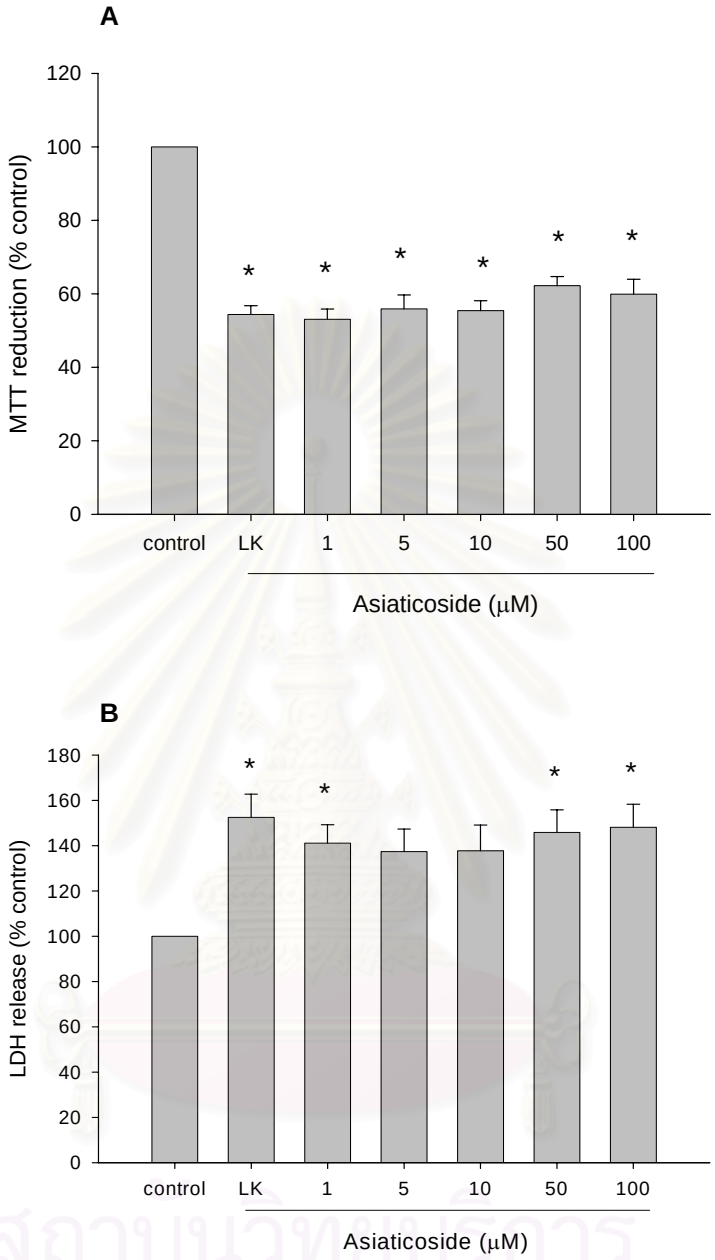

Figure 14 Effect of coexposure with asiaticoside on potassium deprivation-induced cell death in cultured cerebellar granule neurons. Neurons were switched from culture medium containing 25 mM KCl to serum-free medium containing 5 mM KCl without asiaticoside (LK) or with various concentrations of asiaticoside. Control represents survival in serum-free medium containing 25 mM KCl. Cell viability was quantified by MTT reduction assay (A) and LDH release assay (B) 24 hr after the treatment. Each bar represents mean ± SEM of eight independent experiments with duplicate samples.

*P < 0.05 vs control cultures by one-way ANOVA followed by Tukey's test.

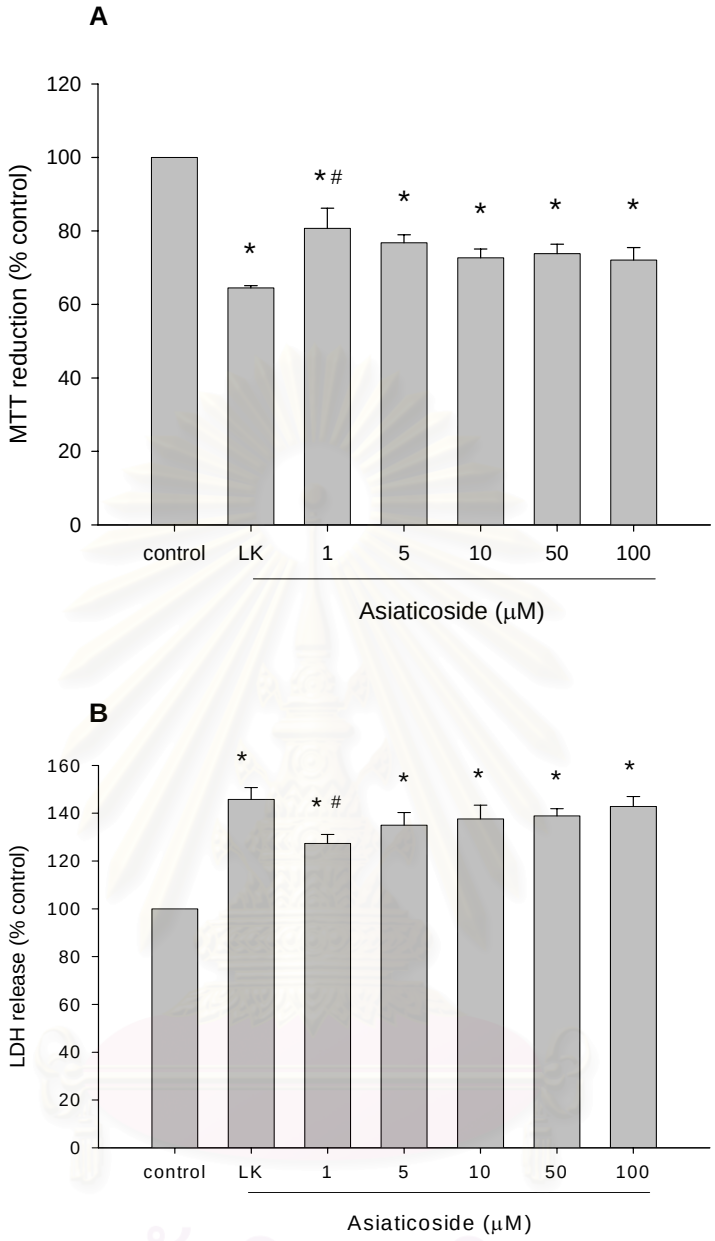

Figure 15 Effect of 24-hr preexposure with asiaticoside on potassium deprivation-induced cell death in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days. Asiaticoside was added at concentrations ranging from 1-100 μM 24 hr before switching to serum-free medium containing 5 mM KCl. Control cultures were maintained in serum-free medium with 25 mM KCl. Cell viability was determined 24 hr later by MTT reduction assay (A) and LDH release assay (B). Results represent mean ± SEM from eight independent experiments with duplicated samples.

$^{\#}P < 0.05$ vs LK cultures by one-way ANOVA followed by Tukey's test.

$^{*}P < 0.05$ vs control cultures by one-way ANOVA followed by Tukey's test.

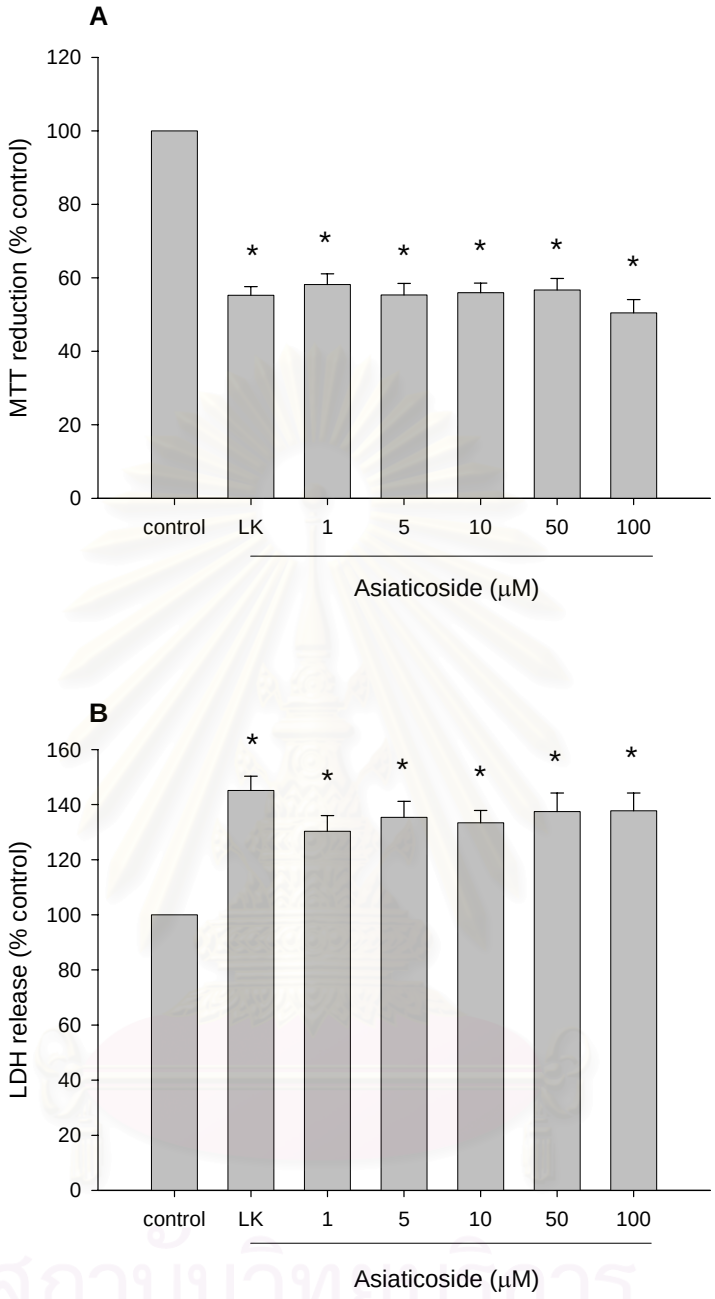

Figure 16 Effect of 48-hr preexposure with asiaticoside on potassium depriviation-induced cell death in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days. Asiaticoside was added at concentrations ranging from 1-100 μM for 48 hr before switching to serum-free medium containing 5 mM KCl. Control cultures were maintained in serum-free medium with 25 mM KCl. Cell viability was determined 24 hr later by MTT reduction assay (A) and LDH release assay (B). Results represent mean±SEM from eight independent experiments with duplicated samples.

*P < 0.05 vs control cultures by one-way ANOVA followed by Tukey's test.

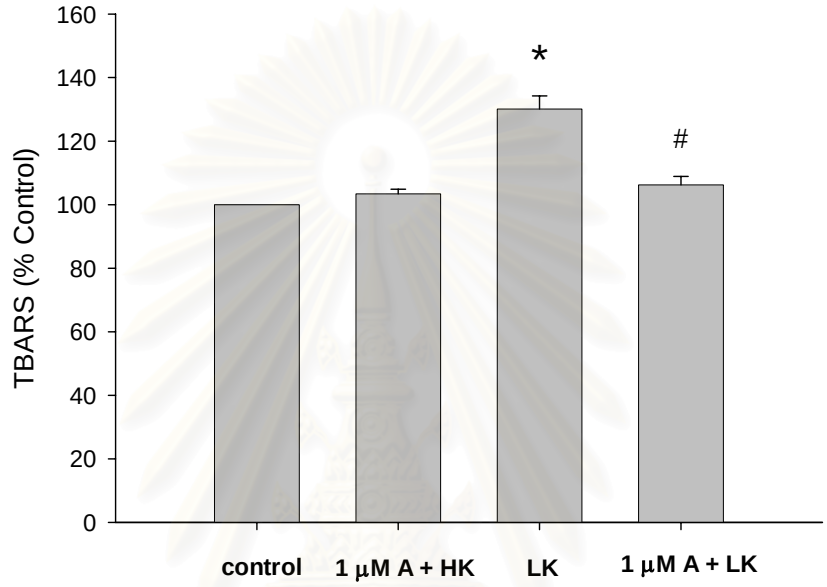

Figure 17 Effect of asiaticoside on potassium deprivation-induced lipid peroxidation in cultured cerebellar granule neurons. Cultured neurons in serum-containing medium were preexposed to 1 μM asiaticoside for 24 hr before switching to serum-free medium containing 5 mM KCl (1 μMA+LK) or maintained in serum-free medium containing 25 mM KCl (1 μMA+ HK). Cultured neurons without preexposure to asiaticoside were also switched to serum-free low K^+ medium (LK). Control cultures were maintained in serum-free high K^+ medium. Cellular lipid peroxidation was determined 24 hr later by TBARS assay. Results represent mean ± SEM from eight independent experiments with duplicate samples.

$^{\#}P < 0.05$ vs LK by one-way ANOVA followed by Tukey's test.

$^{*}P < 0.05$ vs control by one-way ANOVA followed by Tukey's test.

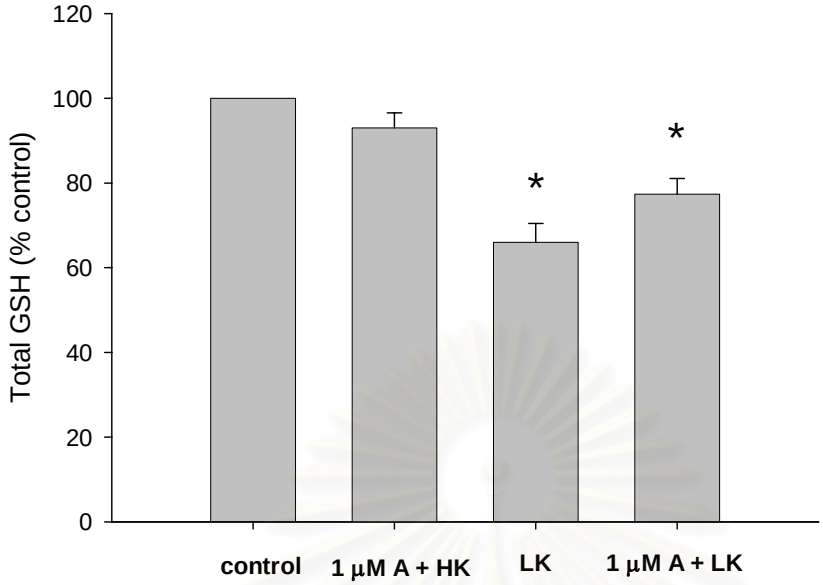

Figure 18 Effect of preexposure with asiaticoside on potassium deprivation-induced glutathione diminution in cultured cerebellar granule neurons. Cultured neurons in serum-containing medium were preexposed to 1 μM asiaticoside for 24 hr before switching to serum-free medium containing 5 mM KCl (1 μM A+LK) or maintained in serum-free medium containing 25 mM KCl (1 μM A+HK). Cultured neurons without preexposure to asiaticoside were also switched to serum-free low K^+ medium (LK). Control cultures were maintained in serum-free high K^+ medium. Total GSH was determined 24 hr later. Results represent mean ± SEM from eight independent experiments with duplicate samples.

*P < 0.05 vs control by one-way ANOVA followed by Tukey's test

CHAPTER V

DISCUSSION AND CONCLUSION

Asiaticoside is a triterpene ester glycoside isolated from *Centella asiatica* (Linn.) Urban, family Apiaceae, a herbal plant that has been known as Gotu kola and pennywort. Asiaticoside has been reported to increase enzyme and non-enzymatic antioxidant (superoxide dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid in newly synthesized tissue and decrease lipid peroxide content (Shukla et al., 1999). In addition, aqueous extract of *Centella asiatica* (CA) has cognition enhancing properties with an associated decrease in the brain oxidative stress parameters of the normal rats (Veerendra et al., 2002). Antioxidant property of CA was also evidenced by decreasing MDA levels and increasing glutathione levels induced by PTZ kindling (Gupta et al., 2003).

Considering available information on antioxidant property and potential protective activities of asiaticoside, it was speculated that asiaticoside might possess some neuroprotective effects that it may be useful in preventing neuronal damages in various neurodegenerative disorders

In the present study, cultured rat cerebellar granule neurons were used to investigate beneficial effects of asiaticoside on potassium deprivation-induced neuronal cell death. These particular neuronal cultures are widely used as a model system for studying neuronal apoptosis. Granule neurons are usually cultured and matured in medium containing 25 mM potassium (high K^+). Postmitotic granule neurons can be readily maintained *in vitro* in their fully differentiated state for several weeks if depolarized with high concentrations of K^+ (Gallo et al., 1987). This depolarization promotes survival of these neurons by preventing their programmed death via apoptosis. After maturation, changing to medium containing 5 mM potassium (low K^+) induces neuronal cell death in which the cells show morphological and biochemical characteristics of apoptosis (D'Mello et al., 1993; Yan et al., 1994; Galli et al., 1995) including cytoplasmic blebbing, condensation or aggregation of nuclear chromatin and internucleosomal DNA fragmentation. The

induction of apoptosis by low K^+ (non-depolarizing) culture conditions is greatly attenuated by inhibitors of RNA and protein synthesis. In addition to depolarizing concentrations of K^+, low (subtoxic) concentrations of glutamate, as well as both NMDA and non-NMDA receptor agonists, also prevent apoptosis of cerebellar granule neurons cultured in low K^+ medium. In addition, this culture system provides a large homogeneous neuronal population. Therefore, these neurons are widely used as a primary cell culture system to investigate the biochemical and molecular mechanisms underlying neuronal apoptosis in the central nervous system.

After maturation by culturing in serum-supplemented medium containing high K^+ for 8 days, changing to serum-free medium containing low K^+ rapidly induces neuronal cell death, evidently by apoptosis. Then approximate 50% of granule cells die within 24 hr. However, the molecular mechanisms by which the low K^+-induced apoptosis occurs in cultured cerebellar granule cells remain unclear. It was found that p38 MAP kinase (p38) was an important factor for low K^+-induced apoptosis (Yamagishi et al., 2001, 2003). After changing to low K^+ medium, p38 was markedly activated. In addition, a specific inhibitor of p38, strongly inhibited the phosphorylation and expression of c-Jun in low K^+-induced apoptosis of cultured cerebellar granule cells. *In vitro* kinase assay showed that p38 directly phosphorylated c-Jun. These results indicate that p38 regulates low K^+-induced apoptosis of cerebellar granule neurons.

Bcl-2 and Bcl-xL protect neurons from apoptosis, while Bax and Bcl-xS may act as proapoptotic proteins. These members of the Bcl-2 family may be involved in the cytochrome-c release to the cytosol. Cytochrome-c is able to form a complex with other proteins to activate a cascade of proteases (Lossi and Merighi, 2003). Recently, it was suggested that the Bcl-2 family members, caspases activation and cytochrome-c release are involved in cerebellar granule cell death induced by low K^+ and their participation in this process could be different depending on neuronal maturation in culture (Alevez, Pedroza, and Moran, 2003).

Recently, low K^+-induced apoptosis in cultured cerebellar granule neurons has been used as an *in vitro* neurodegenerative model for the screening or study of potential neuroprotective agents. In this study, the foundational basis for investigating a potential neuroprotective effect of asiaticoside was due to the reported antioxidant

property and wound healing effect of asiaticoside (Shukla et al., 1999a, 1999b; Cheng et al., 2004).

A line of evidence has shown that asiaticoside possesses good wound healing activity. Enhanced healing activity has been attributed to increased collagen formation and angiogenesis. The activity of asiaticoside has been studied in normal as well as delayed-type wound healing. In guinea pig punch wounds topical applications of asiaticoside produced increase in hydroxyproline, increase in tensile strength, increased collagen content and better epithelialization. In streptozotocin diabetic rats, where healing is delayed, topical application of asiaticoside over punch wounds increased hydroxyproline content, tensile strength, collagen content and epithelialization thereby facilitating the healing. Asiaticoside was active by the oral route also at 1 mg/kg dose in the guinea pig punch wound model. It promoted angiogenesis in the chick chorioallantoic membrane model at 40 μg/disk concentration. These results indicate that asiaticoside exhibits significant wound healing activity in normal as well as delayed healing models (Shukla et al., 1999a).

Since antioxidants have been reported to play a significant role in the wound healing process, Shukla et al. (1999b) studied the effect of asiaticoside on the levels of certain antioxidants in the wound so as to explore the possible involvement of such a mechanism in the asiaticoside induced wound healing. Topical application of asiaticoside for 7 days to cutaneous wounds in rats led to increased enzymatic and non-enzymatic antioxidants, including superoxide dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid in newly formed tissues. It also resulted in a several fold decrease in lipid peroxide levels. However, continued application for 14 days showed no significant difference in these antioxidants compared with control wound tissue. It appears that asiaticoside enhanced induction of antioxidant levels at an initial stage of healing which may be an important contributory factor in the healing properties of this substance.

Apparently, *in vitro* experimental results suggest that asiaticoside exerts a certain degree of neuroprotective effect to cultured cerebellar granule neurons. However, this beneficial effect of asiaticoside is limited and dependent on a particular set of experimental conditions. Significant protection was found only at the preexposure to asiaticoside at a low concentration (1 μM) for 24 hr while coexposure

for 24 hr or preexposure for 48 hr with asiaticoside exerted no significant protection. Preexposure of cultured neurons with 1 μM asiaticoside for 24 hr in high K^+ medium did not decrease basal cellular lipid peroxidation levels but effectively prevented low K^+-induced increase in lipid peroxidation. Moreover, this preexposure had no effect on basal total glutathione content and displayed a marginal not significant preventive effect on low K^+-induced decrease in total glutathione content.

It is quite possible that protection by asiaticoside against potassium deprivation-induced apoptotic cell death in cultured granule neurons may be marginal, not directly involve its antioxidant property, and unlikely to mediate via inhibition of apoptotic mechanisms because the beneficial effect of asiaticoside is clearly evident only under specific condition. Presumably, asiaticoside preexposure may induce certain cellular protective mechanisms by increasing of mitochondrial function, decreasing of cellular lipid peroxidation and marginal increasing of glutathione levels in cultured granule neurons that are still functioning after its absence and under low K^+-condition.

Asiaticoside derivatives were tested for potential protective effects against Aβ-induced cell death. Of 28 derivatives tested, asiatic acid, asiaticoside 6, and SM2 showed strong inhibition of Aβ-induced death of B103 cells at 1 μM. These three asiaticoside derivatives were further tested for their effects on free radical injury and apoptosis. All three derivatives reduced H_2O_2-induced cell death and lowered intracellular free radical concentration, but asiatic acid showed the strongest protection. In contrast, SM2 was the most effective blocker of staurosporine-induced apoptosis. These results suggest that the three asiaticoside derivatives block Aβ toxicity by acting through different cellular mechanisms (Mook-Jung et al., 1999). In this connection, neuroprotective mechanisms of asiaticoside may be distinct from those of its derivatives. Modification of asiaticoside's molecular structures may provide potential candidates for *in vivo* and *in vitro* preclinical studies in the future.

It was notable that when cultured granule neurons subjected to high concentrations of asiaticoside (200-400 μM) or a prolonged duration of exposure (72 hr), remarkable signs of neuronal injury and death were seen. These findings may conceivably suggest that asiaticoside possesses biphasic effects on cell viability,

beneficial effects at lower concentrations or short duration of exposure but detrimental effects at higher concentrations of prolonged duration of exposure.

It is quite interesting that asiaticoside-induced neuronal cell death observed in a prolonged duration of exposure may mediate through a mixture of apoptosis and necrosis. On one hand, with 72 hr exposure to asiaticoside concentrations up to 100 μM, mitochondrial metabolic activity (measured by MTT reduction) was preserved or even increased while neuronal cell death (measured by LDH release) gradually progressed. Therefore, under these exposure conditions, neuronal cell death may undergo via apoptotic pathway which requires active mitochondrial function. On the other hand, with 72 hr exposure to high concentrations of asiaticoside (200-400 μM), mitochondrial metabolic activity was markedly disrupted with more pronounced neuronal cell death. This phenomenon is a suggestive sign of necrotic cell death. It was evident that neurons rapidly lost their mitochondrial membrane potential, energy charge, and subsequently the ability to metabolize MTT before proceeding to necrosis (Ankracrona et al., 1995). The conclusive mechanisms for the different mode of cell death induced by asiaticoside in cerebellar granule neurons are still unknown and suggested for further investigation.

In conclusion, asiaticoside by itself displayed both neuroprotective and neurotoxic effects on cultured cerebellar granule neurons, depending on the concentration and duration of exposure. Neuroprotective effect is clearly evident only under specific conditions of exposure, namely, preexposure to 1 μM asiaticoside for 24 hr. This particular preexposure can prevent low potassium medium induced cell death in cultured rat cerebellar granule neurons, as indicated by an increasing of mitochondrial function, decreasing of cellular lipid peroxidation, and marginal restoring of glutathione diminution. The mechanism of action underlying biphasic effects of asiaticoside on cultured neurons is still unclear at the present time.

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APPENDICES

Table 1 Effect of Asiaticoside on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 24 hr)

Asiaticoside	%control									
(μM)	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
0.05	103	102	98	87	104	90	89	93	95.75	2.42
0.1	102	88	97	101	95	93	96	92	95.50	1.64
0.5	106	96	95	89	96	102	99	88	96.38	2.15
1	107	121	93	99	115	90	92	108	103.13	4.04
5	89	95	106	93	112	108	97	111	101.38	3.14
10	126	99	98	109	106	123	127	110	112.25	4.13
50	112	110	98	147	123	126	96	107	114.88	5.91
100	114	110	118	107	121	115	150	182	127.13	9.13
200	56	67	92	44	68	62	65	74	66.00	4.90
400	86	76	61	57	79	51	68	52	66.25	4.63

N = number of experiments (duplicated)

Table 2 Effect of Asiaticoside on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 48 hr)

Asiaticoside	%control									
(μM)	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
0.05	95	85	96	86	90	96	104	101	94.13	2.39
0.1	89	90	82	95	89	104	97	96	92.75	2.36
0.5	99	91	106	95	104	98	110	105	101.00	2.24
1	104	113	131	128	119	100	111	120	115.75	3.84
5	111	117	130	115	118	109	100	109	113.63	3.09
10	124	122	144	122	128	102	101	118	120.13	4.92
50	126	149	156	118	137	105	119	121	128.88	6.07
100	156	160	193	161	167	158	170	148	164.13	4.76
200	82	72	88	82	70	69	85	73	77.63	2.63
400	75	68	61	68	64	73	56	61	65.75	2.28

N = number of experiments (duplicated)

Table 3 Effect of Asiaticoside on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 72 hr)

Asiaticoside (μM)	%control									
	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
0.05	82	85	94	94	104	93	97	94	92.88	2.41
0.1	87	82	99	93	91	89	87	94	90.25	1.84
0.5	99	96	103	101	91	93	97	86	95.75	1.97
1	93	79	77	87	85	90	84	76	83.88	2.17
5	91	77	69	62	86	77	81	85	78.50	3.35
10	95	94	93	88	94	82	95	87	91.00	1.69
50	91	89	97	98	95	87	85	89	91.38	1.69
100	90	85	94	87	88	91	80	83	87.25	1.61
200	70	70	70	91	82	73	81	75	76.50	2.68
400	50	38	50	68	62	53	66	49	54.50	3.58

N = number of experiments (duplicated)

Table 4 Effect of Asiaticoside on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 24 hr)

Asiaticoside	%control									
(μM)	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
0.05	106	107	103	97	101	99	95	99	100.88	1.48
0.1	118	81	95	89	92	88	102	94	94.88	3.94
0.5	98	83	89	97	86	93	100	95	92.63	2.15
1	119	92	108	84	105	102	95	86	98.88	4.19
5	106	100	92	87	101	99	88	102	96.88	2.47
10	92	97	91	92	87	94	105	100	94.75	2.02
50	104	95	92	105	96	89	87	103	96.38	2.46
100	80	84	100	96	87	101	94	91	91.63	2.67
200	109	111	105	110	115	121	125	122	114.75	2.54
400	121	125	158	120	138	142	135	141	135.00	4.52

N = number of experiments (duplicated)

Table 5 Effect of Asiaticoside on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 48 hr)

Asiaticoside	%control									
(μM)	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
DMSO	89	100	103	99	96	94	96	105	97.75	1.81
0.05	102	98	87	101	92	93	92	92	94.63	1.83
0.1	102	95	95	104	103	95	90	98	97.75	1.73
0.5	101	103	102	95	85	100	90	84	95.00	2.74
1	77	80	83	78	79	93	95	71	82.00	2.82
5	69	91	85	79	67	102	91	68	81.50	4.57
10	68	105	104	101	90	93	89	60	88.75	5.86
50	84	76	79	85	62	87	98	67	79.75	4.06
100	93	100	113	92	84	95	97	92	95.75	2.96
200	122	116	128	114	132	111	121	113	119.63	2.65
400	177	136	153	161	167	148	145	147	154.25	4.70

N = number of experiments (duplicated)

Table 6 Effect of Asiaticoside on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 72 hr)

Asiaticoside	%control									
(μM)	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
DMSO	106	87	108	115	117	104	112	105	106.75	3.28
0.05	104	95	111	131	130	128	108	112	114.88	4.71
0.1	108	102	136	113	115	118	111	117	115.00	3.52
0.5	112	106	115	134	142	148	122	125	125.50	5.24
1	107	100	127	126	128	131	113	119	118.88	3.96
5	107	101	132	143	146	153	122	117	127.63	6.70
10	107	99	130	129	171	148	117	132	129.13	8.09
50	117	104	130	137	167	129	131	135	131.25	6.38
100	110	104	120	122	141	130	138	129	124.25	4.55
200	122	121	117	120	157	126	143	123	128.63	4.94
400	132	145	125	148	165	126	157	147	143.13	5.10

N = number of experiments (duplicated)

Table 7 Effect of Potassium deprivation on MTT reduction assay in Cultured Cerebellar Granule Neurons.

Low K^+ hrs.	%control									
	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
0	100	100	100	100	100	100	100	100	100.00	0.00
6	93	93	97	92	97	94	96	86	93.58	1.22
12	94	90	96	90	85	87	82	88	89.06	1.65
18	72	69	71	70	73	70	72	72	70.99	1.46
24	59	49	51	48	49	54	56	56	52.75	1.41
36	35	36	43	40	35	44	47	39	39.86	1.59
48	18	15	13	24	18	21	28	22	19.86	1.70
72	12	16	11	8	10	10	10	14	11.43	0.91

N = number of experiments (duplicated)

Table 8 Effect of Potassium deprivation on LDH release assay in Cultured Cerebellar Granule Neurons.

Low K^+ hrs.	%control									
	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6	N = 7	N = 8	mean	SEM
0	100	100	100	100	100	100	100	100	100.00	0.00
6	93	98	93	100	106	108	97	103	99.56	2.01
12	114	116	113	111	114	118	114	115	114.40	0.81
18	133	139	129	124	138	122	134	138	132.16	2.26
24	146	166	168	147	142	146	150	164	153.46	3.22
36	175	176	177	200	186	183	186	184	183.46	2.88
48	236	229	238	247	245	247	239	239	239.91	2.19
72	290	287	291	301	294	300	296	300	294.88	1.88

N = number of experiments (duplicated)

Table 9 Effect of Coexposure with Asiaticoside on Potassium deprivation induced cell death in Cultured Cerebellar Granule Neurons. Determination by MTT reduction assay.

Asiaticoside (μM)	% control									
	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0
low K^+	58	49	47	51	51	60	67	52	54.38	2.38
1	63	61	53	54	69	67	66	46	53.06	2.77
5	76	59	50	65	75	63	67	45	55.89	3.82
10	61	58	58	51	73	70	65	54	55.44	2.67
50	62	57	61	54	73	70	71	64	62.22	2.47
100	79	47	47	48	64	48	49	59	59.89	4.09

N = number of experiments (duplicated)

Table 10 Effect of Coexposure with Asiaticoside on Potassium deprivation induced cell death in Cultured Cerebellar Granule Neurons. Determination by LDH release assay.

Asiaticoside (μM)	% control									
	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0
Low K^+	110	135	207	134	155	168	166	145	152.50	10.27
1	104	127	121	134	159	157	170	157	141.13	8.12
5	90	116	116	132	157	165	167	156	137.38	9.96
10	89	126	100	130	155	166	180	156	137.75	11.35
50	94	124	148	129	161	170	177	164	145.88	9.97
100	101	135	130	131	160	166	191	171	148.13	10.21

N = number of experiments (duplicated)

Table 11 Effect of Preexposure with Asiaticoside on Potassium deprivation induced cell death in Cultured Cerebellar Granule Neurons. Determination by MTT reduction assay. (Incubation time = 24 hr)

Asiaticoside (μM)	% control									
	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
Control	100	100	100	100	100	100	100	100	100.00	0.00
Low K^+	67	64	62	63	63	66	66	65	64.50	0.63
1	92	69	100	91	64	98	69	65	80.69	5.51
5	76	84	78	80	82	81	68	68	76.75	2.18
10	81	68	74	81	60	76	71	72	72.69	2.40
50	85	63	78	80	68	78	70	71	73.81	2.57
100	80	63	73	86	62	83	63	69	72.06	3.40

N = number of experiments (duplicated)

Table 12 Effect of Preexposure with Asiaticoside on Potassium deprivation induced cell death in Cultured Cerebellar Granule Neurons. Determination by LDH release assay. (Incubation time = 24 hr)

Asiaticoside (μM)	% control									
	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0
low K^+	144	131	150	167	137	152	135	150	145.75	5.00
1	134	113	135	128	115	140	118	136	127.38	3.74
5	144	115	138	140	120	157	120	146	135.00	5.29
10	141	120	168	131	121	149	127	144	137.63	5.74
50	146	124	140	143	130	151	139	138	138.88	3.04
100	156	132	142	144	128	163	138	140	142.88	4.12

N = number of experiments (duplicated)

Table 13 Effect of Preexposure with Asiaticoside on Potassium deprivation induced cell death in Cultured Cerebellar Granule Neurons. Determination by MTT reduction assay. (Incubation time = 48 hr)

Asiaticoside	% control									
(μM)	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0
low K^+	58	55	50	47	51	60	68	53	55.25	2.36
1	67	48	58	54	67	62	66	46	58.19	2.91
5	46	47	53	65	63	58	67	45	55.31	3.17
10	50	45	59	51	66	58	65	54	55.94	2.62
50	47	43	60	54	62	57	69	64	56.69	3.14
100	69	40	41	48	48	51	49	59	50.44	3.65

N = number of experiments (duplicated)

Table 14 Effect of Preexposure with Asiaticoside on Potassium deprivation induced cell death in Cultured Cerebellar Granule Neurons. Determination by LDH release assay. (Incubation time = 48 hr)

Asiaticoside	% control									
(μM)	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
low K^+	123	129	148	152	147	158	167	137	145.13	5.21
1	104	120	130	131	133	149	154	122	130.38	5.65
5	115	111	137	149	130	148	158	135	135.38	5.82
10	118	123	132	132	130	151	154	127	133.38	4.50
50	112	121	139	141	123	169	157	138	137.50	6.71
100	118	116	141	159	124	160	154	130	137.75	6.46

N = number of experiments (duplicated)

Table 15 Effect of Preexposure with Asiaticoside on Potassium deprivation induced Lipid peroxidation in Cultured Cerebellar Granule Neurons. Determination by TBARS assay. (Incubation time = 24 hr)

Asiaticoside	%control									
1μM	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
LK	138	133	117	149	135	133	121	115	130.13	4.10
A	98	97	104	110	106	102	106	104	103.38	1.52
A+LK	111	111	108	118	94	106	101	101	106.25	2.64

N = number of experiments (duplicated)

Table 16 Effect of Preexposure with Asiaticoside on Potassium deprivation induced Glutathione Diminution in Cultured Cerebellar Granule Neurons. Determination by Total GSH assay. (Incubation time = 24 hr)

Asiaticoside	%control									
1μM	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8	Mean	SEM
control	100	100	100	100	100	100	100	100	100.00	0.00
LK	82	55	53	61	70	51	79	77	66.00	4.44
A	91	85	80	93	88	110	105	92	93.00	3.53
A+LK	87	72	60	78	83	67	91	81	77.38	3.69

N = number of experiments (duplicated)

VITAE

Miss Srirat Supawantanakul was born in December 5, 1973 in Chonburi,Thailand. She graduated with a Bachelor Degree of Pharmacy in 1996 from the Faculty of Pharmacy, Chulalongkorn University, Bangkok, Thailand. After Graduation, She worked as a pharmacist in Siridhorn Health Public College Phitsanuloke, Phitsanulok for one year. Then she worked as a pharmacist in Samuthprakarn Hospital, Samuthprakarn during 1997-2001 before enrolling the Master's Degree in Pharmacology Program, Faculty of Pharmaceutical sciences, Chulalongkorn University.