ผลของเบอร์จินินต่อการเกิดพิษต่อเซลล์ประสาทจากกลูตาเมทในเซลล์แกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาว

นางสาววรวรรณ บุญโญ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF BERGENIN ON GLUTAMATE-INDUCED NEUROTOXICITY IN

CULTURED RAT CEREBELLAR GRANULE CELLS

Miss Worawan Boonyo

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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เบอร์จินินเป็นสารซึ่งแยกได้จากพืชหลายชนิด นิยมใช้เป็นยาพื้นบ้านในหลายประเทศแถบเอเชีย การศึกษานี้มุ่งศึกษาผลของเบอร์จินินต่อเซลล์แกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาวและต่อการเกิดพิษจากกลูตาเมทต่อเซลล์ประสาทเพาะเลี้ยงดังกล่าว โดยใช้การอยู่รอดของเซลล์ การเกิดกระบวนการไลปิดเปอร์ออกซิเดชัน และการเปลี่ยนแปลงปริมาณกลูตาไทโอนเป็นตัวชี้วัด ทั้งนี้โดยใช้เซลล์ประสาทแกรนูลเพาะเลี้ยงอายุ 8 วัน ในการทดลองทุกอย่าง ในการศึกษาขั้นต้น เมื่อให้เบอร์จินินความเข้มข้นต่างๆ (10–100 ไมโครโมลาร์) สัมผัสกับเซลล์ประสาทแกรนูลเพาะเลี้ยงในช่วงเวลาต่างกัน พบว่าการสัมผัสกับเบอร์จินินความเข้มข้น 100 ไมโครโมลาร์ นาน 48 ชั่วโมง มีผลลดการทำงานของไมโตคอนเดรีย การศึกษาผลของเบอร์จินินต่อการเกิดพิษจากกลูตาเมทต่อเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมแบ่งเป็น 2 ส่วน โดยส่วนแรกเป็นการทดลองผลเมื่อให้สัมผัสกับเบอร์จินินความเข้มข้นต่างๆ (10 – 100 ไมโครโมลาร์) ก่อน นาน 48 ชั่วโมง จากนั้นจึงแทนด้วยกลูตาเมทความเข้มข้น 500 ไมโครโมลาร์ นาน 8 ชั่วโมงแก่เซลล์ประสาทแกรนูล ส่วนที่สองเป็นการให้สัมผัสกับเบอร์จินินความเข้มข้นต่างๆ (10-100 ไมโครโมลาร์) และกลูตาเมท 500 ไมโครโมลาร์พร้อมกันนาน 8 ชั่วโมง ผลจากการทดลองแสดงให้เห็นว่าทั้งการสัมผัสกับเบอร์จินินก่อนและพร้อมกับกลูตาเมทไม่มีผลป้องกันพิษต่อเซลล์ประสาทแกรนูลเพาะเลี้ยงจากกลูตาเมท ในทางตรงข้ามเบอร์จินินกลับแสดงแนวโน้มว่ามีผลเสริมความเป็นพิษต่อเซลล์ของกลูตาเมทโดยเพิ่มผลกดการทำงานของไมโตคอนเดรีย (แม้ว่าผลดังกล่าวจะไม่มีนัยสำคัญทางสถิติก็ตาม) เบอร์จินินไม่เปลี่ยนแปลงระดับการเกิดไลปิดเปอร์ออกซิเดชั่นและอาจมีผลฟื้นฟูระดับของกลูตาไทโอนที่ลดลงจากกลูตาเมทได้บ้าง (แม้จะไม่มีนัยสำคัญทางสถิติก็ตาม) ทั้งในการทดลองที่ให้สัมผัสเบอร์จินินก่อนและสัมผัสพร้อมกับกลูตาเมท โดยสรุปผลการทดลองแสดงให้เห็นว่าเบอร์จินินในความเข้มข้นสูงมีผลโดยตรงลดการทำงานของไมโตคอนเดรียในเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมและในความเข้มข้นต่ำไม่แสดงผลที่เป็นประโยชน์ต่อเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัม ที่เกิดพิษจากกลูตาเมท เป็นที่น่าสังเกตว่าเบอร์จินินมีแนวโน้มเกิดผลเสริมความเป็นพิษของกลูตาเมท ซึ่งผลไม่พึงประสงค์ที่อาจเป็นไปได้ดังกล่าวของเบอร์จินินและกลไกการออกฤทธิ์ต่อเซลล์ประสาทเพาะเลี้ยงยังไม่เป็นที่ทราบชัดในขณะนี้

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WORAWAN BOONYO: EFFECTS OF BERGENIN ON GLUTAMATE–INDUCED NEUROTOXICITY IN CULTURED RAT CEREBELLAR GRANULE CELLS. THESIS ADVISOR: ASST. PROF. SURACHAI UNCHERN, Ph.D., 71 pp. ISBN 974-17-9783-4

Bergenin is a compound isolated from several plants used in folk medicine in many Asian countries. In this study, effects of bergenin on cultured rat cerebellar granule cells and on glutamate–induced neurotoxicity in these cells were investigated by using cell viabillity, lipid peroxidation and glutathione content, as the measuring endpoints. Eight-day cultured cerebellar granule cells were used in all experiments. In preliminary experiment, cultured cells were exposed to bergenin (10–100 μM) for different time intervals. The results showed that mitochondrial function of cells exposed to bergenin (100 μM) for 48 hr was decreased. To study effects of bergenin on glutamate–induced neurotoxicity on cultured cerebellar granule cells, experiments were divided into two parts. The first part was performed by preexposing cerebellar granule cells to different concentrations of bergenin for 48 hr, then replaced by 500 μM glutamate for 8 hr. The second part was performed by coexposing cultured cells to bergenin (10–100 μM) and 500 μM glutamate for 8 hr. Experimental results showed that both preexposure and coexposure with bergenin did not prevent glutamate-induced neurotoxicity in cultured cerebellar granule cells. Instead, bergenin tended to aggravate cytotoxicity of glutamate by intensifying the suppression of mitochondrial function. However, there was no statistically significant difference among glutamate-treated alone or preexposure or coexposure to bergenin groups. Bergenin did not alter cellular lipid peroxidation levels but might exert marginal, although statistically nonsignificant, restorative effects on glutamate-induced glutathione diminution in both preexposure and coexposure experiments. In conclusion, bergenin decreased mitochondrial activity of cultured cerebellar granule neurons at high concentrations while at lower concentrations did not showed any beneficial effects on glutamate–induced neurotoxicity in cultured rat cerebellar granule cells. It was notable that bergenin showed a tendency to intensify glutamate-induced neurotoxicity, although the difference was not statistically significant. This tentative adverse effect of bergenin and its mechanism of action on cultured neurons are still unclear at the present time.

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Contents

Page

Abstract (Thai)……………………………………………………………………………………iv

Abstract (English)………………………………………………………………………………..v

Acknowledgements………………………………………………………………………………vi

Contents…………………………………………………………………………………………..vii

List of Tables……………………………………………………………………………………..ix

List of Figures……………………………………………………………………………………xi

List of Abbreviations…………………………………………………………………………….xii

Chapter

I Introduction………………………………………………………………………..1

II Literature review……………………………………………………………………...3

The Cerebellum……………………………………………………………...3

Glutamate……………………………………………………………………7

Bergenin……………………………………………………………………12

III Materials and Methods ……………………………………………………………17

Materials………………………………………………………………..17

1. Experimental Animals…………………………………………………17

2. Chemicals………………………………………………………………17

3. Instruments…………………………………………………………….19

Methods…………………………………………………………………….20

1. Preparation of culture media, buffers and solutions…………20

2. Cerebellar granule neuron cultures…………………………………21

3. MTT reduction assay……………………………………………………24

4. Lactate dehydrogenase (LDH) release assay………………..25

5. Thiobarbituric acid reactive substances (TBARS) assay..…..26

6. Reduced plus oxidized glutathione (total glutathione) assay.27

IV Results……………………………………………………………………………..31

V Discussion and conclusion………………………………………………………….45

Page

References…………………………………………………….…………………………...51
Appendix……………………………………………………………………………………60
Vitae……..………………………………………………….…….………………………71

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

List of Tables

Table Page

1. Properties of excitatory amino acid receptors…..……..…….………..…………………...…8

2. The part of plants that contains bergenin………………………………...……………12

3. Effects of Bergenin on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 12 hr)……………………………………………………………………..61

4. Effects of Bergenin on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 24 hr)………. ……………………………………………………………61

5. Effects of Bergenin on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 48 hr)………. …………………………………………………………..62

6. Effects of Bergenin on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 12 hr)……………………………………………………………………..…62

7. Effects of Bergenin on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 24 hr)……………………………………………………………………..63

8. Effects of Bergenin on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 48 hr)……………………………………………………………………..…63

9. Effects of Glutamate on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 4 hr)………………………………………………………………………64

10. Effects of Glutamate on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 8 hr)……………………………………...……………...…64

11. Effects of Glutamate on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 4 hr)………………………………………………………………………65

12. Effects of Glutamate on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 8 hr)………………………………………………………………………65

13. Effects of Coexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by MTT reduction assay……………...……66

14. Effects of Coexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by LDH release assay ………………………...66

Table Page

15. Effects of Preexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by MTT reduction assay……...……………….67

16. Effects of Preexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by LDH release assay ..……………………….67

17. Effects of Bergenin on Lipid Peroxidation in Cultured Cerebellar Granule Neurons. Determined by TBARS assay. (Incubation time = 48 hr)……………………………………68

18. Effects of Coexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons. Determined by TBARS assay. …………………...68

19. Effects of Preexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons. Determined by TBARS assay……………………69

20. Effects of Bergenin on Glutathione Content in Cultured Cerebellar Granule Neurons. Determined by Total GSH assay. (Incubation time = 48 hr)………………………………..69

21. Effects of Coexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons.Determined by Total GSH assay…………………70

22. Effects of Preexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons.Determined by Total GSH assay………………..…...70

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

List of Figures

Figure Page

1. Anatomy of the brain, showing position of cerebellum……………………………….………4

2. Cerebellar granule cells in culture for 8 days in DMEM-high K^+ (25 mM KCl) medium……………………………………………………………………….…………………5

3. Synaptic organization of the basic cerebellar circuit module…..…………………...……6

4. Mechanisms of excitotoxicity………………………………..…………………………...11

5. The structure of bergenin………………..………………..……………………………13

6. Preparation of cerebellar granule cell cultures…………….…………….………………23

7. Molecular structure of MTT and its corresponding reaction products…………….……...24

8. The reaction of lactate dehydrogenase (LDH) catalyzes….……………………………25

9. The reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA)…..…………...27

10. The reaction of GSH, GSSG and GSSG reductase……………………..………………28

11. Effects of Bergenin on Cell Survival in Cultured Cerebellar Granule Neurons………35

12. Effects of Glutamate on Cell Survival in Cultured Cerebellar Granule Neurons……..36

13. Effects of Coexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. ………………………………………………………………37

14. Effects of Preexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. ………………………………………………………………38

15. Effects of Bergenin on Lipid Peroxidation in Cultured Cerebellar Granule Neurons……………………………………………………………………………………...39

16. Effects of Coexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons……………………………………………………….40

17. Effects of Preexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons..…………………………………………………….41

18. Effects of Bergenin on Glutathione Content in Cultured Cerebellar Granule Neurons……………………………………………………………………………………..42

19. Effects of Coexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons……………………………………………………...43

Figure | Page

20. Effects of Preexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons……………………………………..……………….44

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

List of Abbreviations

β	= beta
°C	= degree Celcius
μg	= microgram
μl	= microliter
μM	= micromolar
AA	= arachidonic acid
AMPA	= α-amino-3-hydroxy-5-methyl-ioxyzole-4-propionic acid
$BaCl_2$	= barium chloride
BDL	= bile duct ligation
Ca^{2+}	= calcium ion
CCl_4	= carbon tetrachloride
CNS	= central nervous system
DMEM	= Dulbecco's modified Eagle's medium
DMSO	= dimethylsulfoxide
DNA	= deoxyribonucleic acid
DNase	= deoxyribonuclease
DPBS	= Dulbecco's phosphate buffered saline
DPPH	= 1,1-diphenyl-2-picrylhydrazyl
DTNB	= 5,5'-dithiobis (2-nitrobenzoic acid)
ER	= endoplasmic reticulum
et al.	= et alii (and other)
FBS	= fetal bovine serum
Galc	= D-galactosamine
GSH	= glutathione
GSSG	= glutathione disulfide
HIV	= human immunodeficiency virus
HYP	= hydroxyproline
IC_{50}	= median inhibition concentration

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

K^+	= potassium ion
KCl	= potassium chloride
kg	= kilogram
L	= liter
LDH	= lactate dehydrogenase
MDA	= malondialdehyde
mg	= milligram
mM	= millimolar
MTT	= 3-(4,5–dimethylthiazol–2-yl)–2,5–diphenyltetrazolium bromide
mV	= millivolt
Na^+	= sodium ion
NADP	= nicotinamide adenine dinucleotide phosphate
NADPH	= nicotinamide adenine dinucleotide phosphate (reduced form)
nm	= nanometer
NMDA	= N-methyl-D-aspartate
nmol	= nanomole
NOS	= nitric oxide synthase
PBS	= phosphate buffered saline
RNA	= ribonucleic acid
ROS	= reactive oxygen speices
s-ALP	=serum levels of alkaline phosphatase
s-ALT	= serum levels of alanine aminotransferase
s-AST	= serum levels of aspartate aminotransferase
SEM	= standard error of mean
SOD	= superoxide dismutase
TBA	= thiobarbituric acid
TBARS	= thiobarbituric acid reactive substance
TLC	= thin layer chromatography
TNB	= 5-thio-2-nitrobenzoate

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

In mammalian central nervous system, glutamate (L-glutamic acid) is the major excitatory neurotransmitter. Glutamate not only mediates excitatory neurotransmission but also plays a crucial role in synaptic plasticity such as long-term potentiation (LTP), which plays a central role in learning and memory (Kawasaki et al., 1997; Rang et al., 2000).

However, excessive activation of glutamate receptor is neurotoxic, leading to neuronal degeneration and death. In many systems, including primary cultures of cerebellar neurons, glutamate neurotoxicity is mediated by excessive activation of NMDA receptors, leading to increased intracellular Ca^{2+}, which induces the neurotoxic process. (Llansola et al., 2000). This phenomenon, glutamate is highly toxic to neurons, dubbed exicitotoxicity (Rang et al., 2000). Glutamate neurotoxicity has been implicated in pathogenesis of several central nervous system disorders, including stroke, trauma and neurodegenerative diseases (Chen et al., 2000). The understanding of mechanism of glutamate neurotoxicity and of possible mechanisms to prevent it would be, therefore, of great interest for the treatment of central nervous system disease.

Bergenin is a C-glucoside of 4-O-methyl galic acid that has been isolated from a number of plants such as *Bergenia crassifolia*, *Corylopsis spicata* and *Shorea leprosula* etc. (Hay and Haynes, 1958). Bergenin has been used as a folk oriental medicine for treatment and therapy of gastrointestinal diseases such as gastritis, gastric ulcer, diarrhea and constipation (Chung et al., 2001). In addition, it has been reported that bergenin has antiinflammatory effect, antitussive effect and hypolipidaemic activity (Kim et al., 2000). Interestingly, the hepatoprotective effects of bergenin and its mechanism have been reported. The hepatoprotective effect of bergenin was evidenced by elevating the activities of glutathione S-transferase (GST) and glutathione reductase

(GR), and content of glutathione in carbon tetrachloride (CCl_4)–intoxicated hepatocytes (Kim et al., 2000). In addition, Bergenin also significantly prevented the elevation of hepatic malondialdehyde formation and depletion of reduced gluththione content in the liver of CCl_4–intoxicated rats (Lim et al., 2000a). The results of these studies implied that bergenin exerted antihepatotoxicity against CCl_4–induced cytotoxicity through glutathione-mediated detoxification as well as free radical scavenging activity.

From these activities of bergenin, it is interesting to investigate the effects of bergenin on glutamate-induced neurotoxicity in cultured rat cerebelar granule cells. Moreover, the effects of bergenin on primary cultures of cerebellar granule neurons have not been reported yet.

Therefore, the purposes of this study were to examine effects of bergenin in primary cultured rat cerebellar granule cells and to explore effects of bergenin on glutamate-induced neurotoxicity in cultured rat cerebellar granule cells by using biochemical analysis 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.
3. Thiobarbituric acid reactive substances (TBARS) assay – an indication of lipid peroxidation reaction.
4. Reduced plus oxidized glutathione (total glutathione) assay – an indication of cellular antioxidation.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

LITERATURE REVIEW

Cerebellum

Cerebellum is attached to the back of upper portion of brain stem, lying underneath occipital lobe of the cortex (Figure 1). It is concerned primarily with motor activity, yet like the basal nuclei, it does not have any direct influence on the efferent motor neurons. It functions indirectly by modifying the output of major motor systems of the brain.

Specifically, cerebellum contributes to the maintenance of balance, enhances muscle tone, and coordinates skilled, voluntary movements. When cortical motor areas send messages to muscles for the execution of a particular movement, the cerebellum is also informed of the intended motor command. In addition, this region receives inputs from peripheral receptors that apprise it of what is actually taking place regarding body movement and position. Cerebellum essentially acts as "middle management" comparing the "intentions" or "orders" of the higher centers with the "performance" of the muscles and then correcting any "errors" or deviations from the intended movement. The cerebellum even appears to be able to predict the future position of a body part in the next fraction of a second and to make adjustments accordingly. These adjustments are especially important for rapidly changing (phasic) activities like typing, playing the piano, or running (Sherwood, 1991).

The cerebellum constitutes only 10% of the total volume of the brain but contains more than half of all its neurons. Granule cells, the excitatory neurons, are one type neuron of cerebellar cortex (Kandel, Schwartz, and Jessell, 2000).

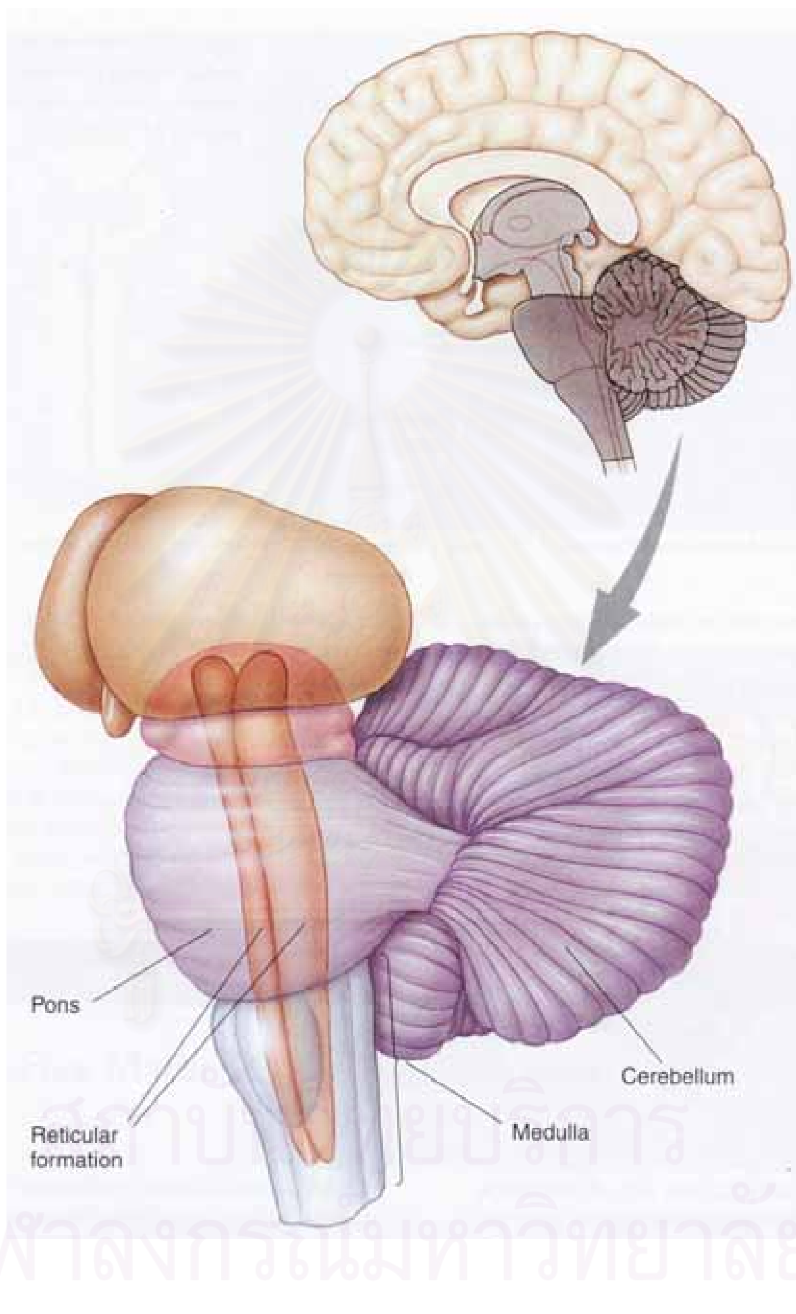

Figure 1. Anatomy of the brain, showing position of cerebellum (Pinel, 2000).

Granule cells

The granule cells are small and closely packed together in the deepest cortical layer. Each cell has a spherical nucleus with a coarse chromatin pattern, and the scanty cytoplasm lacks clumps of Nissl substance (Figure 2). The short dendrites have clawed–like endings that are contacted by mossy fibers. The unmyelinated axon enters the molecular layer, where it bifurcates and runs parallel with the folium. Because of the density of granule cell population, the whole molecular layer contains closely arranged parallel fibers. Each granule cell axon traverses the dendritic trees of some 450 Purkinje cells, making synaptic contacts with their dendritic spines. These axons also synapse with dendrites of stellate, basket, and Golgi cells in the molecular layer. Synaptic transmission of granule cells is a part of the circuit modules that is crucial for motor adaptation and learning (Figure 3) (Kiernan, 1998).

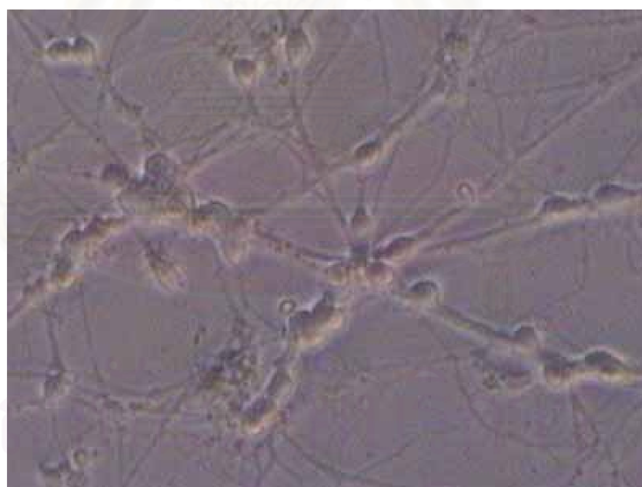

Figure 2. Cerebellar granule cells in culture (8 days) in DMEM–high K^+ (25 mM KCl) medium.

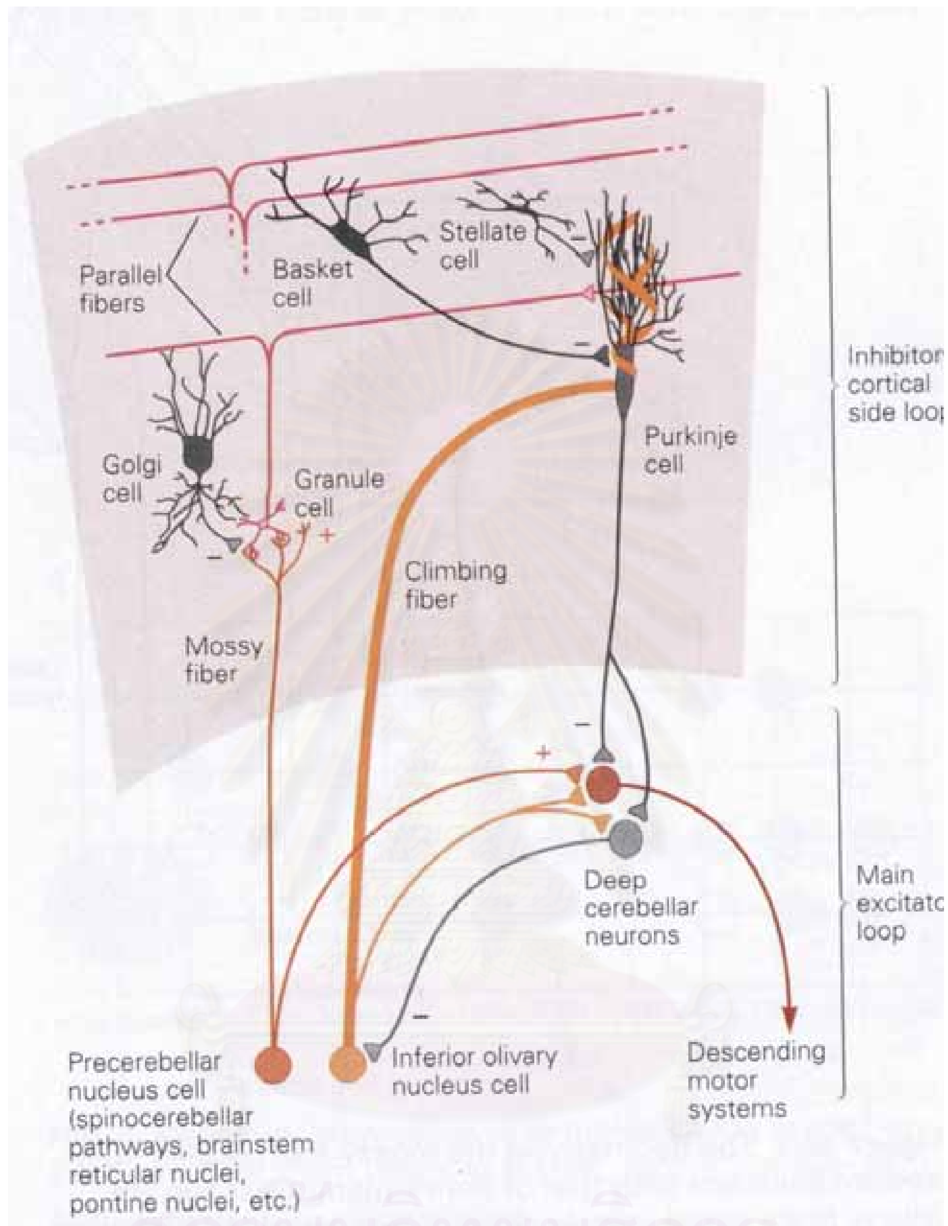

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Figure 3. Synaptic organization of the basic cerebellar circuit module. Mossy and climbing fibers convey input to the cerebellum via a main excitatory loop through the deep nuclei. This loop is modulated by an inhibitory side-loop passing through the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

Glutamate

Glutamate (L–Glutamic acid) is the most abundant excitatory neurotransmitter in the mammalian CNS, accounting for perhaps one–third of all rapid excitatory synapses in the CNS. Glutamate plays an important function as a neurotransmitter, in numerous excitatory local circuits in the cortex, hippocampus cerebellum and many other brain regions (Cotman et al., 1987, cited in Doble, 1999). Glutamate not only mediates excitatory neurotransmission but also is involved in other phenomena such as neuronal plasticity and cell death (neuroexcitotoxicity) (Kawasaki et al., 1997).

Glutamate receptors

There are four main subtypes of glutamate receptors, namely NMDA, AMPA, kainate and metabotropic receptors (Table 1). The first three (often-called ionotropic receptors) are ligand–gated ion channels according to their specific agonists and they have a pentameric structure. Other glutamate receptors (metabotropic) are monomeric G-protein–coupled receptors, linked to intracellular second messenger systems (e.g., phospholipase C or adenylyl cyclase) (Rang et al., 2000).

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Table 1. Properties of excitatory amino acid receptors (Rang et al., 2000).

	NMDA			AMPA		Kainate	Metabotropic
	Receptor site	Modulatory site (glycine)	Modulatory site (polyamine)	Receptor site	Modulatory site		
Endogenous agonists	Glutamate Aspartate	Glycine	Spermine Spermidine	Glutamate	??	Glutamate	Glutamate
Other agonists	NMDA	D - serine		AMPA Quisqualate	Cyclothiazide Aniracetam "Ampakine"*	Kainate Quisqualate	D - AP4 ACPD
Antagonists	AP - 5, AP – 7 CGS 19755 CPP SDZ EAA 494	Kynurenate Chloro - kynurenate HA - 466	Ifenprodil	NBQX CNQX		-	MCPG
Chanel blockers	Dizoclipine (MK801) Phencyclidine Ketamine Dextromethorphan Mg^{2+}			-		-	Not applicable
Effector mechanisms	Ligand - gated cation Channel (slow kinetics, High Ca^{2+} permeability)			Ligand - gated cation Channel (fast kinetics, low Ca^{2+} permeability)		Ligand - gated cation Channel (fast kinetics, low Ca^{2+} permeability)	G-protein-coupled (IP_3 formation and release of Ca^{2+})
Location	Postsynaptic (also glial) Wide distribution			Postsynaptic		Pre-and postsynaptic	Pre-and postsynaptic
Function	Slow EPSP Synaptic plasticity (LTP, LTD) Excitotoxicity			Fast EPSP Wide distribution		Fast EPSP ? presynaptic inhibition Limited distribution	Synaptic modulation Excitotoxicity

ACPD = 1-aminocyclopentane-1,3-dicarboxylic acid; AP-5 = 2-amino-5-phosphonopentanoic acid; AP-7 = 2-amino-7-phosphonoheptanoic acid; CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione; CPP = 3-(2-carboxypirazin-4-yl)-propyl-1phosphonic acid; NBQX = 2,3-dihydro-6-nitro-7-sulfamoyl-benzouinoxaline; MCPG =α-methyl-4-carboxyphenylglycine.

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Functional role of glutamate receptors

AMPA receptors are mainly responsible for fast excitatory synaptic transmission in the CNS. In some regions kainate receptors may also serve this role, but the evidence is incomplete. NMDA receptors (which often coexist with AMPA receptors) contribute a slow component to the excitatory synaptic potential, the magnitude of which varies in different pathways. Metabotropic glutamate receptors are linked either to IP_3 production and release of intracellular Ca^{2+} or to inhibition of adenylyl cyclase. They are located both pre- and postsynaptically, as well as on non-neuronal cells. Their effects on transmission are modulatory, rather than direct, comprising mainly postsynaptic excitatory effects (by inhibition of potassium channels) and presynaptic inhibition (by inhibition of calcium channels).

NMDA and metabotropic glutamate receptors participate in various adaptive and pathophysiological events. Three such roles which are now generally accepted are synaptic plasticity, the pathogenesis of epilepsy and excitotoxicity (Rang et al., 2000).

Excitotoxicity

Excitotoxicity is a phenomenon that neuronal cell death resulting from the toxic effects of the excitatory neurotransmitter glutamate (Nicholls and Ward, 2000). It is associated mainly with activation of NMDA receptors, but other types of glutamate receptors also contribute.

Calcium overload is the essential factor in excitotoxicity. The mechanisms by which this occurs and leads to cell death are as follows (Figure 4) (Rang et al., 2000):

- Glutamate activates NMDA, AMPA and metabotropic receptors (Sites 1, 2 and 3). Activation of AMPA receptors depolarizes the cell, which unblocks the NMDA-channels, permitting calcium entry. Depolarization also opens voltage-activated Ca^{2+} channels (Site 4), releasing more glutamate. Metabotropic receptors cause the release of intracellular Ca^{2+} from the

endoplasmic reticulum. Sodium entry further contributes to Ca^{2+} entry by stimulating Ca^{2+}/Na^{+} exchange (Site 5). Depolarization inhibits or reverses glutamate uptake (Site 6), thus increasing the extracellular glutamate concentration.

- The mechanisms that normally operate to counteract the rise in $[Ca^{2+}]_i$ include the calcium efflux pump (Site 7) and, indirectly, the sodium pump (Site 8).
- The mitochondria and endoplasmic reticulum act as capacious sinks for Ca^{2+}, and normally keep $[Ca^{2+}]_i$ under control. Loading of the mitochondrial stores beyond a certain point, however, disrupts mitochondrial function, reducing ATP synthesis, thus reducing the energy available for the membrane pumps and for Ca^{2+} accumulation by the endoplasmic reticulum. Formation of reactive oxygen species (ROS) is also enhanced. This represents the danger point at which positive feedback exaggerates the process.
- Raised $[Ca^{2+}]_i$ affects many processes, the chief ones relevant to neurotoxicity being:
  - Increased glutamate release
  - Activation of proteases (calpains) and lipases, causing membrane damage
  - Activation of nitric oxide synthase (NOS), which, together with ROS, generates peroxynitrite and hydroxyl free radicals, which react with several cellular molecules, including membrane lipids, proteins and DNA
  - Increased arachidonic acid release, which increases free radical production, and also inhibits glutamate uptake (Site 6).

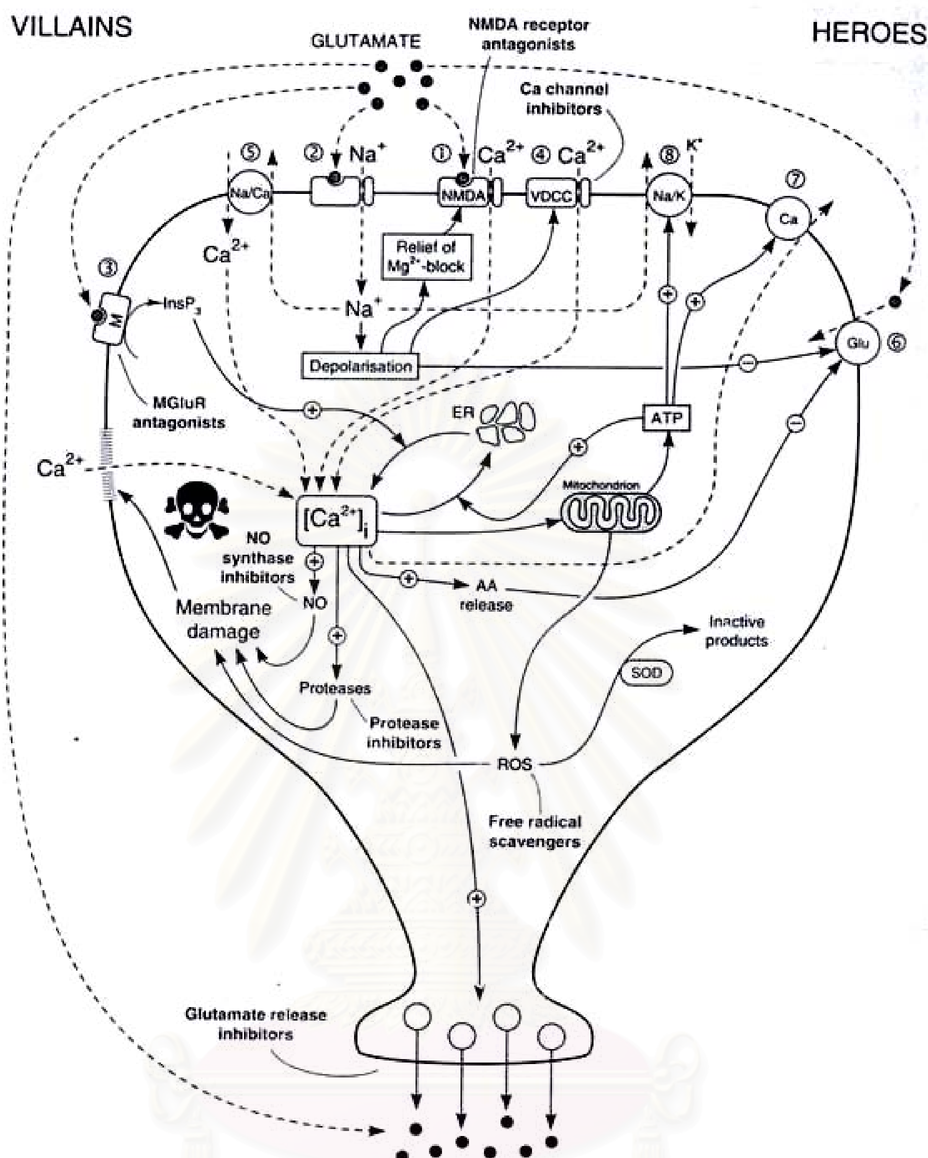

Figure 4. Mechanisms of excitotoxicity. Membrane receptors, ion channels and transporters, identified by number 1-8, are discussed in the text. Mechanisms on the left (villains) are those, which favor cell death, while those on the right (heroes), are protective. (ER = endoplasmic reticulum; AA = arachidonic acid; ROS = reactive oxygen species; SOD = superoxide dismutase) (Rang et al., 2000).

Bergenin

Bergenin occurs widely in a number of plants and has been found as an ingredient in plant extracts used in folk medicine to treat many diseases. It was isolated from many parts of trees that summarized in Table 2.

Table 2. The part of plants that contains bergenin.

Plants	References
the roots of *Bergenia crassifolia*	Sadikov and Guthner, 1927, cited in Hay and Haynes, 1958
the bark of *Corylopsis spicata*	Hattori, 1929, cited in Hay and Haynes, 1958
the heartwood of *Shorea leprosula*	Carruthers, Hay, and Haynes, 1957, cited in Hay and Haynes, 1958
the bark of *Macaranga peltata*	Ramaiah et al., 1979
the bark and cortex of *Mallotus japonicus*	Yoshida et al., 1982; Lim et al., 2000a; Lim et al., 2000c; Chung et al., 2001.
The roots of *Bergenia pupurascenes*	Min et al., 1987
The leaves of *Allophyllus edulis* var. *edulis*	Hoffmann-Bohm et al., 1992
The flowers of *Peltophorum pterocarpum* Back. Ex K. Heyne known as "Non Sree" (Thai name)	Siriwan Hirunyaphisutthikul, 1995
The aerial parts of *Ardisia japonica*	Piacente et al., 1996
The rhizomes of *Astilbe thunbergii* known as "Hong Shengma" (Chinese name) and "Aka-Shouma" (Japanese name)	Li et al., 1997, cited in Han et al., 1998

Table 2. The part of plants that contains bergenin (continue)

Plants	References
The leaves and roots of *Fluggea microcarpa* and *luvangetin*	Goel et al., 1997
The leaves of *Securinega melanthesoids*	Schütz, 1998
The bark of *Mallotus polyadenos*	Setzer, 1999
Bergenia ligulata	Chaunhan et al., 2000
The dried fruits of *Ardisia colorata* Roxb.	Sumino et al., 2002
The aerial parts of *Fluggea virosa* Roxb.ex Wild	Pu et al., 2002

Bergenin is dihydroisocoumarin derivative characterized by a β-D–glucosyl residue C–linked to a hydroxylated phenylcarboxylic acid ortho to the carboxyl group (Figure 5). In addition, the carboxyl group is esterified with the C–2-hydroxyl group of the glucosyl moiety to form a δ-lactone ring (Piacente et al., 1996). It has a melting point of 258 °C (decomposed), $[\alpha]^{24}_{D}$: -30° (C = 0.53,H_2O), UV, IR, FAB–MS, ^{1}H– and ^{13}C–NMR data were in accordance with published data (Murthy and Jairay, 1988, cited in Pu et al., 2002).

Figure 5. The structure of bergenin (Han et al., 1998).

Biological activities of bergenin

Bergenin is considered to be an active component of various plant species, which are employed in folklore medicine. For example, Malloti Cortex water extract, containing about 11-18% of bergenin, has been used as a folk medicine for treatment and therapy of gastrointestinal diseases such as gastritis, gastric ulcer, diarrhea and constipation (Okada et al., 1973; Abe et al., 1980). In addition, several studies on the biological activity of bergenin (e.g., Piegen, 1980; Swarnalakshmi et al., 1984; Jahromi et al., 1992) indicated a wide variety of effects such as anti–HIV activity, antiulcer activity, lipolytic effect, antiarrhythmic effect and hepatoprotective effect.

Anti–HIV activity of bergenin

Bergenin and related compound, norbergenin, that have been extracted from the aerial parts of *Adrisia japonica* showed the in vitro HIV inhibition in infected c8166 cells. Although they did not inhibit HIV replication, bergenin and norbergenin showed significant anti–HIV activity (Piacente et al., 1996).

Antiulcer activity of Bergenin

Bergenin and norbergenin have, moreover, antiulcer activity. Goel and others (1997) had pointed out that these compounds have protective activity against pylorus–ligated and aspirin–induced gastric ulcers in rats and guinea pigs. The study on prostaglandin release by human colonic mucosal incubates had indicated that gastroprotective effect of bergenin and norbergenin could be due to increased prostaglandin production (Goel et al., 1997).

The lipolytic effect of bergenin

Bergenin, isolated from the rhizomes of *Astilbe thunbergii*, has been shown to enhance norepinephrine–induced lipolysis at the concentrations of 1–1,000 μg/ml in a

concentration–dependent fashion in fat cells of 5 weeks old Wistar rats, while bergenin by itself did not cause lipolysis. Furthermore, this compound slightly stimulated adrenocorticotrophic hormone–induced lipolysis and inhibited insulin–induced lipogenesis from glucose (Han et al., 1998).

The antiarrhyhmic effect of bergenin

Anti-arrhythmic effects of bergenin, isolated from the aerial parts of *Fluggea virosa* Roxb. ex Wild, was investigated. Bergenin showed distinct therapeutic effects on $BaCl_2$-induced arrhythmias in rats and significantly countered arrhythmias induced by ligation and reperfusion of the coronary artery. Additionally, at 0.8 mg/kg, bergenin elevated the atrial fibrillation threshold in rabbits from 1.34 mV to 1.92 mV. These suggest that bergenin has good potential to treat cardiac arrhythmias (Pu et al., 2002).

The hepatoprotective effect of bergenin

Pharmacological studies indicated that bergenin and its derivatives have protective effects on D–galactosamine (Galc) and CCl_4–intoxicated rat hepatocytes (Hoffman–Bohm et al., 1991; Lim et al., 2000a; Lim et al., 2000c). Hoffman–Bohm and others (1991) found that bergenin and 11–o–galloylbergenin, found in the leaf extracts of *Allophyllus edulis*, exhibited moderate antihepatotoxic activity against CCl_4 and Galc cytotoxicity in primary cultured rat hepatocytes (Hoffman–Bohm et al., 1991). In addition, several studies had revealed that bergenin has many hepatoprotective mechanisms. Bergenin significantly reduced the activities of glutamic pyruvic transaminase and sorbitol dehydrogenase released from primary cultured rat hepatocytes subjected to CCl_4–induced cytotoxicity. The antihepatotoxicity of bergenin was also evidenced by elevating the activities of GST and GR, and content of glutathione in the CCl_4–intoxicated hepatocytes. It is assumed that bergenin exerted antihepatotoxicity against CCl_4–induced cytotoxicity through glutathione–mediated detoxification as well as free radical scavenging activity (Kim et al., 2000). It appeared that bergenin showed hepato-protective effects against Galc–intoxicated rat hepatocytes by inhibiting the release of

glutamic pyruvic transminase and sorbitol dehydrogenase as well as by increasing RNA synthesis (Lim et al., 2000c). Moreover, bergenin also significantly prevented the elevation of hepatic malondiadehyde formation and depletion of reduced glutathione content in the liver of rats with CCl_4–induced hepatic damage (Lim et al., 2000a). Acetylbergenin, one of the derivative of bergenin, has hepatoprotective effects against Galc–induced hepatotoxicity by inhibiting lipid peroxidation and maintaining an adequate level of GSH for the detoxification of xenobiotics as underlying hepatoprotective mechanisms (Lim et al., 2000b). It also has potent hepatoprotective activity against CCl_4–induced hepatic damage in rats by glutathione–mediated detoxification as well as having free radical scavenging activity (Lim et al., 2001).

Recently, Chung and others (2001) studied the effects of bergenin and acetylbergenin on liver fibrosis induced by bile duct ligation (BDL) in rats. They found that bergenin and acetylbergenin decreased towards normal the accumulated levels of hydroxyproline (HYP) (a marker of collagen accumulation) in the liver and the elevated serum levels of alanin aminotransferase (s-ALT), aspartate aminotransferase (s-AST) and alkaline phosphatase (s-ALP). These results indicated that bergenin and acetylbergenin ameliorated the liver damage induced by BDL in rats (Chung et al., 2001).

The antioxidant activity and cytotoxic effect

In the course of screening for antioxidants in Thai medicinal plants, bergenin that extracted from the fruits of *Adrisia colorata* Roxb. showed relative scavenging activity towards 1,1–diphenyl–2–picrylhydrazyl (DPPH) radicals in TLC autographic assay. Bergenin showed 131 μM of IC_{50} value and 0.3 mM of trolox equivalent value. Bergenin also showed weak activity (IC_{50} : 44 μM) in cytotoxicity against the murine breast cancer cell line, FM3A (Sumino et al., 2002).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental Animals

Postnatal day 8, Wistar rat (both sexes) were used in this study. They were obtained from time–pregnant rats of embryonic day 18 purchased from the National Laboratory Animal Center, Salaya, Mahidol University, Bangkok. 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. Yanawa, Bangkok, Thailand) and tap water *ad libitum*.

2. Chemicals

The following chemicals were used in the experiments:

Bergenin [Sigma]

n-Butanol [BDH]

Butylated hydroxytoluene [Sigma]

Calcium chloride [Ajax chemicals]

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 (DMEM) without phenol red [Sigma]

Dulbecco's phosphate buffered saline (DPBS) without calcium chloride [Sigma]

Equin serum [Hyclone]

Ethanol [Merck]

Fetal bovine serum (FBS) [Hyclone]

D–glucose, monohydrate [Unilab]

Glutathione, reduced form [Sigma]

Glutathione reductase [Sigma]

HEPES [Sigma]

Hydrochloric acid [Merck]

Insulin [Sigma]

In vitro Toxicology Assay Kit (Lactate Dehydrogenase Based) [Sigma]

L-Glutamic acid [Sigma]

Magnesium chloride [Merck]

Magnesium sulphate heptahydrate [BDH]

Methanol [BDH]

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]

Potassium dihydrogen phosphate [Sigma]

Poly-D-lysine hydrobromide (MW 15,000–30,000) [Sigma]

Progesterone [Sigma]

Putrescine [Sigma]

Pyruvic acid sodium salt [Fluka]

Sodium chloride [Ajax finechem]

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]

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Sodium selenite [Sigma]

Streptomycin Sulfate [Sigma]

Sulfosalicylic acid

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 [Labsystems]

Aluminium 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]

Cell strianer : 40 μm Nylon [Becton Dickinson]

Centrifuge [Kokusan]

Conical tube : 15 ml, 50 ml [Nunc]

Disposable glass Pasteur pipette: 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, Axiovert 135 [Zeiss]

Latex-free syringe: 10 ml [Becton Dickinson]

Laminar air flow hood [Hepaco]

96-well microtiter plate [Nunc]

Microplate reader [Biorad model 3550]

pH meter [Beckman Instruments]

Pipette tip: 1–200 μl, 200–1000 μl, 1–5 ml [Labsystems]

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]

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 nonpyrogenic distilled water followed by filter sterilization through a 0.22 μm membrane filter. They were kept at 4°C until used.

1.1 Culture media

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 medium was adjusted to pH 7.3 using 1 N NaOH and 1 N HCl.

1.2 Dulbecco's phosphate buffered saline (without Ca_2Cl) (DPBS)

DPBS was purchased from Sigma. It was used for the preparation of cerebellar granule cell culture and 0.5% triton–X 100. For 1 liter preparation, it consisted of $MgCl_2.6H_2O$ 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.

1.3 Hank 's balanced salt solution

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.

1.4 Locke's solution

Locke's solution was used for the exposure of cerebellar granule neuron cultures to glutamic acid. It contained 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose and 5 mM HEPES. The solution was adjusted to pH 7.4 using 1 N NaOH and 1 N HCl.

1.5 0.1 M Sodium phosphate buffer

0.1 M Sodium phosphate buffer was used for dissolving 5-5'- dithiobis-(2-nitrobenzoic acid) (DTNB) and NADPH. 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 pH 7.5 using 1 N NaOH and 1 N HCl.

2. Cerebellar granule neuron cultures (Figure 6)

Cultures of cerebellar granule cells were prepared from pooled cerebellar of 8–day old Wistar rat pups, a time when many of the granule neurons were still at an early post–mitotic stage of differentiation. Postnatal rats were decapitated under ether anesthesia by scissors. Cerebellar were removed aseptically from the brain.

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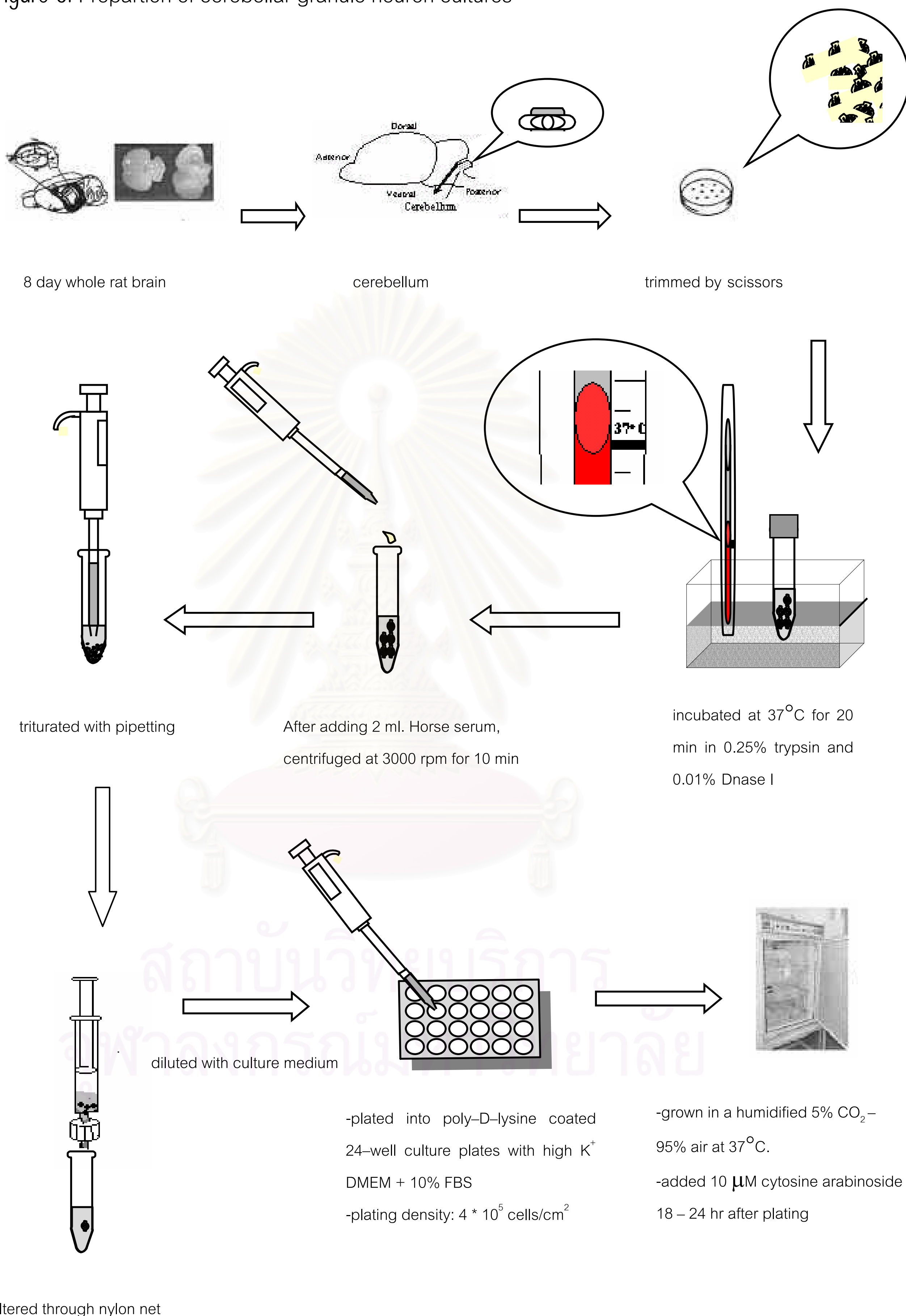
After removal of the meninges, the tissue was cut into cubes of about 0.4 mm side dimensions, and incubated for 20 min at 37 °C with 0.25% trypsin and 0.01% DNase I in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution. The incubation was terminated by the addition of horse serum and tissue fragments were centrifuged at 3,000 rpm for 10 min. The tissue pellet was gently rinsed and resuspended in high K^{+} (25 mM KCl) DMEM containing 10% FBS. The single cells were dissociated by gently 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 4×10^{5} 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. One-third of volume of 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 or Locke's solution plus desired concentrations of bergenin and/or glutamate.

Figure 6. Prepartion of cerebellar granule neuron cultures



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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 product in living cell but not in dead cells or their lytic debris (Figure. 7) (Mosman, 1983). The MTT assay is though to measure the ability of MTT to be reduced by electrons flowing through the mitocondrial electron transport chain and therefore reflects early redox changes within the cell. MTT is also reduced by superoxide that may be generated from mitochondrial oxidative metabolism by xanthine oxidase (Behl et al.,1994). The intracellular purple formazan, solubilized in dimethyl sulfoxide, is easily measured with a microplate reader (Skaper et al., 1998).

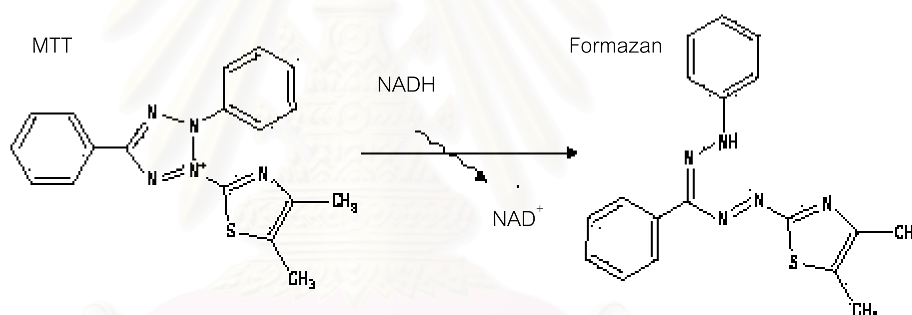

Figure 7. Molecular structure of MTT and their corresponding reaction products. (Methods for studying cell proliferation and viability in cell populations : assays that measure metabolic activity, n.d.)

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 or Locke's solution in each well (final concentrations were 100 μg/ml). Cultures were incubated in a CO_2 incubator for 1 h 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 thorough 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 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 8).

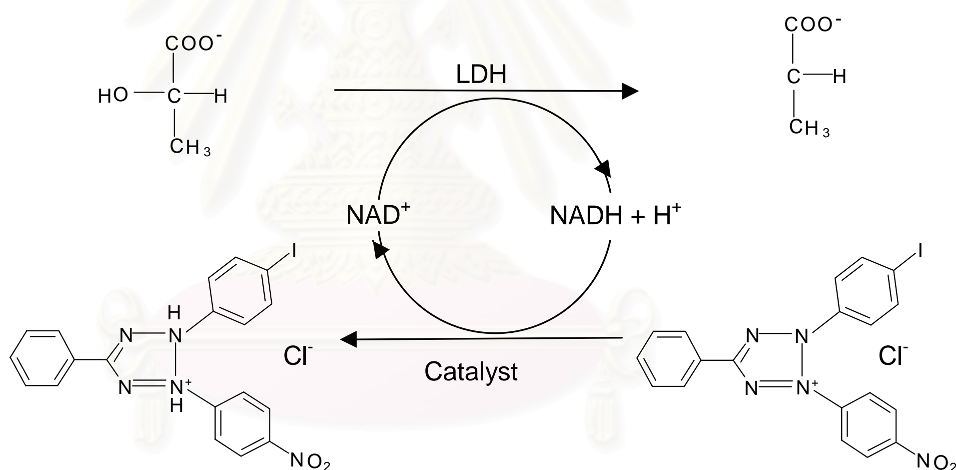

Figure 8. The reactions in lactate dehydrogenase (LDH) assay. (Cytotoxicity detection kit (LDH) : instruction manual version 5, 1999)

Cell viability was determined by assaying the medium from each well for LDH activities 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 or Locke's solution from each well into a 96–well microtiter plate. Cellular LDH in cultures was measured by carefully removed the culture medium from the adherent cells and solubilized cells with 1 ml of 0.5 Triton X-100 in PBS after which 100 μl aliquots were

pipette 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 and the reference wavelength was measured at 610 nm with a BIO–RAD 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:

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

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

5. Thiobarbituric acid reactive substance (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.

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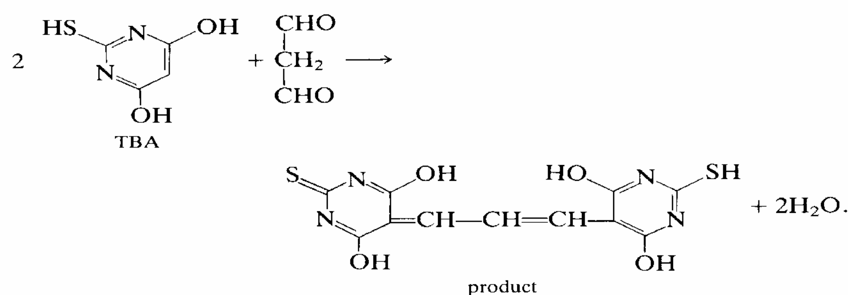

Figure 9. The reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA). (Halliwell and Gutteridge, 1989).

TBARS was measured using a technique modified from Ohkawa et al. (1979) and Storch et al. (2000). In this study, granular cultures grown in 24–well plate were lysed with 160 μl of 2% sodium dodecyl sulfate for 30 min. The lysates from six 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 are expressed in term of the percentage of control. 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 the 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.

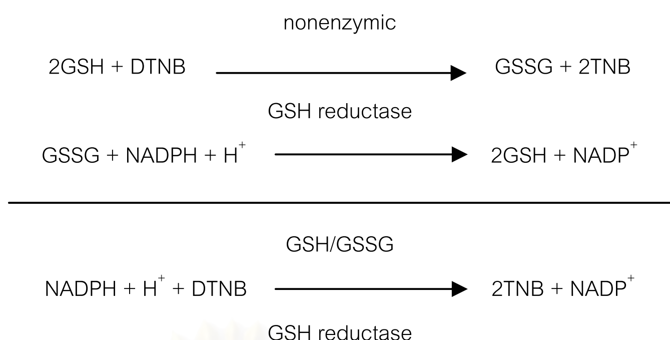

Figure 10. The reaction of GSH, GSSG and GSH reductase. (Akerboom and Sies, 1981)

In this study, granule cells were washed twice with 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. An aliquot 20 μl of cell extract was added to PBS and adjusted to 100 μl. Then an aliquot was added into the well of a 96-well microplate. The assay was initiated by addition of 100 μl of GSH reductase solution in 0.1 M sodium phosphate buffer. This 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 percentage of control. The cellular content of GSSG was typically less than 2% of GSH level and was not considered.

Experimental procedure

The study was divided into 4 parts as follows:

1. Determining effects of bergenin on cultured cerebellar granule cells
2. Determining glutamate neurotoxicity on cultured cerebellar granule cells
3. Determining effects of coexposure with bergenin on glutamate–induced cytotoxicity in cultured cerebellar granule cells

4. Determining effects of preexposure to bergenin on glutamate–induced cytotoxicity in cultured cerebellar granule cells

1. Determination of effects of bergenin on cultured cerebellar granule cells

Effects of begenin on cerebellar granule neurons were assay after 7 days of culture. Culture medium was removed. Cells were then replenished with serum-free high K^+ DMEM containing N2 supplement in the presence of bergenin. Bergenin was dissolved in 0.5% dimethylsulfoxide (DMSO) and then diluted to final concentrations of 0–100 μM in culture medium. The cells were incubated at 37 °C in 5% CO_2 atmosphere. Cell viability was measured 12, 24 and 48 hr later, by MTT cell viability assay and LDH release assay to determine the effects of time and concentration of bergenin exposure. TBARS assay and total glutathione assay were measured at 48 hr for clarifying the mechanism of bergenin's effects.

2. Determination of glutamate neurotoxicity on cultured cerebellar granule cells

Culture medium was removed on the experimental day. Cells were exposed to glutamate (0, 500 μM, 1 mM and 2 mM) in Locke's solution in the presence of 1 μM glycine. Glycine was added to fully activate the NMDA–sensitive glutamate recognition sites (Johnson and Ascher, 1987, cited in Atlante et al, 1999). Cells were incubated for 4 and 8 hr at 37 °C in 5% CO_2 atmosphere. Cell viability was determined by MTT cell viability assay and LDH release assay to determine the effects of time and concentration of glutamate exposure. TBARS assay and total glutathione assay were measured at 48 hr for clarifying the mechanism of glutamate's effects.

3. Determination of effects of coexposure to bergenin on glutamated–induced cytotoxicity in cultured cerebellar granule cells

On the day of the experiment, the medium was removed. Cells were then replenished with Locke's solution containing 500 μM glutamate and 1 μM glycine with

different concentration of bergenin (0–100 μM). They were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 8 hr. After the incubation period, MTT cell viability assay, LDH release assay, TBARS assay and total glutathione assay were conducted.

4. Determination of effects of preexposure to bergenin on glutamated–induced cytotoxicity in cultured cerebellar granule cells

On the day of the experiment, the medium was removed. Cells were then incubated with serum-free high K^+ DMEM containing N2 supplement in the presence of bergenin (0–100 μM) for 48 hr at 37 °C in a humidified atmosphere of 5% CO_2. After the incubation period, the medium was removed and replaced with Locke's solution in the presence of 500 μM glutamate and 1 μM glycine. MTT cell viability assay, LDH release assay, TBARS assay and total glutathione assay were conducted 8 h later.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) from groups of samples with n ≥ 6 separate experiments. Differences between control and treatment groups were analyzed using 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.

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CHAPTER IV

RESULTS

1. Effects of Bergenin on Cell Survival of Cultured Cerebellar Granule Neurons

Exposure of cultured cerebellar granule neurons for 12-24 hr to bergenin at different concentrations (10-100 μM) did not induce any significant changes in cell metabolic activity (MTT reduction) or cell death (LDH release). However, after an exposure for 48 hr to bergenin at 10-100 μM, cultured neurons showed a trend of cell damage at bergenin concentration of 75 μM (73% of control) and a certain degree of cell injury at 100 μM (60%, $p < 0.05$) (Fig. 11A). However, all concentrations of bergenin did not induce significant changes in the extent of cell death (Fig. 11B).

2. Effects of Glutamate on Cell Survival of Cultured Cerebellar Granule Neurons

Cultured cerebellar granule neurons were exposed to glutamate at different concentrations (500-2,000 μM) for 4 hr and 8 hr, at which times cell dysfunction and death were assessed using two different assays, MTT reduction and LDH release assays. Glutamate was toxic to cerebellar granule neurons in a time- and concentration-dependent manner. Using MTT reduction assay, exposure of cerebellar granule neurons to high concentrations of glutamate (1,000-2,000 μM) resulted in a decrease of MTT reduction to 85% – 89 % ($p < 0.05$) of control levels after 4 hr incubation period. This toxic effect on mitochondrial function gradually progressed to 45% - 63% of control at 8 hr of exposure to glutamate (500–2,000 μM) (Fig 12A). The pattern of LDH release following exposure to increasing concentrations of glutamate (500-2,000 μM) for 4 and 8 hr mirrored the pattern of decrease in MTT reduction. Glutamate significantly increased levels of LDH at 1,000-2,000 μM (to ~ 170% of control) at 8 hr (Fig 12B). Therefore, exposure to 500 μM of glutamate for 8 hr was used as an insulting condition in subsequent experiments of this study.

3. Effects of Coexposure to Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons

Coexposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) and glutamate at 500 μM for 8 hr did not show any beneficial effects on cell metabolic activity (MTT reduction) (Fig. 13A) or cell death (LDH release) (Fig 13B). Instead, the simultaneous exposure to both compounds aggravated the suppression of cell metabolic activity (to ~55% - 65% of control, $p < 0.05$) without any significant effects on cell death to cultured cerebellar granule neurons. However, the cytotoxic effect of coexposure on neurons did not differ from that of glutamate (500 μM) exposure alone.

4. Effects of Preexposure to Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons

Preexposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) for 48 hr before submission to glutamate exposure at 500 μM for 8 hr did not show any beneficial effects on cell metabolic activity (MTT reduction) (Fig.14A) or cell death (LDH release) (Fig. 14B). Instead, the preexposure to bergenin enhanced the suppression of cell metabolic activity (to ~ 55% - 63% of control, $p < 0.05$) without any significant effects on cell death to cultured cerebellar granule neurons. In addition, preexposure of cerebellar granule neurons to a low concentration of bergenin (10 μM) plus glutamate (500 μM) significantly augmented neuronal injury (56% of control) compared with glutamate-treated neurons (75% of control). But, there were no significant differences between preexposure with other bergenin concentrations (25 – 100 μM) and glutamate-treated neurons in cell metabolic activity (MTT reduction) and cell death (LDH release).

5. Effects of Bergenin on Lipid Peroxidation in Cultured Cerebellar Granule Neurons

Exposure of cultured cerebellar granule neurons with bergenin at different concentrations ranging from 10-100 μM for 48 hr before the determination of lipid peroxidation by TBARS assay revealed no significant effects on cellular lipid peroxidation (Fig 15).

6. Effects of Coexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons

Lipid peroxidation determinations in cultured cerebellar granule neurons coexposed with bergenin (at different concentrations ranging from 10-100 μM) and glutamate at 500 μM for 8 hr are shown in figure 16. No significant alterations in lipid peroxidation levels could be detected in cerebellar granule neurons.

7. Effects of Preexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons

Preexposure of cultured cerebellar granule neurons to bergenin (at different concentrations ranging from 10-100 μM) for 48 hr before submission to glutamate exposure at 500 μM for 8 hr showed no significant effects on cellular lipid peroxidation (Fig 17).

8. Effects of Bergenin on Glutathione Content in Cultured Cerebellar Granule Neurons

Exposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) for 48 hr before determination of total glutathione content revealed no significant effects on cellular GSH levels (Fig 18).

9. Effects of Coexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons

Glutamate (500 μM) treatment for 8 hr resulted in a 26% decrease ($p < 0.05$) in cellular GSH content (Fig 19) of cultured cerebellar granule neurons. This glutamate-induced glutathione diminution was partially restored towards control levels (81% - 96% of control) by coexposing cultured neurons with bergenin (at different concentrations ranging from 10-100 μM) and glutamate at 500 μM for 8 hr. However, this beneficial effect was marginal and did not reach statistically significant level.

10. Effects of Preexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons

Preexposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) for 48 hr before submission to glutamate exposure at 500 μM for 8 hr partially restored glutamate-induced glutathione diminution towards control levels (Fig 20). However, this beneficial effect was marginal and did not reach statistically significant level.

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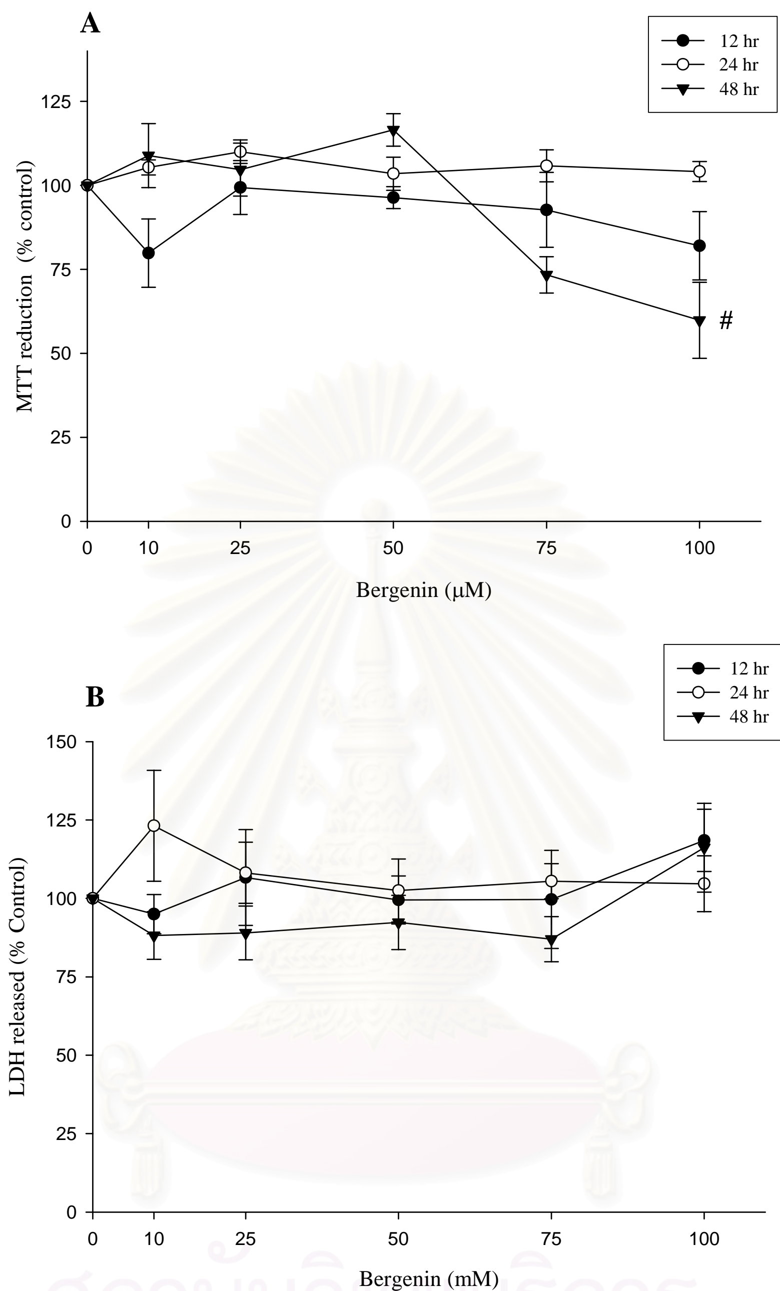

Figure 11. Effects of bergenin on viability of cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then treated with various concentrations of bergenin (0-100 μM) for 12, 24, and 48 hr. Cell viability was determined by MTT cell viability assay (A) and LDH release assay (B). Data are presented as mean ± SEM from six independent experiments (duplicate). $^{\#}p < 0.05$ vs. control.

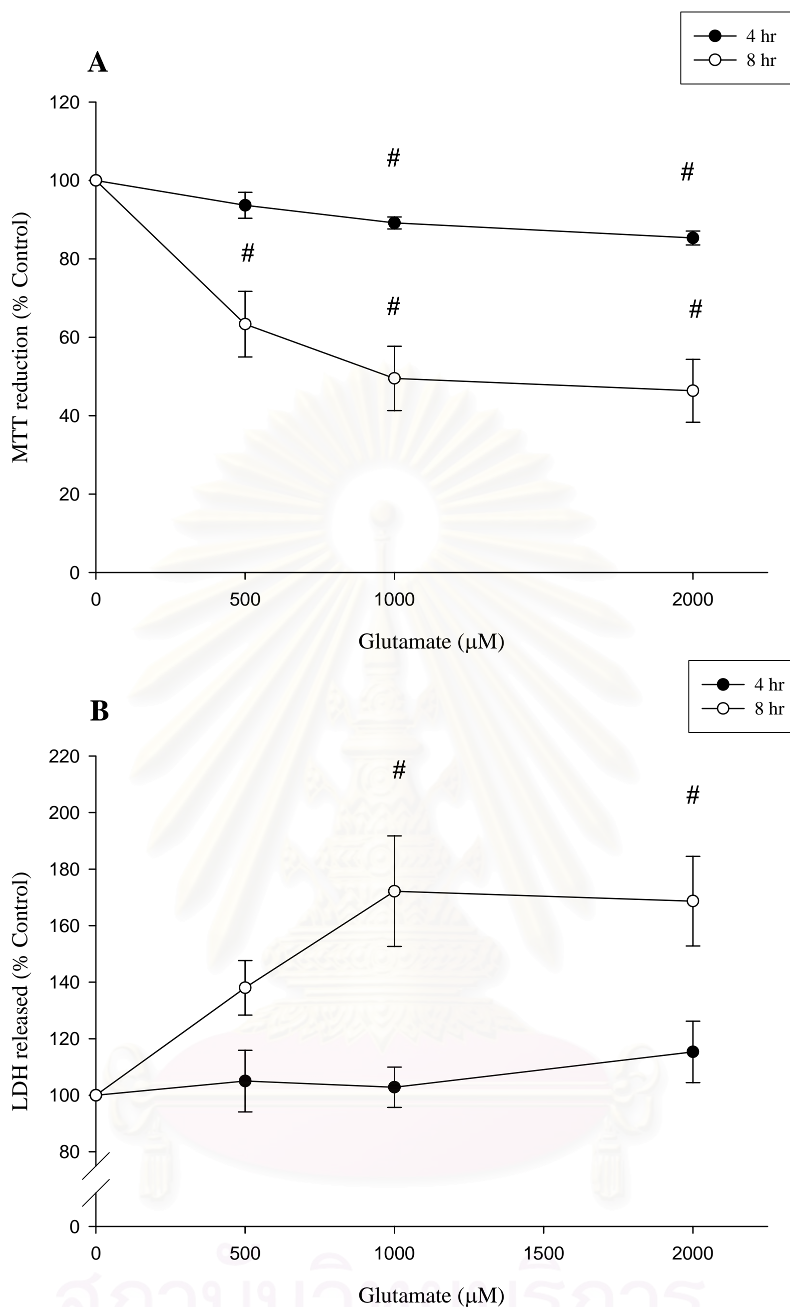

Figure 12. Effects of glutamate on cell survival of cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then exposed to the indicated concentrations of glutamate for 4 hr and 8 hr. Levels of MTT reduction by the cells (A) and LDH release (B) in the culture medium were shown in this figure. Data are mean ± SEM values of six separate experiments (duplicate cultures in each experiment). $^{\#}p < 0.05$ compared to control cultures.

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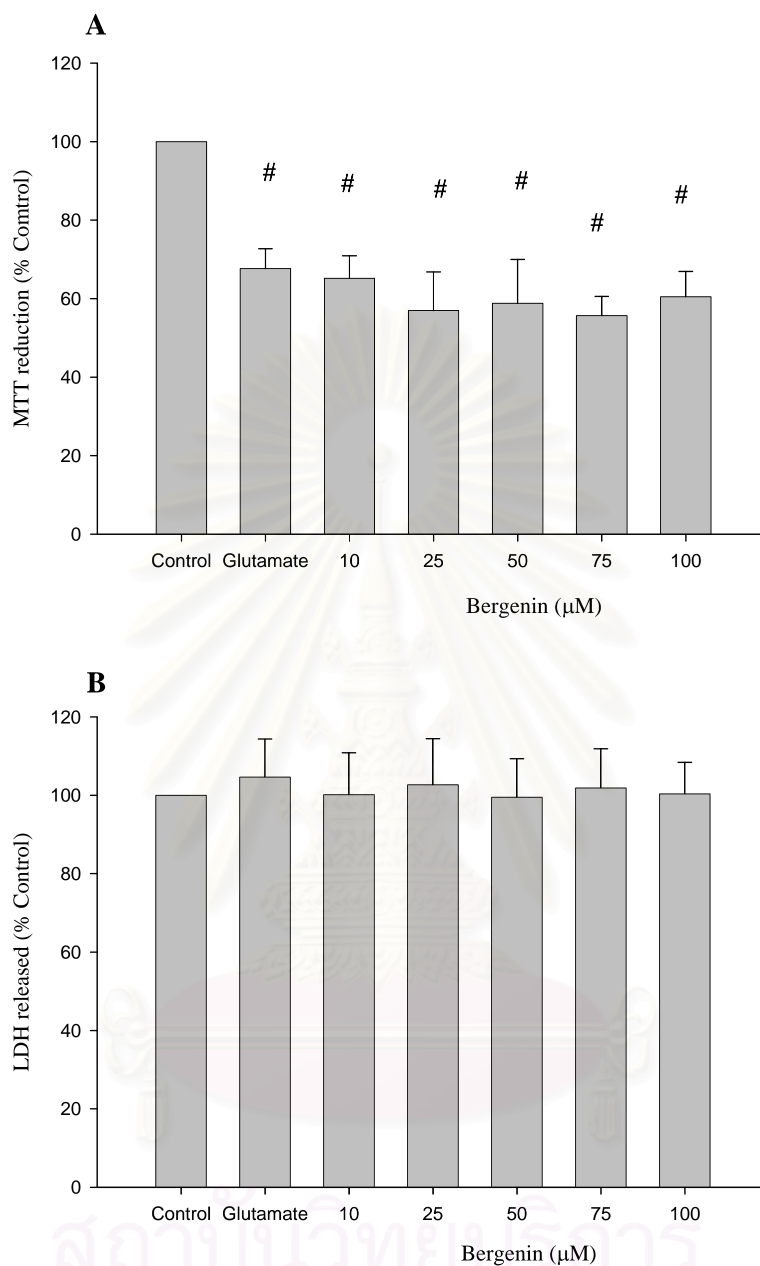

Figure 13. Effects of coexposure with bergenin on glutamate–induced cytotoxicity in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then exposed to glutamate (500 μM) for 8 hr with or without bergenin. Viability was then determined by MTT assay (A) and LDH release assay (B). Data are mean ± SEM from six independent experiments (duplicate). $^{\#}p < 0.05$ compared to control cultures.

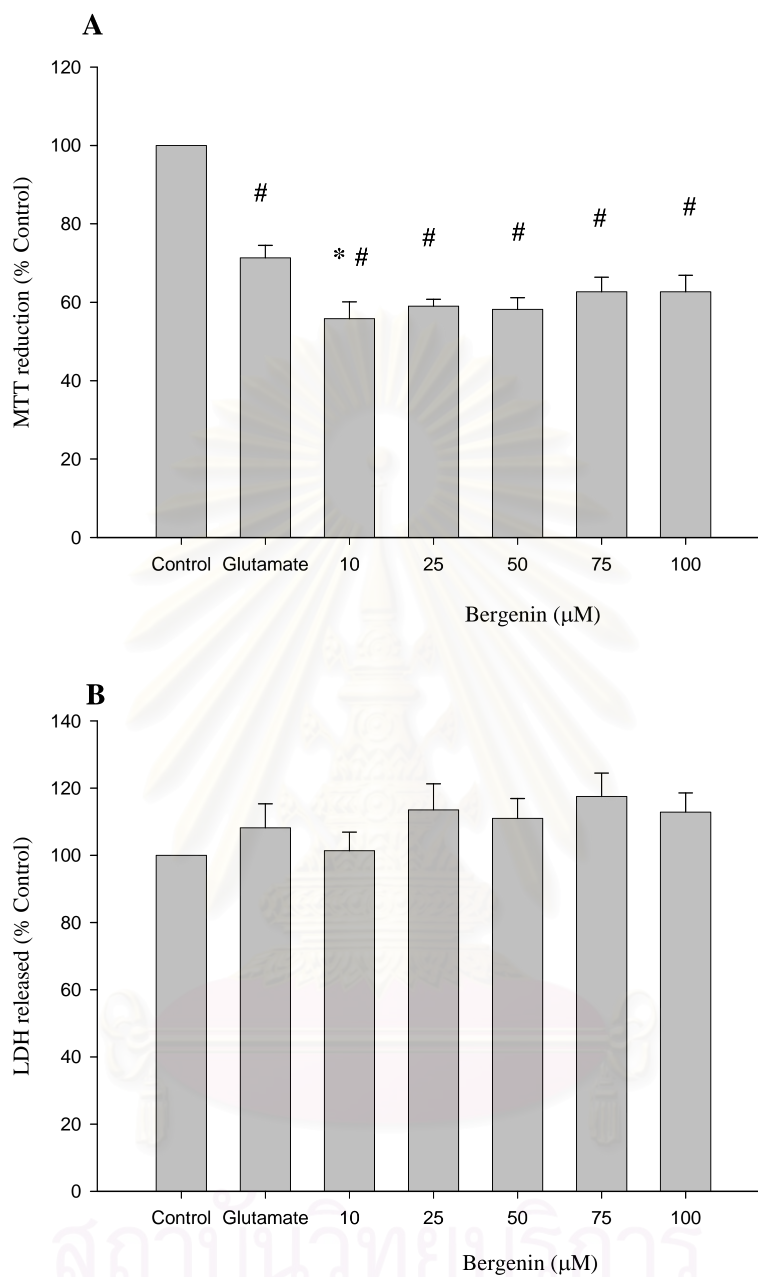

Figure 14. Effects of preexposure with bergenin on glutamate–induced cytotoxicity in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days. Bergenin was added at concentrations ranging from 10-100 μM 48 hr prior to glutamate exposure. Viability was determined by MTT assay (A) and LDH release assay (B). Data are mean ± SEM from six independent experiments (duplicate). $^{\#}p < 0.05$ compared to control cultures. $^{*}p < 0.05$ compared to glutamate-treated cultures.

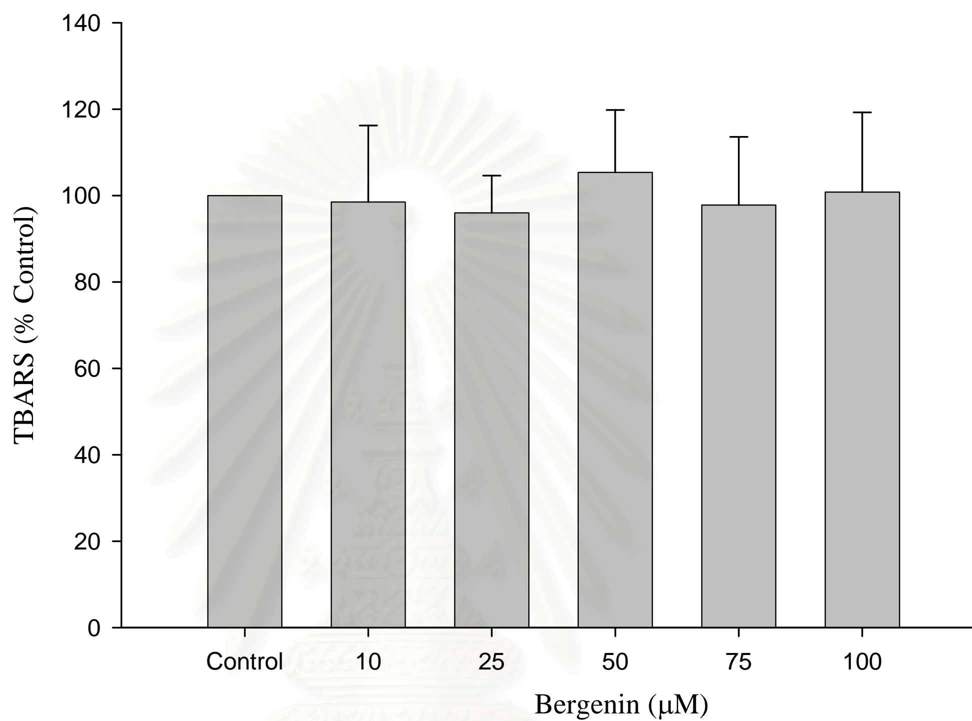

Figure 15. Effects of bergenin on lipid peroxidation in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then incubated with or without different concentrations of bergenin (0-100 μM) for 48 hr. Lipid peroxidation was measured by TBARS assay. Data are mean ± SEM from independent experiments (n = 6).

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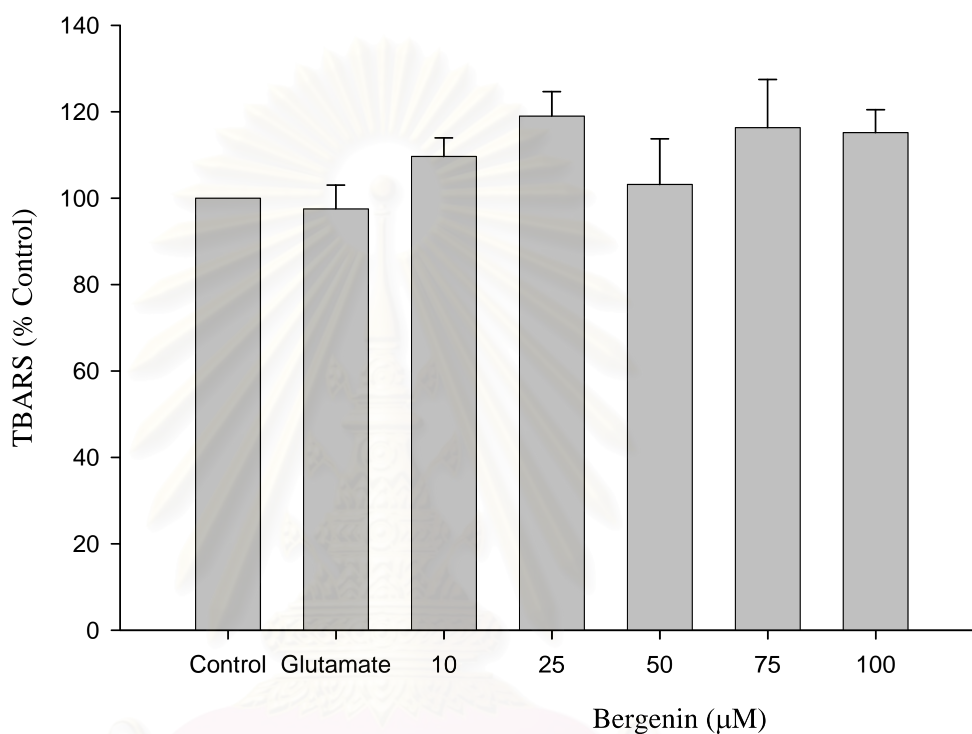

Figure 16. Effects of coexposure to bergenin on glutamate–induced lipid peroxidation in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then exposed to glutamate (500 μM) for 8 hr with or without bergenin. Lipid peroxidation was measured by TBARS assay. Data are mean ± SEM from independent experiments (n = 6).

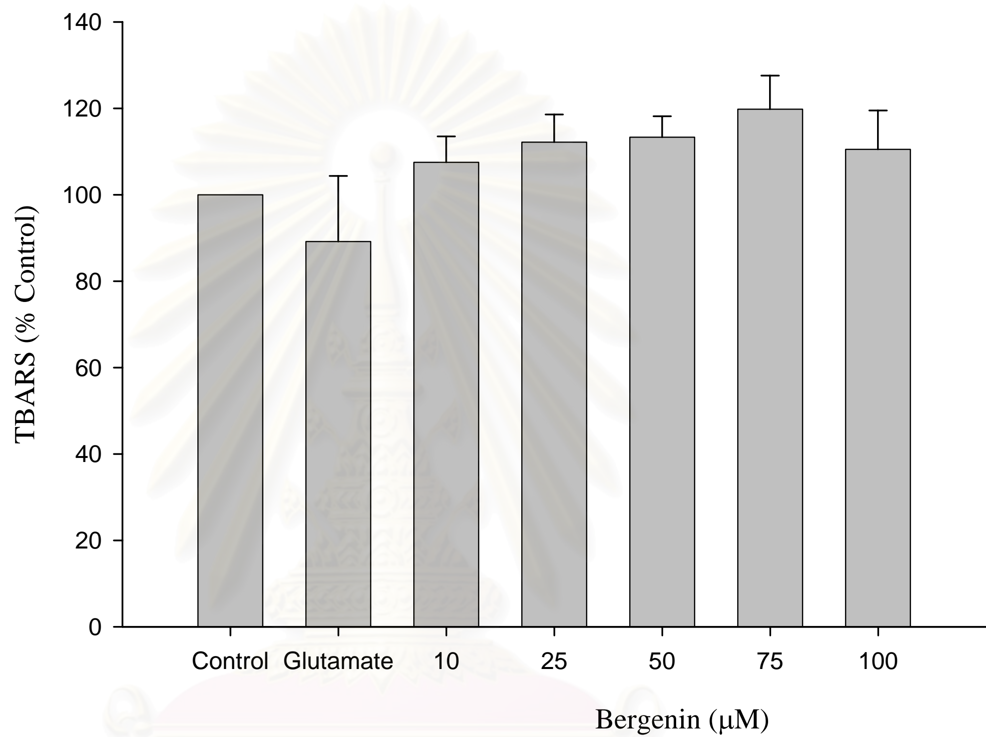

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Figure 17. Effects of preexposure to bergenin on glutamate–induced cytotoxicity in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days. Cells were preincubated with bergenin at concentrations ranging from 10-100 μM 48 hr before replacement with 500 μM glutamate for 8 hr. Lipid peroxidation was measured by TBARS assay. Data represent mean ± SEM from six independent experiments.

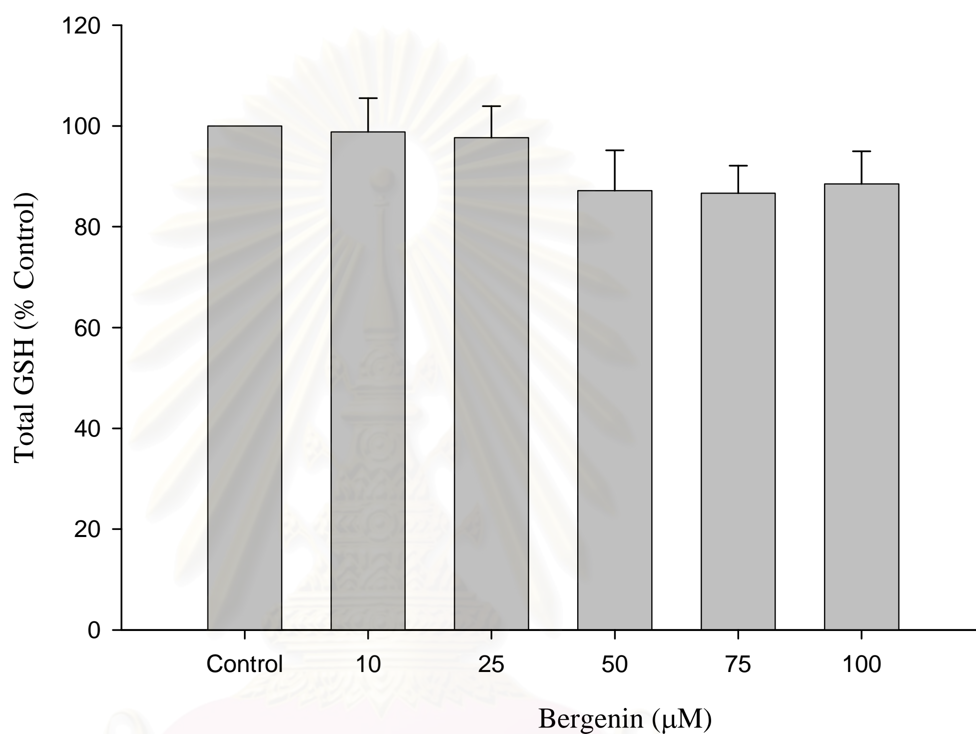

Figure 18. Effects of bergenin on glutathione content in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then were incubated with or without different concentrations of bergenin (0-100 μM) for 48 hr. Data represent mean ± SEM from six independent experiments.

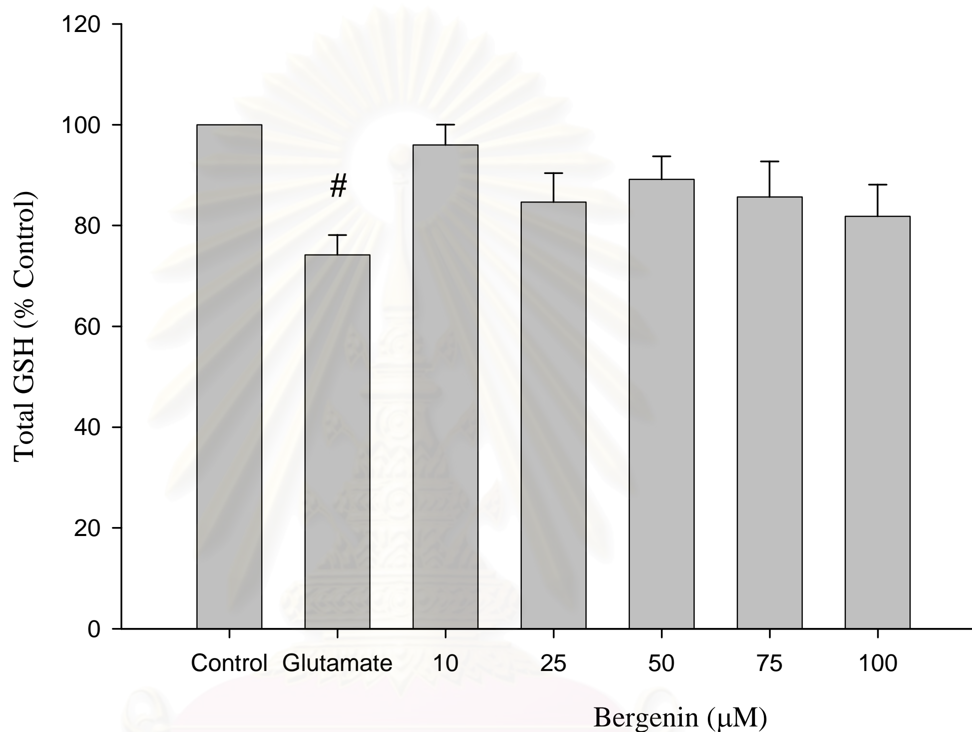

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Figure 19. Effects of coexposure with bergenin on glutamate–induced glutathione diminutuon in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days and then exposed to glutamate (500 μM) for 8 hr with or without bergenin. Data are mean ± SEM from six independent experiments (duplicate). $^{\#}p < 0.05$ compared to control cultures.

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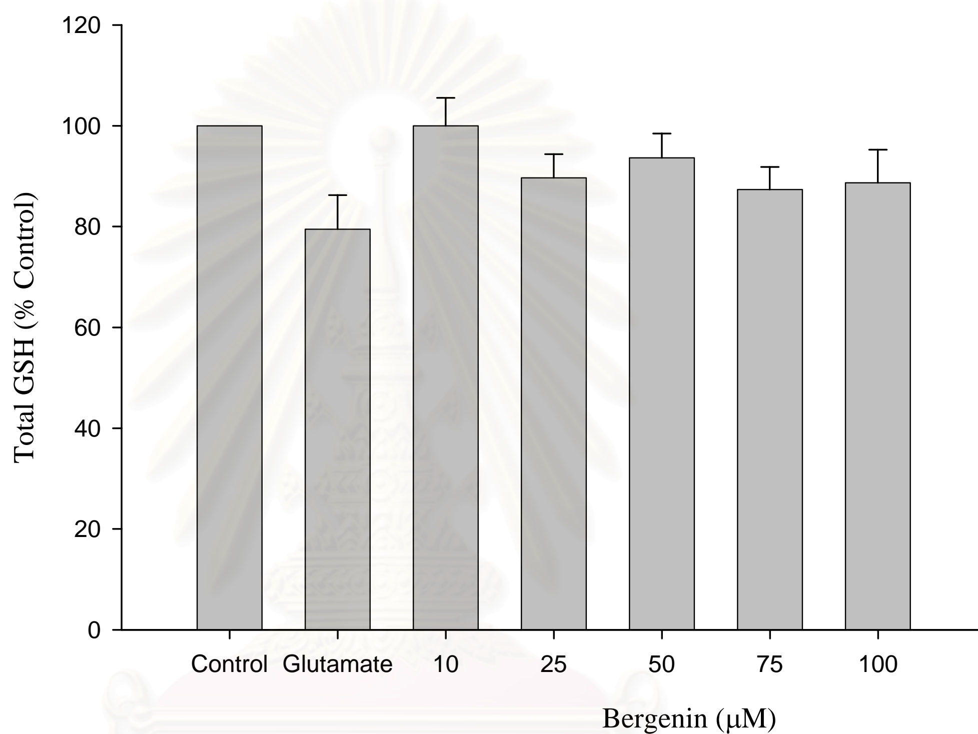

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Figure 20. Effects of preexposure with bergenin on glutamate–induced glutathione diminution in cultured cerebellar granule neurons. Cerebellar granule neurons were cultured for 8 days. Bergenin was added at concentrations ranging from 10-100 μM 48 hr prior to glutamate treatment. Data are mean ± SEM from six independent experiments (duplicate).

CHAPTER V

DISCUSSION AND CONCLUSION

Bergenin, a major constituent of *Mallotus japonicus*, had been reported to have a potent hepatoprotective action against carbon tetrachloride (CCl_4)-induced hepatic damage in rats (Lim et al., 2000c) and CCl_4-induced cytotoxicity in primary cultured rat hepatocytes (Kim et al., 2000). Bergenin significantly reduced the activities of glutamic pyruvic transaminase and sorbitol dehydrogenase released from the CCl_4-intoxicated hepatocytes. The antihepatotoxicity of bergenin was also evidenced by elevating the activities of glutathione S-transferase and glutathione reductase, and content of glutathione in the CCl_4-intoxicated hepatocytes. It was assumed that bergenin exerted antihepatotoxicity against CCl_4-induced cytotoxicity through glutathione-mediated detoxification as well as free radical suppressing activity. In addition, bergenin also showed hepatoprotective effect against D-galactosamine (GalN)-induced injury in primary cultured rat hepatocytes (Lim et al., 2000a). It was suggested that bergenin showed hepatoprotective effects against galactosamine-intoxicated rat hepatocytes by inhibiting the release of glutamic pyruvic transaminase and sorbitol dehydrogenase as well as by increasing RNA synthesis.

Considering available information on hepatoprotective effect of bergenin, this study was designed to investigate the effects of bergenin against glutamate-induced neurotoxicity on primary cultured rat cerebellar granule cells. It was speculated that bergenin might possess some cytoprotective effects to chemically induced neurotoxicity *in vitro*. However, the experimental results from this study did not support this speculation. On the other hand, they suggested the possibility that bergenin might potentiate glutamate-induced neurotoxicity by, at the present time, unclarified mechanisms. These findings suggest that bergenin may exert different actions on

different cell types, at least in *in vitro* conditions, as illustrated different effects on primary cultured hepatocytes and granule neurons.

Effects of bergenin on cultured rat cerebellar granule cells and on glutamate–induced neurotoxicity in these cells were investigated by using cell metabolic activity and cell viability (MTT reduction and LDH release assays), lipid peroxidation (TBARS assay) and content of glutathione (GSH), as the measuring endpoints.

MTT reduction assay was used to quantify mitochondrial metabolic activity by measuring the formation of a dark blue formazan product formed by the reduction of tetrazolium ring of MTT. The reduction of MTT is though to mainly occur in the mitochondria through the action of succinate dehydrogenase, thereby providing a measure of mitochondrial function (Lobner, 2000). In addition, Takahashi and other (2002) showed the substrate–preference for MTT reduction in cultures of rat type 1 neurons. It was indicated that pyruvate dehydrogenase supports MTT reduction more effectively than glucose or lactate even though both of these substrates can produce NADH and pyruvate (Takahashi et al., 2002).

Eight-day cultured cerebellar granule cells were used in all experiments. Exposure of cultured cerebellar granule neurons for 12-24 hr to bergenin at different concentrations (10-100 μM) did not induce any significant changes in cell metabolic activity (MTT reduction) or cell death (LDH release). However, after an exposure for 48 hr to bergenin at 10-100 μM, cultured neurons showed a trend of cell injury at bergenin concentrations of 75-100 μM. However, all concentrations of bergenin did not induce significant changes in the extent of cell death. By incubating primary cultured rat hepatocytes with 1 - 1,000 μM of bergenin for 1.5 hr (Kim et al., 2000) and 14 hr (Lim et al., 2000a), a certain degree of adverse effect was apparent at bergenin concentrations higher than 300 μM. This line of evidence is in accordance with results of the present study. At higher concentrations, especially with prolonged exposure, bergenin may exert nonspecific cytotoxic effects on cellular functions thereby suppressing mitochondrial

metabolic acitivity. However, this harmful effect of bergenin is not drastic enough to induce neuronal cell death.

Glutamate is the main excitatory neurotransmitter in mammals. However, excessive activation of glutamate receptors is neurotoxic, leading to neuronal degeneration and death. In many systems, including primary cultures of cerebellar neurons, glutamate neurotoxicity is mediated by excessive activation of NMDA receptors, leading to increased intracellular [Ca^{2+}], which induces the neurotoxic process (Llansola et al., 2000).

Glutamate was toxic to cerebellar granule neurons in a time- and concentration-dependent manner. In this study, exposure to 500 μM of glutamate for 8 hr was used as an insulting condition in subsequent experiments because this condition gave rise to approximately 50% decrease in MTT reduction activity of cultured neurons.

To study effects of bergenin on glutamate–induced neurotoxicity on cultured cerebellar granule cells, experiments were divided two parts. The first part was done by preexposing cerebellar granule cells with different concentrations of bergenin, before exposing to glutamate. The second part was done by simultaneous coexposing cultured cells with bergenin and glutamate. Experimental results showed that both preexposure and coexposure with bergenin did not prevent glutamate-induced neurotoxicity in cultured cerebellar granule cells.

Coexposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) and glutamate at 500 μM for 8 hr did not show any beneficial effects on cell metabolic activity (MTT reduction) or cell death (LDH release). Instead, the simultaneous exposure to both compounds tended to aggravate the suppression of cell metabolic activity without any significant effects on cell death to cultured cerebellar granule neurons. However, the cytotoxic effect of coexposure on neurons was not statistically different from that of glutamate (500 μM) exposure alone.

Preexposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) for 48 hr before submission to glutamate exposure at 500 μM for 8 hr did not show any beneficial effects on cell metabolic activity or cell death, in a similar fashion to the result of coexposure experiments. The only difference was that preexposure of cerebellar granule neurons to a low concentration of bergenin (10 μM) plus glutamate (500 μM) significantly augmented neuronal injury as compared to glutamate-treated neurons.

These findings were somewhat contradictory to the reported hepatoprotective activity of bergenin in primary cultured hepatocytes and rats. However, this discrepancy might be due to differences in cell types tested, insulting agent used, and conditions of bergenin exposure. Neurons are nondividing cells, highly demanding for metabolic energy, sensitive to insulting conditions, and relatively lack of effective antioxidative capacity. It is possible that hepatoprotective mechanisms of bergenin in cultured hepatocytes may involve some targets that are unavailable or ineffective in cultured neurons, e.g., glutathione (GSH) replenishing system.

Bergenin, by itself or in preexposure and coexposure with glutamate, did not alter cellular lipid peroxidation levels and total GSH content. Exposure of cultured cerebellar granule neurons with bergenin at different concentrations ranging from 10-100 μM for 48 hr, before the determination of lipid peroxidation by TBARS assay or total GSH content assay, revealed no significant effects on cellular lipid peroxidation or GSH content.

Preexposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) for 48 hr before submission to glutamate exposure at 500 μM for 8 hr showed no significant effects on cellular lipid peroxidation. Results of coexposure experiments were also in the same manner with those found in preexposure experiments.

In this study, the exposure of cultured cerebellar granule neurons with glutamate at 500 μM for 8 hr showed no significant effects on cellular lipid peroxidation. On one hand, this might be due to the non-intensive nature of the insulting condition which allowed cellular antioxidant capacity to counteract membrane damages. On the other hand, this might be due to the fact that small amounts of MDA formed in cell culture system were in a lower limit of the assay's sensitivity thereby making the quantitation inaccurate. However, it has been hypothesized that one of the principal causes of glutamate-induced neuronal injury is lipid peroxidation by free radical derivatives of glutamate (Skaper et al., 1998; Llansola, 2000).

In a state of oxidative stress, GSH is converted to GSSG and GSH depletion leading to lipid peroxidation. Therefore, the role of GSH as a marker for the evaluation of oxidative stress is reasonable (Recknagel et al., 1991 cited in Lim et al., 2000a). Exposure with glutamate at 500 μM for 8 hr showed a signigicant decrease in cellular GSH content in accordance with previous studies.

Preexposure of cultured cerebellar granule neurons with bergenin (at different concentrations ranging from 10-100 μM) for 48 hr before submission to glutamate exposure at 500 μM for 8 hr revealed a tentative restorative effect on glutamate-induced diminution of GSH content. Results of coexposure experiments were also in accordance with those found in preexposure experiments. These findings are in agreement with a previously reported findings in which antihepatotoxicity of bergenin was evidenced by elevating the activities of glutathione S-transferase and glutathione reductase, and content of glutathione in the CCl_4-intoxicated hepatocytes (Kim et al., 2000). However, the restorative effect of bergenin on glutamate-induced GSH diminution was only marginal (with statistical nonsignificance) and might not be effective enough to rescue neurons from glutamate-induced neurotoxicity.

Acetylbergenin, synthesized from acetylation of bergenin, showed increased lipophilic and physiological activities. It had hepatoprotective effects against GalN-induced hepatotoxicity by inhibiting lipid peroxidation and maintaining an adequate level

of GSH for the detoxification of xenobiotics. It was notable that lipophilic acetylbergenin showed more activity in the hepatoprotection than that of the much less lipophilic bergenin (Lim et al., 2000b).

Norbergenin, an O-demethyl derivative of bergenin, has recently been found to show moderate antioxidant activity (IC_{50} 13 μM in DPPH radical scavenging; 32 μM in superoxide anion scavenging). In this connection, norbergenin 11-caproate was the most potent derivative which not only exhibited stronger antioxidant activity but also prevented neuronal death at 10 μM on the primary culture of rat cortical neurons in DMEM supplemented with N2 (Takahashi et al., 2003).

These experimental findings suggest that bergenin, by itself, may not be a potential candidate for neuroprotective agent due to its low antioxidant activity and difficulty in entering the target cells. Modification of bergenin's molecular structure is currently undertaking in different chemical laboratories and may provide potential candidates for *in vivo* and *in vitro* preclinical studies in the future.

In conclusion, bergenin by itself decreased mitochondrial activity of cultured cerebellar granule neurons at high concentrations and at lower concentrations did not showed any beneficial effects on glutamate–induced neurotoxicity in cultured rat cerebellar granule cells. It was notable that bergenin showed a tendency to intensify glutamate-induced neurotoxicity, although the difference was not statistically significant. This tentative adverse effect of bergenin and its mechanism of action on cultured neurons are still unclear at the present time.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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APPENDIX

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3 Effects of Bergenin on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 12 hr)

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00 %	0
10	92%	105%	75%	51%	51%	105%	79.83 %	10
25	112%	116%	81%	82%	82%	123%	99.33 %	8
50	95%	99%	109%	89%	87%	99%	96.33 %	3
75	101%	42%	124%	100%	99%	90%	92.67 %	11
100	94%	43%	114%	88%	89%	64%	82.00 %	10

N = number of experiments (duplicated)

Table 4 Effects of Bergenin on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 24 hr)

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	111%	102%	109%	103%	110%	93%	105.335	2
25	109%	129%	112%	105%	108%	125%	110.00%	4
50	115%	107%	112%	109%	111%	90%	103.44%	5
75	110%	125%	114%	104%	113%	101%	105.78%	5
100	101%	120%	98%	111%	98%	96%	104.11%	3

N = number of experiments (duplicated)

Table 5 Effects of Bergenin on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 48 hr)

Bergenin	% Control							
(μM)	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	126%	92%	143%	82%	116%	94%	108.83%	10
25	112%	101%	93%	83%	100%	139%	104.67%	8
50	119%	115%	125%	116%	95%	129%	116.50%	5
75	91%	78%	83%	57%	60%	71%	73.33%	5
100	88%	93%	32%	50%	28%	68%	59.83%	11

N = number of experiments (duplicated)

Table 6 Effects of Bergenin on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 12 hr)

Bergenin	% Control							
(μM)	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	97%	100%	108%	68%	109%	88%	95.00%	6
25	71%	131%	118%	106%	157%	57%	106.67%	15
50	104%	99%	119%	64%	109%	102%	99.50%	8
75	61%	103%	109%	48%	133%	144%	99.67%	15
100	102%	122%	127%	94%	105%	161%	118.50%	10

N = number of experiments (duplicated)

Table 7 Effects of Bergenin on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 24 hr)

Bergenin	% Control							
(μM)	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	94%	102%	157%	99%	196%	91%	123.17%	17
25	89%	98%	110%	98%	155%	99%	108.17%	9
50	100%	108%	116%	70%	139%	82%	102.50%	10
75	94%	106%	126%	99%	117%	91%	105.50%	5
100	141%	118%	81%	88%	101%	99%	104.67%	8

N = number of experiments (duplicated)

Table 8 Effects of Bergenin on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 48 hr)

Bergenin	% Control							
(μM)	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	77%	61%	95%	82%	101%	113%	88.17%	7
25	86%	61%	100%	71%	97%	119%	89.00%	8
50	107%	59%	96%	75%	102%	115%	92.33%	8
75	88%	62%	111%	72%	96%	93%	87.00%	7
100	165%	82%	152%	85%	108%	105%	116.17%	14

N = number of experiments (duplicated)

Table 9 Effects of Glutamate on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 4 hr)

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
500	89%	88%	103%	87%	105%	90%	93.67%	3
1000	92%	93%	90%	85%	84%	91%	89.17%	1
2000	92%	79%	85%	84%	88%	84%	85.33%	1

N = number of experiments (duplicated)

Table 10 Effects of Glutamate on MTT reduction assay in Cultured Cerebellar Granule Neurons. (Incubation time = 8 hr)

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
500	90%	82%	48%	61%	35%	64%	63.33%	8
1000	76%	62%	41%	29%	27%	62%	49.50%	8
2000	76%	42%	65%	37%	28%	30%	46.33%	8

N = number of experiments (duplicated)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 11 Effects of Glutamate on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 4 hr)

Bergenin	% Control							
(μM)	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
500	98%	112%	95%	155%	79%	91%	105.00%	10
1000	91%	107%	92%	114%	83%	130%	102.83%	7
2000	91%	145%	104%	114%	88%	150%	115.33%	10

N = number of experiments (duplicated)

Table 12 Effects of Glutamate on LDH release assay in Cultured Cerebellar Granule Neurons. (Incubation time = 8 hr)

Bergenin	% Control							
(μM)	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
500	153%	99%	123%	163%	138%	152%	138.00%	9
1000	230%	99%	162%	193%	141%	208%	172.17%	19
2000	214%	108%	162%	180%	146%	202%	168.67%	15

N = number of experiments (duplicated)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 13 Effects of Coexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by MTT reduction assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	43%	73%	76%	81%	58%	60%	65.17%	5
25	42%	87%	50%	51%	85%	27%	57.00%	9
50	22%	82%	83%	72%	67%	27%	58.83%	11
75	58%	64%	71%	57%	46%	38%	55.67%	4
100	40%	61%	85%	59%	69%	49%	60.50%	6
Glutamate (500 μM)	66%	82%	75%	62%	74%	47%	67.67%	5

N = number of experiments (duplicated)

Table 14 Effects of Coexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by LDH release assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00	0
10	137%	97%	75%	76%	89%	127%	100.17	10
25	152%	87%	83%	75%	120%	99%	102.67	11
50	145%	83%	82%	84%	101%	102%	99.50	9
75	138%	87%	79%	80%	103%	124%	101.83	10
100	131%	91%	81%	82%	102%	115%	100.33	8
Glutamate (500 μM)	144%	104%	78%	85%	100%	117%	104.67	9

N = number of experiments (duplicated)

Table 15 Effects of Preexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by MTT reduction assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	64%	58%	39%	67%	59%	48%	55.83%	4
25	58%	61%	61%	65%	56%	53%	59.00%	1
50	60%	56%	69%	63%	51%	50%	58.17%	2
75	66%	57%	76%	62%	66%	49%	62.67%	3
100	65%	62%	73%	74%	54%	48%	62.67%	4
Glutamate (500 μM)	77%	66%	78%	80%	65%	62%	71.33%	3

N = number of experiments (duplicated)

Table 16 Effects of Preexposure with Bergenin on Glutamate-Induced Cytotoxicity in Cultured Cerebellar Granule Neurons. Determined by LDH release assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00	0
10	97%	98%	103%	124%	82%	104%	101.33	5
25	115%	105%	138%	133%	89%	101%	113.50	7
50	126%	87%	115%	121%	116%	101%	111.00	5
75	113%	126%	118%	145%	108%	95%	117.50	6
100	128%	113%	104%	128%	112%	92%	112.83	5
Glutamate (500 μM)	100%	120%	117%	132%	93%	87%	108.17	7

N = number of experiments (duplicated)

Table 17 Effects of Bergenin on Lipid Peroxidation in Cultured Cerebellar Granule Neurons. Determined by TBARS assay. (Incubation time = 48 hr)

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00	0
10	50%	108%	52%	100%	115%	166%	98.50	17
25	71%	101%	71%	111%	123%	99%	96.00	8
50	84%	104%	49%	141%	143%	112%	105.33	14
75	72%	96%	38%	129%	144%	108%	97.83	15
100	78%	86%	29%	127%	151%	134%	100.83	18

N = number of experiments

Table 18 Effects of Coexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons. Determined by TBARS assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100%	0
10	117%	121%	108%	116%	103%	93%	109.67%	4
25	120%	104%	109%	116%	144%	122%	119.00%	5
50	118%	100%	109%	115%	124%	51%	103.17%	10
75	115%	94%	111%	124%	165%	89%	116.33%	11
100	115%	97%	115%	122%	135%	107%	115.17%	5
Glutamate (500 μM)	103%	100%	89%	119%	79%	95%	97.50%	5

N = number of experiments

Table 19. Effects of Preexposure to Bergenin on Glutamate-Induced Lipid Peroxidation in Cultured Cerebellar Granule Neurons. Determined by TBARS assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00	0
10	134%	100%	112%	103%	105%	91%	107.50	5
25	137%	94%	109%	110%	123%	100%	112.17	6
50	125%	118%	122%	104%	117%	94%	113.33	4
75	139%	136%	129%	106%	119%	90%	119.83	7
100	149%	107%	103%	113%	110%	81%	110.50	8
Glutamate (500 μM)	125%	59%	29%	99%	110%	113%	89.17	15

N = number of experiments

Table 20. Effects of Bergenin on Glutathione Content in Cultured Cerebellar Granule Neurons. Determined by Total GSH assay. (Incubation time = 48 hr)

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	93%	109%	125%	100%	85%	81%	98.83%	6
25	96%	109%	119%	85%	77%	100%	97.67%	6
50	119%	78%	69%	69%	100%	88%	87.17%	8
75	111%	84%	75%	92%	77%	81%	86.67%	5
100	85%	75%	81%	115%	100%	75%	88.50%	6

N = number of experiments (duplicated)

Table 21. Effects of Coexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons.Determined by Total GSH assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	113%	93%	84%	91%	100%	95%	96.00%	4
25	67%	86%	80%	78%	88%	109%	84.67%	5
50	87%	93%	80%	78%	88%	109%	89.17%	4
75	67%	86%	84%	83%	76%	118%	85.67%	7
100	80%	79%	80%	61%	82%	109%	81.83%	6
Glutamate (500 μM)	67%	71%	80%	65%	71%	91%	74.17%	3

N = number of experiments (duplicated)

Table 22. Effects of Preexposure to Bergenin on Glutamate-Induced Glutathione Diminution in Cultured Cerebellar Granule Neurons.Determined by Total GSH assay.

Bergenin (μM)	% Control							
	N= 1	N= 2	N= 3	N= 4	N= 5	N= 6	Mean	SEM
Control	100%	100%	100%	100%	100%	100%	100.00%	0
10	100%	87%	100%	118%	112%	83%	100.00%	5
25	83%	93%	86%	109%	92%	75%	89.67%	4
50	92%	100%	86%	105%	104%	75%	93.67%	4
75	83%	93%	90%	95%	96%	67%	87.33%	4
100	75%	87%	90%	109%	104%	67%	88.67%	6
Glutamate (500 μM)	75%	93%	57%	73%	104%	75%	79.50%	6

N = number of experiments (duplicated)

CURRICULUM VITAE

Miss Worawan Boonyo was born in September 14, 1976 in Phitsanuloke, Thailand. She graduated with a Bachelor of Pharmacy in 1999 from the Faculty of Pharmacy, Chiangmai University, Chiangmai, Thailand. After graduation, she worked as a pharmacist in Sirinthorn Health Public College Phitsanuloke, Phitsanuloke for one year before enrolement into the Master of Science course in Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University..

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