ผลของสารสกัดบัวบกต่อการบาดเจ็บจากโฮโมซีสเทอีนในเซลล์ประสาทเพาะเลี้ยง

นางสาวภควดี กิจชนะพานิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

EFFECTS OF *CENTELLA ASIATICA* EXTRACT ON HOMOCYSTEINE-INDUCED INJURIES IN NEURONAL CELL LINE CULTURES

Miss Pakawadee Kidchanapanish

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmacology

Department of Pharmacology and Physiology

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2008

Thesis Title	EFFECTS OF *CENTELLA ASIATICA* EXTRACT ON HOMOCYSTEINE-INDUCED INJURIES IN NEURONAL CELL LINE CULTURES.
By	Miss Pakawadee Kidchanapanish
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Surachai Unchern, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

…….. Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Pramyothin, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Ph.D.)

…….……. Thesis Advisor

(Assistant Professor Surachai Unchern, Ph.D.)

…….. Examiner

(Associate Professor Vimolmas Lipipun, Ph.D.)

…….. Examiner

(Assistant Professor Lt. Pasarapa Towiwat, Ph.D.)

ภควดี กิจชนะพานิชย์ : ผลของสารสกัดบัวบกต่อการบาดเจ็บจากโฮโมซีสเทอีนในเซลล์ประสาทเพาะเลี้ยง. (EFFECTS OF *CENTELLA ASIATICA* EXTRACT ON HOMOCYSTEINE-INDUCED INJURIES IN NEURONAL CELL LINE CULTURES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. สุรชัย อัญเชิญ, 50 หน้า.

เอเซียติโคไซด์เป็นสารไตรเทอร์ปีนสำคัญในบัวบก ซึ่งรู้จักแพร่หลายในแง่ผลสมานแผล ทางด้านแพทย์แผนโบราณมีการใช้สารสกัดบัวบกและเอเซียติโคไซด์เพื่อบำบัดรักษาความผิดปกติต่างๆ ของระบบประสานส่วนกลาง รวมถึงโรคระบบประสาทเสื่อมและความจำบกพร่อง การวิจัยนี้ออกแบบเพื่อศึกษาสมรรถนะที่น่าจะเป็นของสารสกัดบัวบกและเอเซียติโคไซด์ในการป้องกันหรือบรรเทากระบวนการเสื่อมของระบบประสาท โดยใช้การบาดเจ็บของเซลล์เพาะเลี้ยง neuroblastoma N1E-115 ที่เกิดจากโฮโมซีสเทอีนเป็นแบบทดสอบนอกร่างกาย ตัวชี้วัดผลที่ใช้ได้แก่ การอยู่รอดและการตายของเซลล์ (วัดโดย MTT reduction และ LDH release) ปริมาณกลูตาไธโอน และรูปแบบการตายของเซลล์ การเลี้ยงเซลล์ N1E-115 ในสารสกัดบัวบกหรือเอเซียติโคไซด์ความเข้มข้น 1-100 ไมโครกรัมต่อมิลลิลิตร เป็นเวลา 24 หรือ 48 หรือ 72 ชั่วโมง ไม่ปรากฏผลต่อการอยู่รอดของเซลล์ การเลี้ยงเซลล์ในโฮโมซีสเทอีนซึ่งเป็นอะมิโนแอซิดที่มีส่วนประกอบของซัลเฟอร์ เหนี่ยวนำการบาดเจ็บและการตายของเซลล์ประสาทในลักษณะที่สัมพันธ์กับความเข้มข้นที่ใช้ โดยทำให้เซลล์ประมาณ 50% บาดเจ็บเมื่อใช้โฮโมซีสเทอีนความเข้มข้น 10 มิลลิโมลาร์เป็นเวลา 72 ชั่วโมง การเลี้ยงเซลล์ล่วงหน้าในสารสกัดบัวบก 100 ไมโครกรัมต่อมิลลิลิตร หรือเอเซียติโคไซด์ 50-100 ไมโครกรัมต่อมิลลิลิตร และการเลี้ยงเซลล์ร่วมในสารสกัดบัวบก 75-100 ไมโครกรัมต่อมิลลิลิตร หรือเอเซียติโคไซด์ 75-100 ไมโครกรัมต่อมิลลิลิตร พร้อมกับโฮโมซีสเทอีนความเข้มข้น 10 ไมโครโมลาร์เป็นเวลา 72 ชั่วโมง เพิ่มการอยู่รอดที่ลดลงจากผลของโฮโมซีสเทอีน และลดการตายของเซลล์ที่ตามมา นอกจากนั้นสารสกัดบัวบกและเอเซียติโคไซด์ความเข้มข้น 100 ไมโครกรัมต่อมิลลิลิตร ทำให้ปริมาณกลูตาไธโอนทั้งหมดที่ลดลงจากผลของโฮโมซีสเทอีนกลับเพิ่มขึ้น ตลอดจนลดการตายของเซลล์ประสาทแบบอะพอพโตสิสที่เกิดจากโฮโมซีสเทอีน

โดยสรุป การศึกษานี้ชี้แนะว่าสารสกัดบัวบก และเอเซียติโคไซด์อาจมีคุณสมบัติปกป้องเซลล์ประสาทต่อการบาดเจ็บของเซลล์อันเกิดจากโฮโมซีสเทอีนในระบบการทดสอบนอกร่างกาย โดยกลไกการปกป้องอาจเกี่ยวข้องโดยตรงกับคุณสมบัติต้านออกซิเดชันหรือโดยอ้อมกับการเหนี่ยวนำระบบต้านออกซิเดชันของเซลล์ประสาท นอกจากนั้นยังอาจยับยั้งการตายของเซลล์ประสาทแบบอะพอพโตสิสได้อีกด้วย อย่างไรก็ตามกลไกการออกฤทธิ์โดยละเอียดยังไม่เป็นที่เข้าใจอย่างถ่องแท้และต้องมีการวิจัยต่อไป

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2551

4876591033: MAJOR PHARMACOLOGY

KEYWORDS : ASIATICOSIDE / NEUROBLASTOMA CELL LINE / HOMOCYSTEINE

PAKAWADEE KIDCHANAPANISH : EFFECTS OF *CENTELLA ASIATICA* EXTRACT ON HOMOCYSTEINE-INDUCED INJURIES IN NEURONAL CELL LINE CULTURES. THESIS ADVISOR: ASST. PROF. SURACHAI UNCHERN, Ph.D. 50 pp.

Asiaticoside, a main triterpene of *Centella asiatica*, was well known for wound healing effect. In traditional medicine, *C. asiatica* extracts have been used in the management of central nervous system disorders including neurodegenerative diseases and memory deficit. The present research was designed to investigate the potential ability of *C. asiatica* extract and asiaticoside to prevent or attenuate the process of neurodegeneration in the *in vitro* model of homocysteine-induced injuries in cultured neuroblastoma N1E-115 cells. Cell viability and cell death (assessed by MTT reduction and LDH release respectively), glutathione content, and type of cell death were used as the measuring endpoints. Treatment of cultured N1E-115 cells with 1-100 μg/ml of *Centella asiatica* extract or asiaticoside for 24, 48 or 72 hr had no apparent effect on cell viability. Homocysteine, a non-essential sulphur-containing amino acid, induced neuronal injury and death in a concentration-related manner with approximately 50% cell injury occurred after an exposure to 10 mM homocysteine for 72 hr. Pre-treatment with 100 μg/ml of *Centella asiatica* extract or 50-100 μg/ml of asiaticoside, and co-treatment with 75-100 μg/ml of *Centella asiatica* extract or 75-100 μg/ml of asiaticoside, with homocysteine for 72 hr attenuated homocysteine-induced reduction in cell viability and successive cell death. In addition, *Centella asiatica* extract and asiaticoside at a concentration of 100 μg/ml replenished homocysteine-induced decrease of total glutathione content and attenuated homocysteine-induced apoptotic cell death.

In conclusion, the present study suggested that *Centella asiatica* extract and asiaticoside may possess the marginal *in vitro* cytoprotective property against homocysteine-induced neuronal injuries. The underlying mechanisms might directly involve antioxidant activity or indirectly involve facilitation of cellular antioxidant defense system of *Centella asiatica* extract and asiaticoside. In addition, they might antagonize cellular apoptotic cascade of cell death. However, the detailed mechanisms are not fully understood and remain to be further elucidated.

Department: Pharmacology and Physiology Student's signature……

Field of study: Pharmacology Advisor's signature……

Academic year: 2008

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my advisor, Assistant Professor Dr. Surachai Unchern for his valuable guidance and kind concern throughout my research study which enable me to accomplish this thesis.

I also would like to express my sincere gratitude to the committee members: Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Associate Professor Vimolmas Lipipun, and Assistant Professor Lt. Pasarapa Towiwat, for their worthy comments and suggestions.

I would like to thank all staff members and everyone in the Department of Pharmacology and Physiology. Special thanks are also extended to everyone in the Unit Cell of Pharmaceutical Biothchnology, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their help, friendly relationship, and provision of laboratory facilities.

Finally, I would like to express my deepest appreciation to my family and my friends for their helps, encouragement, and morale that have made me complete this work.

(This study was supported partly by the Graduate School, Chulalongkorn University).

CONTENTS

CONTENTS (CONT.)

Page

LIST OF FIGURES

FIGURE Page

LIST OF FIGURES (CONT.)

Page

LIST OF ABBREVIATIONS

%	=	percent
%v/v	=	percent of volume by volume (ml/100ml)
%w/v	=	percent of weight by volume (g/100ml)
AS	=	Asiaticoside
^{o}C	=	degree Celcius
C. asiatica	=	*Centella asiatica*
CA	=	*Centella asiaitca* extract
CAT	=	catalase
CNS	=	central nervous system
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	dimethyl sulfoxide
DTNB	=	5,5'-dithiobis-2-nitrobenzoic acid
EDTA	=	ethylenediaminetetraacetic acid
e.g.	=	example gratia (for example)
et al.	=	et alii (and other peoples)
etc.	=	et cetera (and other similar things)
FBS	=	Fetal bovine serum
Fig.	=	Figure
g	=	gram
GSH	=	glutathione
GSSG	=	glutathione disulfide
GRx	=	glutathione reductase
GPx	=	glutathione peroxidase
HCY	=	homocysteine
hr	=	hour
L	=	liter
LDH	=	Lactate dehydrogenase
M	=	molar (mole/liter)
MDA	=	malondialdehyde
mg	=	milligram

min	=	minute
ml	=	milliliter
mM	=	millimolar
mol	=	mole
MTT	=	3-[4,5-dimethiazol-2-yl]-2,5=diphenyltetrazolium bromide
NADH	=	nicotinamide adenine dinucleotide
NADPH	=	nicotinamide adenine dinucleotide phosphate
NMDA	=	N-methyl-D-aspartate
nm	=	nanometer
PBS	=	phosphate buffered saline
pH	=	potential of hydrogen
PI	=	propidium iodide
ROS	=	reactive oxygen species
rpm	=	round per minute
sec	=	second
S.E.M.	=	standard error of mean
SDS	=	sodium dodecyl sulfate
SOD	=	superoxide dismutase
USA	=	The United States of America
TBARS	=	thiobarbituric acid reactive substance
vs.	=	versus
μl	=	microliter
μg	=	microgram
μM	=	micromolar
α	=	alpha
β	=	beta
γ	=	gamma

CHAPTER I

INTRODUCTION

Background and Rationales

Alzheimer's disease is the most common cause of cognitive decline in the elderly, and affects around 12 million patients worldwide. Brain regions relevant for learning and memory, e.g., the hippocampus and adjacent areas, are particularly affected in Alzheimer's disease. There is increasing evidence for the involvement of dietary factors such as Vitamin B deficiency and increased homocysteine levels in adult neurological disorders (Christie, et al., 2004).

Homocysteine is a non-protein forming, sulphur-containing amino acid formed by demethylation of the essential amino acid methionine. It is an excitatory amino acid which markedly enhances the vulnerability of neuronal cells to excitotoxic and oxidative injury *in vitro* and *in vivo*. Clinical evidence suggested that medial temporal atrophy progression in patients with Alzheimer's disease was significantly greater among those patients with higher plasma homocysteine levels. A significant relationship between higher plasma homocysteine levels and brain atrophy in healthy elderly subjects, in elderly subjects at risk of Alzheimer's disease, and in patients with alcoholism has been observed (Maler et al., 2003).

Homocysteine has now been implicated in increased oxidative stress, DNA damage, the triggering of apoptosis and excitotoxicity, all important mechanisms in neurodegeneration (Mattson and Shea, 2003). Homocysteine is rapidly taken up by neurons through a specific membrane transporter, leading to high intracellular levels of homocysteine (Grieve et al., 1992). The brain may be particularly vulnerable to high levels of homocysteine in the blood because it lacks two major metabolic pathways for its elimination: betaine remethylation and transsulfuration (Finkelstein, 1998).

Centalla asiatica belongs to the family Umbelliferae and is found almost all over the world. It is herbaceous creeper growing to 50 cm with fan-shaped leaves. The whole plant is collected and dried for use. The major principles in the plant are the polyphenols (Abdul-Hamid et al., 2003) and triterpenes, mainly asiaticoside and asiatic acid (Inamdar et al., 1996). *Centella asiatica* was used in the management of central nervous system, skin and gastrointestinal disorders in Indian system of medicine (Singh and Rastogi, 1968). *Centella asiatica* has been shown to improve memory (Kuppurajan et al., 1978). It was also shown to have wound healing

property (Sugana et al., 1996), anticancer property (Babu et al., 1995), antioxidant property (Abdul-Hamid et al., 2003) and was also shown to have antileprotic property (Sahu et al., 1989).

Extracts of *Centella asiatica* exhibit various pharmacological actions including antipsoriasis (Natarajan and Paily, 1973), antianxiety (Bradwejn et al., 2000), antioxidant, anticancer and wound healing. As evidenced by previous unpublished observations that *Centella asiatica* extract and asiaticoside might be useful in slowing down neurodegenerative disease. The preventive effects might be mediated by their antioxidant properties or reduction of free radical activity.

This study is designed to investigate the potential ability of *Centella asiatica* extract to prevent or attenuate the process of neurodegeneration in the *in vitro* model of homocysteine-induced injuries in neuronal cell line cultures.

Hypothesis

Homocysteine induce cell injuries and death in N1E-115 neuroblastoma cell cultures that can be protected or attenuated by *Centella asiatica* extract.

Objective

1. To investigate toxic effects of homocysteine on N1E-115 neuroblasteoma cell cultures.
2. To examine protective effects of *Centella asiatica* extract on homocysteine-induced injuries in N1E-115 neuroblasteoma cell cultures.
3. To investigate possible mode of action of *Centella asiatica* extract-induced neuroprotection.
4.

Expected Benefit and Application

This study will provide new pharmacological knowledge on effects of homocysteine on neuronal cell line cultures and effects of asiaticoside to prevent or attenuate the process of neurodegeneration. The information from this study will be useful for the further application in herbal and traditional medicine development.

CHAPTER II

LITERATURE REVIEW

Homocysteine and Neurodegenerative Diseases

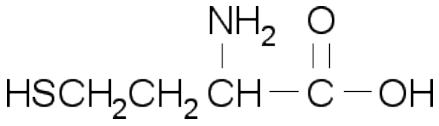


Fig. 1 Homocysteine structure

Homocysteine is a non-essential sulphur-containing amino acid produced as a natural consequence of methionine metabolism (Oldreive and Doherty, 2006), that is totally absent from any dietary source (Kim, et al., 2007). Homocysteine levels are normally maintained low by two major mechanisms. First, homocysteine is remethylated to form methionine by a reaction requiring folate and vitamin B_{12}. Second, homocysteine can be converted to cystathionine by the activity of the enzyme cystathionine-β-synthase (Chung et al., 2003).

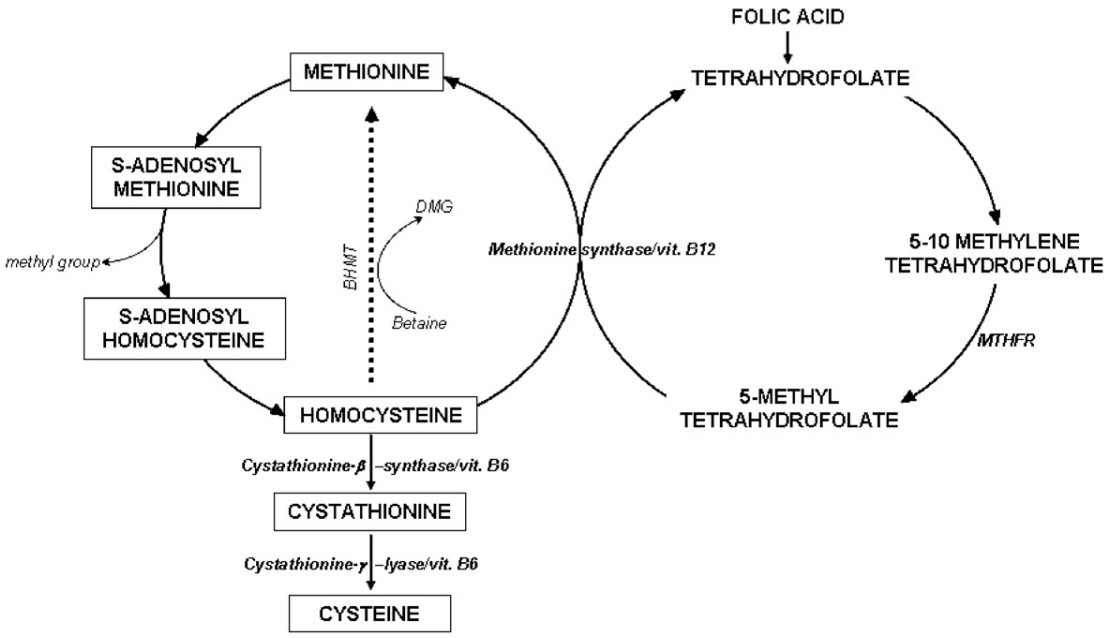


Fig. 2 Metabolic cycle of homocysteine; BHMT: betaine homocysteine methyl transferase; DMG: dymethylglycine; MTHFR: methylene tetrahydrofolate reductase (Martignoni et al., 2007).

CHAPTER II

LITERATURE REVIEW

Homocysteine and Neurodegenerative Diseases

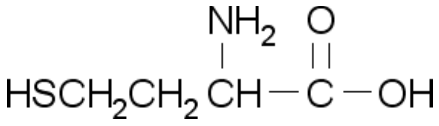


Fig. 1 Homocysteine structure

Homocysteine is a non-essential sulphur-containing amino acid produced as a natural consequence of methionine metabolism (Oldreive and Doherty, 2006), that is totally absent from any dietary source (Kim, et al., 2007). Homocysteine levels are normally maintained low by two major mechanisms. First, homocysteine is remethylated to form methionine by a reaction requiring folate and vitamin B_{12}. Second, homocysteine can be converted to cystathionine by the activity of the enzyme cystathionine-β-synthase (Chung et al., 2003).

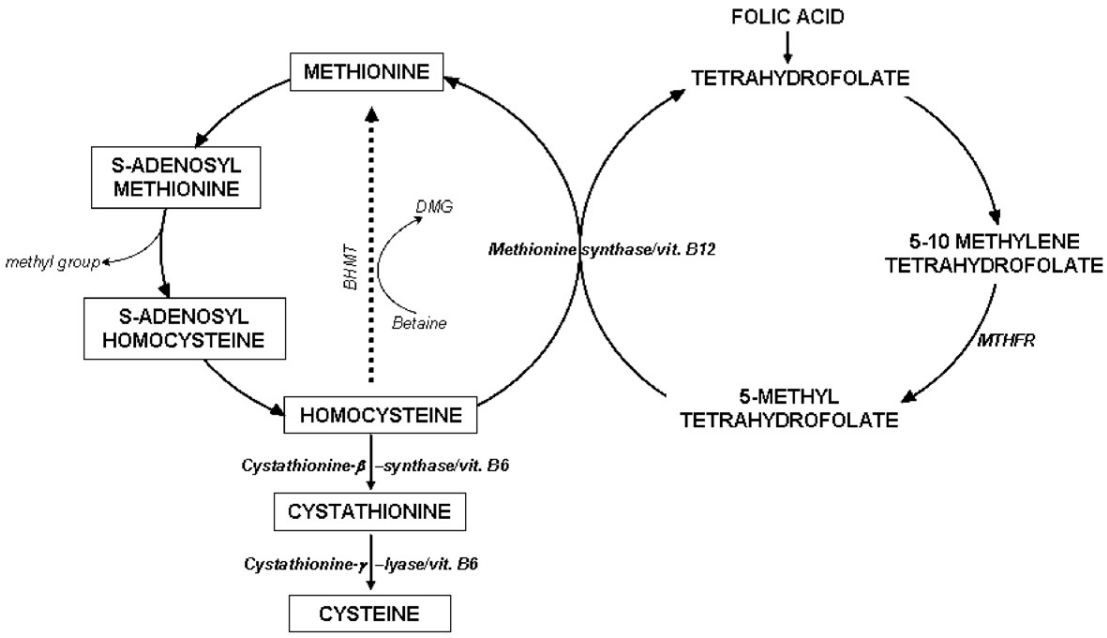


Fig. 2 Metabolic cycle of homocysteine; BHMT: betaine homocysteine methyl transferase; DMG: dymethylglycine; MTHFR: methylene tetrahydrofolate reductase (Martignoni et al., 2007).

CHAPTER II

LITERATURE REVIEW

Homocysteine and Neurodegenerative Diseases

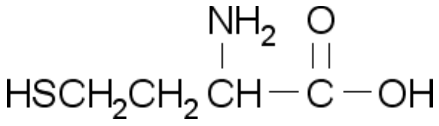


Fig. 1 Homocysteine structure

Homocysteine is a non-essential sulphur-containing amino acid produced as a natural consequence of methionine metabolism (Oldreive and Doherty, 2006), that is totally absent from any dietary source (Kim, et al., 2007). Homocysteine levels are normally maintained low by two major mechanisms. First, homocysteine is remethylated to form methionine by a reaction requiring folate and vitamin B_{12}. Second, homocysteine can be converted to cystathionine by the activity of the enzyme cystathionine-β-synthase (Chung et al., 2003).

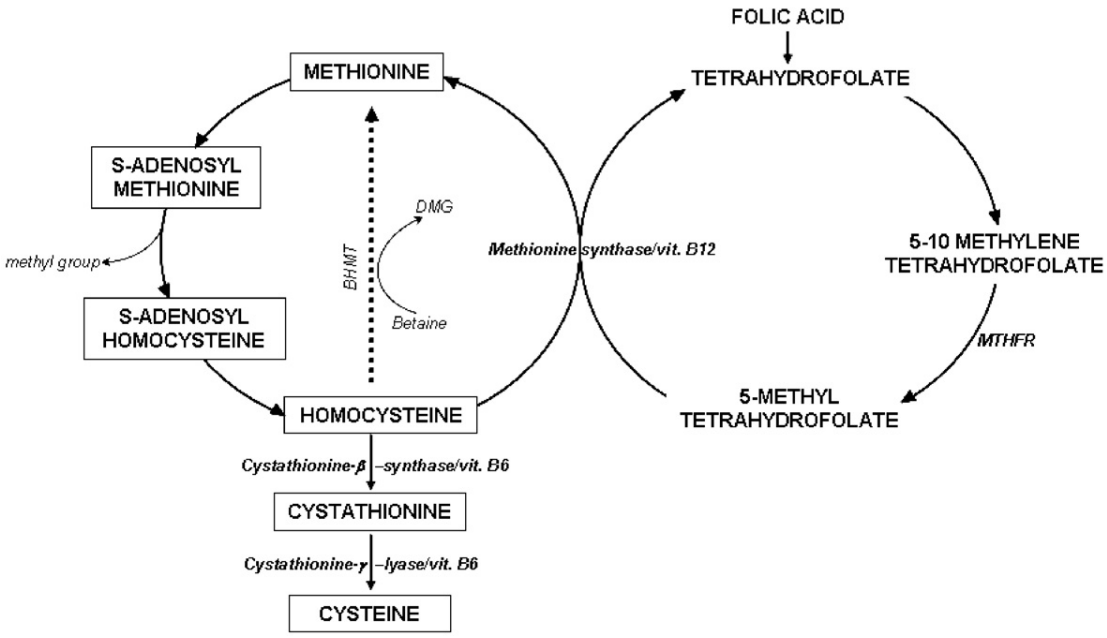


Fig. 2 Metabolic cycle of homocysteine; BHMT: betaine homocysteine methyl transferase; DMG: dymethylglycine; MTHFR: methylene tetrahydrofolate reductase (Martignoni et al., 2007).

In recent years, the detailed effects of homocysteine exposure on the nervous system have begun to be explored. Homocysteine activates an apoptotic cell death pathway in rat hippocampal neurons in culture and its metabolite, homocysteic acid, can induce cell death in the rat hippocampus *in vivo*. Elevated plasma homocysteine levels render mice more vulnerable to the dopaminergic neurotoxin MPTP. Murine cortical neurons are killed by exposure to homocysteine *in vitro* and it has been shown that this agent makes these neurons more sensitive to the effects of amyloid β peptide (Oldreive and Doherty, 2006). In addition, homocysteine is directly neurotoxic (Mattson and Shea, 2003). One of the suggested mechanisms of these effects is NMDA receptor-mediated neurotoxicity (Kim and Pae, 1996; Lipton et al., 1997). Homocysteine functions as an excitatory amino acid by activating group I metabotropic glutamate receptors and N-methyl-D-aspartate receptors. The oxidation and transsulfuration products of homocysteine are also agonists at ionotropic and metabotropic glutamate receptors (Sachdev, 2005).

Apoptosis is a form of spontaneous cell death in which cells shrink, condensation of the chromatin occurs and the cell breaks up and is phagocytosed without an inflammatory response. One mechanism by which homocysteine promotes apoptosis is through DNA breakage because of impaired transmethylation. Cultured neurons eventually are depleted of ATP reserves and the caspase pathway is activated. Furthermore, the oxidative stress caused by homocysteine increases the activity level of NF-kB, a redox-sensitive transcription factor that is important for the control of apoptosis mediation through reactive oxygen species. This results in an influx of Ca^{2+} leading to secondary excitotoxicity. Increased cytosolic Ca^{2+} damages mitochondria and has flow-on effects in reduction of ATP, further Ca^{2+} accumulation, leakage of cytochrome *c*, activation of caspase-3 pathway and apoptosis (Sachdev, 2005).

In vitro studies have reported that homocysteine, at high concentrations, induces cell death in various cell lines. This may be related to the putative pro-oxidant properties of the amino acid, given the well-known role of oxidative stress as a major trigger of cell death (Martignoni et al., 2007). Homocysteine toxicity in the CNS is related to increased cytosolic Ca^{2+} levels (Loureiro, et al., 2008).

The type of cell death induced by homocysteine is largely reported to be apoptotic. Caspase-dependent, apoptotic cell death in response to homocysteine has been reported in human dopaminergic cells, rat hippocampal and mouse cortical neurons. However, high levels of homocysteine have been shown to induce caspase-independent cell death of murine cerebellar

granule cells (Oldreive and Doherty, 2006). High brain concentrations of either homocysteine or its oxidized derivatives might alter neurotransmission or induce excitotoxicity in neurons, particularly those expressing N-methyl D-aspartate type receptors. A second mechanism postulates that homocysteine neurotoxicity is due to one-carbon metabolism which extends to the brain. An accumulation of homocysteine would increase intracellular S-adenosylhomocysteine (SAH), which is a potent inhibitor of many methylation reactions. The third possibility is based on the association of homocysteinemia with occlusive vascular disease which may be mediated through damage to the blood vessel wall or impaired blood coagulation (Troen, 2005).

A potential mechanism for neurotoxicity of homocysteine includes production of reactive oxygen species (ROS) such as H_2O_2, and involves Cu reduction followed by interaction between Cu and H_2O_2, to generate the highly toxic $OH^{-\bullet}$ radical. In addition, homocysteine acts as an agonist at NMDA subtype of the glutamate receptor (Chung et al., 2003), ionotropic and several metabotropic glutamate receptors (Görtz et al., 2003). Thiol auto-oxidation can also generate hydrogen peroxide and hydroxyl radicals (Chung et al., 2003). Homocysteine also suppressed the expression of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase in vascular endothelial cells, suggesting that homocysteine may enhance the cytotoxic effects of agents or conditions that elicit oxidative damage (Kim, H., et al., 2008).

Normally homocysteine concentration in human blood does not exceed 14 μM (Loureiro, et al., 2008). Hyperhomocysteinemia is known to be an independent risk for vascular diseases and is closely correlated to vascular dementia and Alzheimer's disease. The cognitive impairment of patients with hyperhomocysteinemia cannot solely be explained by structural lesions like multi-infarct dementia or cerebral atrophy but may also be caused by an additional functional disturbance. Patients with severe hyperhomocysteinemia may develop acute neurological symptoms such as seizures and psychosis (Görtz et al., 2003). Elevated plasma homocysteine has recently been found to be a risk factor for a variety of central nervous system diseases (Akanji et al., 2007). High homocysteine levels have been suggested as a mediating factor in alcohol-related brain atrophy and implicated in increased oxidative stress, DNA damage, the triggering of apoptosis and excitotoxicity, all important mechanisms in neurodegeneration (Sachdev, 2005).

An abnormal increase in plasma homocysteine might be due to a defect that occurred along the intracellular metabolic pathway. To protect the cell, extruding mechanisms export the excess homocysteine into the circulation and exposes all tissues to the potential toxicity of

homocysteine. Hyperhomocysteinemia is associated with a wide range of clinical manifestations, mostly affecting the central nervous system e.g., mental retardation, cerebral atrophy, epileptic seizures and Alzheimer's disease (Martignoni et al., 2007; Akanji et al., 2007). Increased risk of schizophrenia has been associated with the high homocysteine levels and patients with a family history of psychosis have a trend towards higher total homocysteine values (Akanji et al., 2007). The pathogenic relationship between homocysteine and schizophrenia may be mediated by the role of homocysteine in the damage to neuronal DNA with enhancement of apoptosis and increased sensitivity to excitotoxicity (Akanji et al., 2007). Homocysteine levels elevated in the serum of Parkinson's disease patients which may be a consequence of the use of levodopa to alleviate the symptoms of this disorder (Doherty, 2007). Thus homocysteine might not induce neurotoxicity by itself in such models but might enhance neuronal viability to other neurotoxins (Doherty, 2007).

Hypercystinuria is an inborn error of metabolism caused by severe deficiency of cystathionine β-synthase activity, resulting in tissue accumulation of homocysteine and methionine. Affected patients present alterations in various organs and systems, especially the central nervous system and the vascular system. Homocystinuric patients usually present mental retardation, seizures and atherosclerosis (Streck, et al., 2003).

The excitotoxic effects of homocysteine have been demonstrated in cultured neuronal cells and in whole animals. In whole animal studies, homocysteine administered at very high dosages (plasma levels in millimolar range) induced seizures in rats. In the retina, it has been shown *in vitro* that high levels of homocysteine trigger death of retinal ganglion cells (Moore et al., 2001). Parkinson's disease, Alzheimer-type dementia and other dementing illnesses have been reported to have an association with hyperhomocysteinemia. Plasma homocysteine increases with age independent of vitamin status, and hyperhomocysteinemia is highly prevalent in the elderly (Chung et al., 2003).

The two major proposed mechanisms for neuronal damage in Alzheimer's disease are the accumulation of amyloid β-peptide (Aβ) and the abnormal phosphorylation of tau proteins. Homocysteine promotes both pathways. Homocysteine potentiates the neurotoxicity of Aβ in cultured neurons. Aβ and homocysteine appear to have a synergistic effect in increasing levels of oxidative stress and promoting apoptosis (Sachdev, 2005).

***Centella asiatica* (Linn.) Urban**

Fig. 3 *Centella asiatica* (Linn.) Urban

Synonyms: *Hydrocotyle asiatica*, Gotu kola, Indian pennyworth, Brahmi, Indian water, Navelwort, Mandukaparni

Thai name: Bua Bok, Pak Nhok, Pak Van, Jumpakrue

Family: Apiaceae/Umbelliferae

Habitat: Asia, South Africa

Description of plant: Weedy, creeping, rooting at the nodes, low-growing with round-lobed leaves and tiny pink flowers.

Part Used: Whole plant

Chemical Components: *Centella asiatica* leaves and above-ground parts are rich in terpenoids including asiatic acid, madecassic acid and asiaticoside. Other terpenoids include centelloside, centellic acid; centoic acid, asiaticentoic acid, madecassoside, brahmoside, brahminoside (saponin glycosides). Aglycones are referred to as hydrocotylegenin A-E; compounds A-D are reported to be esters of the triterpene including quercetin, kaempferol and various glycosides; volatile oils including beta-caryophyllene, farnesene, germacrene D (sesquiterpenes) as major components; alpha and beta pinene. The major terpenoid is stated to be unidentified. Other constituents include an alkaloid called hydrocotylin; a bitter principle called vallerine; fatty acids including oleic, linoleic, linolenic, palmitic, stearic acids and lignocene; phytosterols including

campesterol, sitosterol and stigmasterol; resin and tannin. The root region contains 14 different polyacetylenes.

Traditional Uses

Centella asiatica has been widely used in humans for several decades. *Centella asiatica* is used as a nourishing food and a valuable medicine in many cultures. It was used as anxiolytic, memory and brain tonic, antispasmodic, sedative, antibacterial and anti-inflammatory.

Fresh extracts of this plant have been used by the people of Java and the Malay Peninsula for many decades, as both topical and internal agents, for healing of wounds (Kartnig, 1988). In India and Madagascar, this plant was used to treat leprosy (Sahu et al., 1989), while in Malaysia, although this herb is commonly eaten fresh as a vegetable (salad), especially among the Malay communities, it is also said to have beneficial effects in improving memory and in treating mental fatigue, anxiety, and eczema (Goh et al., 1995).

In classical Indian Ayurveda medicine, it is considered to be one of 'Rasayana' (rejuvenator) drugs which is used as a calming and rejuvenating herb, especially for nerve and brain cells. It is used to increase intelligence, longevity, and memory, retarding aging and senility. *Centella asiatica* is also used to reduce anxiety, treat petit mal epilepsy, for rheumatic pain, as diuretic, and for varicose veins. It is often used as a topical wash for skin infections, leprosy, and burns. It is said to improve texture of skin (Cox et al., 1993).

In Chinese medicine, *Centella asiatica* was often used, interchangeably with several other species of low-growing, round-leaved plants under the name of Zhi xue cao, for treating dermatitis, wounds, sores, dysentery, tuberculosis, jaundice, hematuria, and hemoptysis and also as a nerve tonic. The Chinese prescribed the leaves in curing leucorrhea and toxic fever (Kan, 1986).

Pharmacological Properties

Centella asiatica and asiaticoside, a major pentacyclic triterpenoid saponin component, are described to have wound healing, antiulcer, antioxidant and anti-inflammatory activities. Guo et al. (2004) reported that the mechanisms by which asiaticoside exerted its antiulcer and anti-inflammatory effects might be associated with inhibition of NO synthesis by its inhibitory effects on inducible nitric oxide synthase (iNOS).

Extracts of *Centella asiatica* exhibit various pharmacological actions including:

1) Anxiolytic properties

Centella asiatica is a psychoactive medicinal plant that has been used for centuries in Ayurvedic medicine to alleviate symptoms of anxiety and to promote a deep state of relaxation and mental calmness during meditation practices. Recent investigations using human and animal models of anxiety have confirmed that *Centella asiatica* does indeed possess anxiolytic activity. Bradwejn et al. (2000) reported that a single 12 g dose of *Centella asiatica* administered orally was more effective than placebo in decreasing acoustic startle response in healthy humans. This effect was most pronounced 60 min after treatment. In animals, *Centella asiatica* increases pentobarbitone-induced sleeping time and decreases immobility in the forced swim test. *Centella asiatica* also elicits anti-anxiety effects in the elevated plus maze (Wijeweera et al., 2006) and an aqueous extract of *Centella asiatica* was reported to have cognitive-enhancing as well as antioxidant effects in rats (Gupta et al., 2003).

2) Antioxidative properties

Recent evidence suggested that supplementation of *Centella asiatica* was effective in reducing brain regional lipid peroxidation and protein carbonyl levels, and in increasing the antioxidant status. Thus, *Centella asiatica* by acting as a potent antioxidant exerted significant neuroprotective effects and proved efficacious in protecting rat brains against age related oxidative damage (Marimuthu et al., 2005).

Oral treatment with 50 mg/kg/day of crude methanol extract of *Centella asiatica* for 14 days significantly increased the antioxidant enzymes, like superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx), and antioxidants like glutathione (GSH) (Jayashree et al., 2003). Recent study has revealed that *Centella asiatica* extract and powder may ameliorate H_2O_2-induced oxidative stress by decreasing lipid peroxidation via alteration of the antioxidant defense system of the rats (Abdul-Hamid et al., 2005). The antioxidative activity of the ethanol extract, of both roots and leaves of *Centella asiatica*, was found to be as good as α-tocopherol (Abdul-Hamid et al., 2003). It is also found that different parts of *Centella asiatica* (leaf, root, and petiole) contain high phenolic contents, which exhibit strong antioxidative activities (Abdul-Hamid et al., 2003). Besides, it was found to have a challenging role in quenching free radical-induced lipid peroxidation, protein carbonyls and also useful against age-related decline in antioxidant status in aged rat brain regions (Subathra et al., 2005).

3) Anticancer activity

Oral administration of *Centella asiatica* extracts and its partially purified fractions retarded the development of solid and ascites tumors and increased the life span of these tumor-bearing mice (Babu et al., 1995).

4) Radiation ulcer

The extract of *Centella asiatica* has shown an ability of reducing acute radiation reactions by its anti-inflammatory activity (Chen et al., 1999), and it offers good behavioral radioprotection against a conditioned taste aversion in rats during clinical ratiotherapy (Shobi and Goel, 2001).

5) Gastric ulcer

Studies in rats have documented that asiaticoside exhibits a protective action against stress-induced gastric ulcers, following subcutaneous administration, and accelerates the healing of chemical-induced duodenal ulcers, after oral administration (Ravokatra and Ratsimamanga, 1974a; Ravokatra et al., 1974b). In another study, oral administration of *Centella asiatica* extract (0.05 g/kg, 0.25 g/kg and 0.50 g/kg) prevented ethanol-induced gastric mucosal lesions in rats by strengthening the mucosal barrier and reducing the damaging effects of free radicals (Cheng and Koo, 2000).

6) Wound healing, scar healing, and skin diseases

Centella asiatica extract was found to be useful in preventing and treating keloids and hypertrophic scars (Bosse, et al., 1979). A *Centella asiatica* extract containing asiaticoside (40%), asiatic acid (29-30%), madecassic acid (29-30%), and madasiatic acid (1%), was documented to be successful as both a preventive and curative treatment, when given to 227 patients with keloids or hypertrophic scar. The effective dose in adults was reported to be between 60 and 90 mg. Its activity appears to be on the connective tissue by stimulating synthesis of hyaluronidase and chondroitin sulfate. It is also believed to have an effect on keratinization in areas of infection and to stimulate the reticuloendothelial system. Its topical form is claimed to improve tissue healing, particularly in skin, connective tissue, lymph and mucous membranes. It also may stabilize connective tissue growth in scleroderma. These properties have been ascribed to the active principles, asiatic acid, asiaticoside, madecassic acid, and madecassoside, which are pentacyclic triterpenes (Pointel et al., 1987). Madacassol, a formulation based on *Centella asiatica* plant extracts, when applied locally on wounds in rats prompted the proliferation of granulation and increased tensile strength (Vogel et al., 1990). It

decreased the wounds area of the skin necrosis induced by burn (Tsurumi et al., 1973; Manuel and Eduardo, 1976). Asiaticoside has yielded encouraging results in treatment of leprosy (Bailey, 1945; Boiteau, 1949; Chopra et al., 1956; Viala et al., 1977). In addition, it has been shown to enhance the rate of wound healing by promoting fibroblast proliferation and stimulating collagen synthesis (Maquart et al., 1990). Rosen et al. (1967) have also reported wound healing activity of the plant. Titrated extract of asiatic acid and madecassic acid has been reported to be effective in treating systemic scleroderma, abnormal scar formation and keloids (Kiesswetter, 1964; Tallat and Abbas, 1971; Nakajima and Ajiyoshi, 1973) by strongly inhibiting the biosynthesis of acid mucopolysaccharides and collagens in carrageenin granulomas (Sasaki, et al., 1972). Brahmic acid, a biologically active triterpenoid (Singh, 1968) has therapeutic value in ulcerations, extensive wounds, and eczemas (Yoshinori, 1982), and inhibitory effect on the biosynthetic activity of fibroblast cells (Veechai, 1984). Its wound-healing effects may be due to its up-regulation of human collagen I expression (Bonte et al., 1994) and increase in tensile strength of wound (Suguna et al., 1996). In guinea pig punch wounds, topical applications of 0.2% solution of asiaticoside produced 56% increase in hydroxyproline, 57% increase in tensile strength, increased collagen content and better epithelisation. In streptozotocin diabetic rats, where healing is delayed, topical application of 0.4% solution of asiaticoside over punch wounds increased hydroxyproline content, tensile strength, collagen content and epithelisation, thereby facilitating the healing. Asiaticoside was also active by the oral route 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 (Shukla, et al., 1999).

Centella asiatica can be applied as a topical ointment or compressed to the skin, or it can be ingested as dried leaves or an infusion three times a day (each dose should be between 0.33 and 0.68 g).

Since antioxidants have been reported to play a significant role in the wound healing process, the possible involvement of such a mechanism in wound healing induction of antioxidant levels at initial stage of healing was studied (Shukla et al., 1999). Topical application of 0.2% asiaticoside twice daily for 7 days to excision-type cutaneous wounds in rats led to increased enzymatic and non-enzymatic antioxidants, namely SOD (35%), CAT (67%), GPx (49%), vitamin E (77%) and ascorbic acid (36%) in newly formed tissues. It also induced a marked decrease of lipid peroxide levels (69%).

7) Immunomodulatory properties

An ethanol extract of *Centella asiatica* dramatically inhibited NO production through the suppression of TNF-α production (Punturee et al., 2005).

8) Mental and Miscellaneous

Gupta et al. (2003) concluded that *Centella asiatica* significantly prevented the cognitive impairment and attenuated the oxidative stress induced by PTZ kindling. Veerandra and Gupta (2002) have shown that the aqueous extract of whole plant (200 mg/kg for 14 days) showed an improvement in learning and memory in both shuttle box and step through paradigms. The whole plant of *Centella asiatica* has been shown to be beneficial in improving memory (Mukerji, 1953; Vaidyaratnam, 1994) and it is reported to improve general mental ability of mentally retarded children (Appa et al., 1973; Kakkar, 1990). Nalini et al. (1992) have shown that fresh leaf juice improves passive avoidance task in rats. Two glycosides, brahmoside and brahminoside, have been shown to exert sedative and hypoglycemic effects in experimental rats.

Pharmacokinetic Properties

A recent study in twelve healthy human volunteers investigated effects of single or repeated administrations of *Centella asiatica* (30 or 60 mg was administered orally to humans on a single occasion or once daily for consecutive days). However, the assay method was only able to investigate levels of asiatic acid. The elimination half life was 2 to 3 hours irrespective of the dose used. The peak plasma concentration, area under curve (0 to 24 hours) and the plasma half life were significantly increased following repeated administration of *Centella asiatica*. These increases may partly be explained by the fact that asiaticoside is metabolized *in vivo* to asiatic acid (Grimaldi et al., 1990). The active ingredients of *Centella asiatica* extract are reported to be excreted primarily in the feces over a 24 to 76 hour period, with a smaller percentage being eliminated via the kidneys.

Toxicological properties

In mice, a dose of 1 g/kg body weight of an extract of *Centella asiatica* (in 50% ethanol) did not lead to any toxic effects. No mortalities were recorded.

The American Herbal Products Association lists *Centella asiatica* as an herb that may be safely consumed with appropriate use (McGuffin et al., 1997). No signs of toxicity have been

found from *Centella asiatica* in animal studies (Sakina and Dandiya, 1990; Babu et al., 1995), mutagenicity studies (Yen et al., 2001), and in human studies (Brinkhaus et al., 2000). In rats, the acute oral LD_{50} of a 70% ethanolic extract of the leaves was greater than 675 mg/kg and no toxicity was found form chronic oral administration of 150 mg/kg (De Lucia et al., 1997). Allergic skin reactions from topical use of preparations containing *Centella asiatica* or triterpene-rich extracts are rare (Danese et al., 1994; Gonzalo et al., 1996). Asiaticoside is only a weak sensitizer (Hausen, 1993).

Adverse Effects

Centella asiatica seems to be well tolerated when taken by mouth. Stomach discomfort and nausea have been reported in studies. In theory, it may cause drowsiness and may raise blood cholesterol and blood sugar levels. Therefore, people with diabetes should avoid *Centella asiatica*. Animal studies show that *Centella asiatica* may lower the chances of becoming pregnant, but it is not clear if this effect occurs in humans (Ramswamy et al., 1970). Topical administration may cause contact dermatitis in sensitive individuals (Danese, 1994).

Interactions

Interactions with drugs, food supplements and other herbs have not been thoroughly studied. The interactions listed below have been reported in scientific publications.

Interactions with Drugs

Centella asiatica may increase the amount of drowsiness caused by some drugs. Examples include benzodiazepines, such as lorazepam; barbiturates, such as phenobarbital; narcotics, such as codeine; and alcohol. Caution is advised while driving or operating machinery. In theory, *Centella asiatica* may increase cholesterol levels and may work against cholesterol-lowering drugs.

Centella asiatica may raise blood sugar levels and may work against drugs that lower blood sugar levels. Patients taking oral drugs for diabetes or using insulin should be monitored closely by their health care provider while using *Centella asiatica*. Dosing adjustment may be necessary. In theory, dexamethasone and phenylbutazone may decrease any wound-healing abilities of *Centella asiatica*.

Lipid peroxidation

Malondialdehyde is one of the products of peroxidative cleavage of polyunsaturated fatty acids containing at least two methylene-interrupts double bonds. Pryor and Stanley (1975) have proposed a mechanism of formation of malondialdehyde that involves an intermediate bicycloendoperoxide. However, Esterbauer et al. (1991) still propose that the production of malondialdehyde from polyunsaturated fatty acids with more than two double bonds involves formation of hydroperoxides, β cleavage to yield hydroperoxyaldehydes, and finally, via a second β scission to yield malondialdehyde or acrolein radical which combines with OH to form the enol.

The most popular method to quantitate lipid peroxidation is the colorimetric or fluorometric determination of malondialdehyde or malondialdehyde-like materials by thiobarbituric assay.

Glutathione

Glutathione (γ-glutamylcysteinylglycine; GSH) is the major cellular antioxidant and is found in high concentrations in most mammalian cells (1 to 10 mM). Intracellular GSH is maintained in its thiol form by glutathione disulfide (GSSG) reductase, which requires NADPH. GSH has several functions, including roles in metabolism, transport, catalysis (coenzyme), and maintenance of the thiol moieties of proteins and the reduced form of other molecules such as cysteine, coenzyme A, and antioxidants such as ascorbic acid. It is also used in the formation of deoxyribonucleic acids. GSH participates non-enzymatically and enzymatically (GSH S-transferase) in the protection against toxic compounds. Perhaps one of its most important functions is in the protection against oxidative damage caused by reactive oxygen species (ROS), many of which are generated during normal metabolism. GSH can react non-enzymatically with ROS, and GSH peroxidase (and non-Se peroxidase), and catalyzes the destruction of hydrogen peroxide and hydroperoxides.

GSH is synthesized intracellularly by the consecutive actions of γ-glutamylcystein (1) and GSH (2) synthases:

(1) L-Glutamate + L-cysteine + ATP ↔ L-γ-glutamyl-L-cysteine + ADP + Pi

(2) L-γ-glutamyl-L-cysteine + glycine + ATP ↔ GSH + ADP + Pi

The synthesis of GSH is limited by the availability of substrates; cysteine is usually the limiting substrate. γ-glutamylcystein synthetase is nonallosterically feedback inhibited by GSH (K_1 about

1.5 mM). Thus, under physiological conditions, γ-glutamylcystein synthetase is probably not operating at its maximal rate.

The degradation of the γ-glutamyl moiety of GSH (or GSH S-conjugates) is catalyzed by γ-glutamyl transpeptidase, a membrane-bound enzyme whose active site is on the external surface of certain cells. GSH is normally transported out of cells where transpeptidation occurs in the presence of amino acids and the product is formed. This can then be transported into cells where it, but not GSH, is a substrate for γ-glutamyl cyclotransferase and forms amino acids and 5-oxoproline. 5-oxoproloine is ring opened by 5-oxoprolinase to form glutamate. Most L-amino acids participate in transpeptidation, but cystine is one of the best acceptor amino acids. When cystine participates, γ-glutamylcystine is formed, transported, and reduced to cysteine and γ-glutamylcystine. Cysteine can be used for the two-step pathway of GSH biosynthesis and γ-glutamylcystine can be used directly by GSH synthetase to form GSH. This alternative pathway of GSH synthesis serves to conserve cysteine moieties; however, the extent to which it functions physiologically is not yet known.

In Vitro Cellular Model of Neurodegeneration

Neurons are highly specialized cells and are responsible for the reception, integration, transmission, and storage of information. Afferent neuronal pathways carry information into the nervous system for processing; efferent pathways carry commands to the periphery. In addition, there are interneurons that process local information and transfer data within the nervous system. Within the CNS, neurons are segregated into functionally related nuclei that form interconnecting bundles of axonal fibers. Higher organizational levels consisting of several functionally related neurons are frequently called systems, e.g., motor, visual, associative, and neuroendocrine systems. The CNS consists of a number of systems responsible for the coordination of receiving and processing information from the environment, maintaining the balance of all other organ systems, and responding to changes in the environment.

The *in vivo* reactions of neurons to injury vary dramatically. Degeneration can be induced by a direct action on the perikaryon or loss of synaptic target site influences and deprivation of trophic factors. A number of chemicals appear to have distinct cellular specificity for neuronal populations. Although specificity can exist and the pattern of degeneration has been used in diagnostic neuropathology, degeneration of any particular neuronal type cannot necessarily identify the damaging agent. Often this pattern reflects the severity and duration of

injury and the acute or chronic nature of exposure. The degenerative process of the nerve cell can be either relatively quick or a slow, prolonged process, depending on the underlying mechanism (Hary et al., 1998).

In the field of neurobiology, *in vitro* cell culture techniques have been successfully developed and employed to address specific questions of cell biology and nervous system functioning and provide a means to systematically study complex nervous systems. Cell lines are cultures that have been serially transplanted or subcultured through a number of generations and can be propagated for an extended period of time. In neurobiology, *in vitro* methods are not ordinarily considered as general alternatives to *in vivo* procedures. Instead, *in vitro* methods are selected to address specific hypotheses. Therefore, *in vitro* models are used in an attempt to study biological processes in a more isolated context or in the direct investigation of specific biological processes.

Continuous cell lines are transformed cells derived from neuroblastomas, gliomas, and pheochromocytomas with a useful life span of approximately 50 divisions. Cell lines of limited life span often undergo crisis after which their growth potential changes and their life span becomes unlimited. These cell lines are termed immortalized cell lines. The major attributes of continuous clonal cell lines are homogeneity and the ease with which a large quantity of cells can be grown. Therefore neuronal cell lines are practical to use in an *in vitro* model of neurodegeneration.

In the current study, cell cultures of rat neuroblastoma cell line, N1E-115, are used to test *in vitro* neuroprotective effects of *Centella asiatica.* It is conceivable that *Centella asiatica* extract and asiaticoside may have a challenging role in reducing homocysteine-induced neuronal injuries and also be useful against decline in antioxidant status in cultured neurons.

CHAPTER III

MATERIALS AND METHODS

Cell culture

Cell culture of the rat neuroblastoma N1E-115 cell line, clone C1300, (American Type Culture Collection, Manassas, VA, USA) was used in the experiments.

Chemicals

- *Centella asiatica* extract (37.20% madecassoside and 38.56% asiaticoside) (kindly provided by Associate Professor Dr. Ekarin Saifa, Department of Pharmaceutical Botany and Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University)
- 90% Asiaticoside (Changzhou Natural Products Development, China) with the following specifications.

Test Item	Standard	Assay Result
Color	White powder	White powder
TLC	Conforms	Conforms
Loss on drying	$\leq$ 5%	1.39%
Heavy metals	< 10 ppm	< 10 ppm
Sulphated ash	< 0.5%	0.36%
Assay (HPLC)	Asiaticoside 90%	Asiaticoside 90.03%
Granule size	98% pass 100 mesh	98% pass 100 mesh
Total bacteria count	Less than 1000/g	121/g
Yeast/moulds	Less than 100/g	Negative
E. coli	Negative	Negative
Pseudomonas aeruginosa	Negative	Negative
Staphylococcus aureus	Negative	Negative

- 5-5'-dithiobis-(2-nitrobenzoic acid) [Sigma, U.S.A.]
- Dimethyl sulfoxide (DMSO) [BHD, England]
- Dulbecco's Modified Eagle's Medium (DMEM) [Gibco, U.S.A.]

- Dulbecco's Phosphate Buffered Saline (DPBS) [Sigma, U.S.A.]
- Ethylenediaminetetraacetic acid (EDTA) [Sigma, U.S.A.]
- Fetal Bovine Serum (FBS) [Hyclone, U.S.A.]
- Glutathione (reduced form) (GSH) [Sigma, U.S.A.]
- Glutathione reductase (GRx) [Sigma, U.S.A.]
- Hoechst 33342 (Ho 33342) [Sigma, U.S.A.]
- Homocysteine [Sigma, U.S.A.]
- Human transferrin [Sigma, U.S.A.]
- *In vitro* toxicology assay kit (Lactate Dehydrogenase Based) [Sigma, U.S.A.]
- Insulin from bovine pancrease [Sigma, U.S.A.]
- MTT (3-[4,5-Dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide) [Sigma, U.S.A.]
- β-Nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) [Sigma, U.S.A.]
- Penicillin G sodium [Sigma, U.S.A.]
- Progesterone [Sigma, U.S.A.]
- Propidium iodide (PI) [Sigma, U.S.A.]
- Putrescin dihydrochloride [Sigma, U.S.A.]
- Streptomycin sulfate [Sigma, U.S.A.]
- Sulfosalicylic acid [Sigma, U.S.A.]
- Triton X-100 [Sigma, U.S.A.]
- Trypan blue [Sigma, U.S.A.]
- Trypsin [Sigma, U.S.A.]

Materials and Instruments

- 24-well cell culture plate
- 96-well cell culture plate [Nunc, Denmark]
- Cell culture flask: 25 cm^2, 75 cm^2 [
- Conical tube: 15 ml, 50 ml [Nunc, Denmark]
- Sterile Millex-GV (0.22 μm filter unit) [Millipore, U.S.A.]
- Autoclave [Hirayama, Japan]

- Carbon dioxide incubator [Forma Scientific, U.S.A.]
- Centrifuge
- Hemocytometer (Depth 0.100 mm) [Improved Neubauer]
- Inverted microscope, Axiovent 135 [Zeiss, Germany]
- Laminar air flow hood [Hepaco, U.S.A.]
- Microliter pipette: 10 – 100 μl [Labsystems, Finland]
- Microliter pipette: 200 – 1000 μl, 1 – 5 ml [Labsystems, Finland]
- Microplate reader [Anthos htl, Australia]
- Microplate reader [Perkin Elmer, Germany]
- pH meter
- Sonicator [Elma, Germany]
- Vortex mixer [Clay adams, U.S.A.]
- Water bath [Thelco, U.S.A.]

Preparation of *Centella asiatica* Extract, Asiaticoside and Homocysteine Solutions

In all experiments, *Centella asiatica* extract and asiaticoside were dissolved in double distilled water and homocysteine was dissolved in DMSO for the addition into cell culture medium. The final concentration of DMSO in culture medium was less than 0.5%. Control groups were treated with the corresponding amount of their respective solvents.

Preparation of Culture Media

Dulbecco's modified Eagle's medium (DMEM, high glucose) was supplemented with 3.7 mg/ml sodium bicarconate, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate, 10% (v/v) fetal bovine serum (FBS). Serum-free DMEM composed of normal DMEM supplemented with 30 nM selenium, 30 μg/ml human transferrin, 5 μg/ml insulin, 100 μM putrescin and 20 nM progesterone (N2 media supplements).

Preparation of Neuroblastoma Cell Cultures

The cell suspension of rat neuroblastoma cell line, N1E-115, was plated into 75 cm^2 plastic culture flasks and grown in DMEM containing 10% FBS in a humidified incubator maintaining at 37 °C and containing 5%CO_2-95% air atmosphere. When cultures reached confluency, cells were removed from culture flasks by trypsinization. Culture medium was aspirated and confluent cells were incubated with 0.25% trypsin-EDTA solution for 3 minutes at

room temperature, followed by addition of an equal volume of DMEM containing 10% FBS. The cell suspension was centrifuged at 1,250 rpm for 4 minutes at 4 °C and the pellet was suspended in fresh DMEM containing 10% FBS. The cells were counted on hemocytometer using trypan blue exclusion. After appropriate dilution with culture medium, aliquots of 1 ml were plated at a density of 5 x 10^4 cells/well in 24-well culture plates and cultures were incubated in a humidified atmosphere of 5%CO_2-95% air at 37 °C. The medium was changed every 2-3 days and cultured cells were subcultured once they reached 80-90% confluency. After growth to confluency [approximately 48 hours after plating], the cells were used in the experiments.

Experimental conditions

1. Treatment of cultured N1E-115 cells with *Centella asiatica* extract or asiaticoside

   Cultures were treated with 1, 10, 25, 50, 75 and 100 μg/ml of *Centella asiatica* extract or asiaticoside for 24, 48 and 72 hr in serum-free DMEM containing N2 media supplements.

2. Exposure of cultured N1E-115 cells to homocysteine

   Cultures were exposed to homocysteine at final concentrations of 0.5, 1, 2.5, 5, 10 and 20 mM in serum-free DMEM containing N2 media supplements for 24, 48 and 72 hr.

3. Treatment of 10 mM homocysteine-exposed N1E-115 cell cultures with test agents

   1.3.1 Pre-treatment with *Centella asiatica* extract

   1.3.2 Co-treatment with *Centella asiatica* extract

Analytical Methods

MTT reduction colorimetric assay

Mitochondrial dehydrogenase activity, which is considered to reflect cell viability, was measured by the reduction of tetrazolium salt MTT [3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide to formazan. Mitochondrial dehydrogenase in viable cells converts the soluble yellow form of the salt into an insoluble, intracellular purple formazan which is quantitated spectrophotometrically by solubilization in an organic solvent (Desagher et al., 1996).

MTT reduction was analyzed by adding 15 μl of the MTT stock solution [5 mg/ml in phosphate-buffered saline (PBS)] to the medium in each well (final concentration was 100 μg/ml). Cultures were incubated in a CO_2 incubator at 37 °C for 1 hr and the medium in each well was aspirated off without disturbing the formazan precipitate. Then 400 μl of DMSO was

added to each well in order to solubilize the formazan crystals. Following thorough formazan solubilization, 100 µl aliquots of soluble fotmazan were transferred to a 96-well microtiter plate. The cellular reduction of MTT was determined by measuring the absorbance at 570/620 nm with an Anthos Labtec HT2 version 1.21E dual wavelength microplate reader. The results were expressed as percentage of MTT reduction relative to that of control cultures.

Lactate dehydrogenase (LDH) release assay

Released LDH, a biologically stable enzymatic marker that correlates linearly with cell death, was determined by an *in vitro* toxicology assay kit (Sigma, U.S.A.) with the procedure according to the manufacturer's instructions. Briefly, medium LDH was assayed by pipetting 100 µl of culture medium from each well into a 96-well microtiter plate. Cellular LDH was measured by carefully aspirated off the remaining culture medium 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 lactate dehydrogenase assay mixture into each well. The plate was covered with an aluminium foil to protect from light and incubated at room temperature for 20-30 min. The reaction was terminated by the addition 50 µl of 1N HCl into each well. The light absorbance in each well was measured at 510/620 nm with an Anthos Labtee HT2 version 1.21E dual wavelength microplate reader. The LDH release which reflects cell death was presented as percentage of total LDH activity by the following formula:

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

Glutathione assay

Total cellular GSH levels were measured enzymatically by a method that modified from the procedure of Tietze (1969). This method is based on determination of a chromophoric product, 2-nitrosi-5-thiobenzoic acid, resulting from the reaction of 5-5'-dithiobis-(2-nitrobenzoic acid) with GSH. In this assay, GSH is oxidized to GSSG, which is then reconverted to GSH in the presence of GSH reductase (GRx) and NADPH. The rate of 2-nitro-5-thiobenzoic acid formation is followed at 450 nm.

Cells was washed with ice-cold PBS and immediately collected by scraping with 0.5 ml of 1% (w/v) sulfosalicylic acid. Cell lysates were transferred to 1.5 ml Eppendorf tubes and

centrifuged at 12,000 rpm for 5 min at 4 °C. Following cell extraction, 20 µl aliquots of supernatant were transferred into 96-well microtiter plate and the volume was made up to 100 µl with 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.5). The reaction was started by adding 100 µl of reaction mixture [0.15 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.2 mM NADPH, and 1 U/ml glutathione reductase in 0.1 M sodium phosphate (pH 7.5)]. The formation of 2-nitro-5-benzoic was monitored at 10-s intervals for 15 cycles at 450/620 nm with an Anthos Labtee HT2 version 1.21E dual wavelength microplate reader. The slope of the initial rate of reaction will be used for calculating GSH content from a standard curve obtained from known amounts of GSH (1-100 µM).

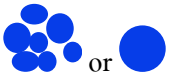
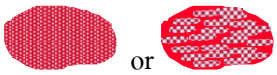
Determination of apoptotic cell death

N1E-115 neurons were cultured in 24-well culture plates (5 x 10^4 cells/well) by the method described previously. Cultures were detected for apoptotic cells by a two-color fluorescence staining with two nuclear fluorochromes, the membrane-permeant bisbenzimide dye Hoechst 33342 (Ho 33342) which labels nuclei of all cells, and the membrane-impermeant dye propidium iodide (PI) which labels only nuclei of dead cells. By this method it was possible to detect, in the same sample and at the same time, intact cells, cells undergoing apoptosis, and dead cells resulting from apoptotic and/or necrotic processes.

Cells were stained according to the one-step staining procedure. The staining involving several dye combinations have been applied to a variety of cell types. By controlling the dye concentrations it is possible to minimize the dye-dye interactions as well as the excessive background fluorescence. Ho 33342 (1 mg/ml stock solution in distilled water) was added to the cultures to give a final concentration of 1 µg/ml. Simultaneously, PI (500 µg/ml stock solution in distilled water) was also added to the cultures, to give a final concentration of 5 µg/ml. Cultures were incubated in a CO_2 incubator for 15 min at 37 °C. After removing the medium, neurons were rinsed once with Hank's balanced salt solution (HBSS, pH 7.2) and kept in cooled HBSS. The stained cells were immediately analyzed under UV fluorescence optics with an inverted microscope and the photographs were obtained. In each culture, six to ten consecutive microscopic fields (200×) were counted. To determine the proportion of dead or apoptotic neurons, at least 200 cells were counted per culture. Numbers of cells in different stages were presented as the percentage of total cell count. Whenever possible, comparisons were made on

matched sister cultures derived from a single plating. The criteria for identification of cells in different states are described in the followings.

Criteria for the simultaneous assessment of apoptotic and necrotic cells by the two-color flurescence DNA staining.

Cell subpopulation	**Ho 33342 fluorescence (blue)**	**PI fluorescence (red)**
Viable 	Intermediate	Negative
Early apoptotic 	Increased, condensed or fragmented	Negative
Late apoptotic 	Decreased, condensed or fragmented	Positive, condensed or fragmented
Dead or Necrotic 	Much decreased	Highly positive

Statistical Data Analysis

Results were expressed as the mean ± standard error of mean (SEM) from 6 independent cultures, with duplicate replications in each experiment. Differences among means were analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc analysis (Scheffe Test). The *P* values of less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

1. Effects of homocysteine (HCY) on cell viability of N1E-115 neuroblastoma cell line.

Upon exposure to 0.5 to 20 mM of HCY for 24, 48 and 72 hr, mitochondrial metabolic activity of cultured N1E-115 cells was markedly decreased accordingly to increasing concentrations when compared with control cultures (Fig. 1). After an exposure of N1E-115 cultures to 10 mM of HCY for 72 hr, mitochondrial metabolic activity of the cultured cells was decreased to nearly 50% compared with controls. Therefore, this experimental setting was arbitrary adopted as the neurotoxic insult in an *in vitro* cellular model of neurodegeneration (Fig. 4).

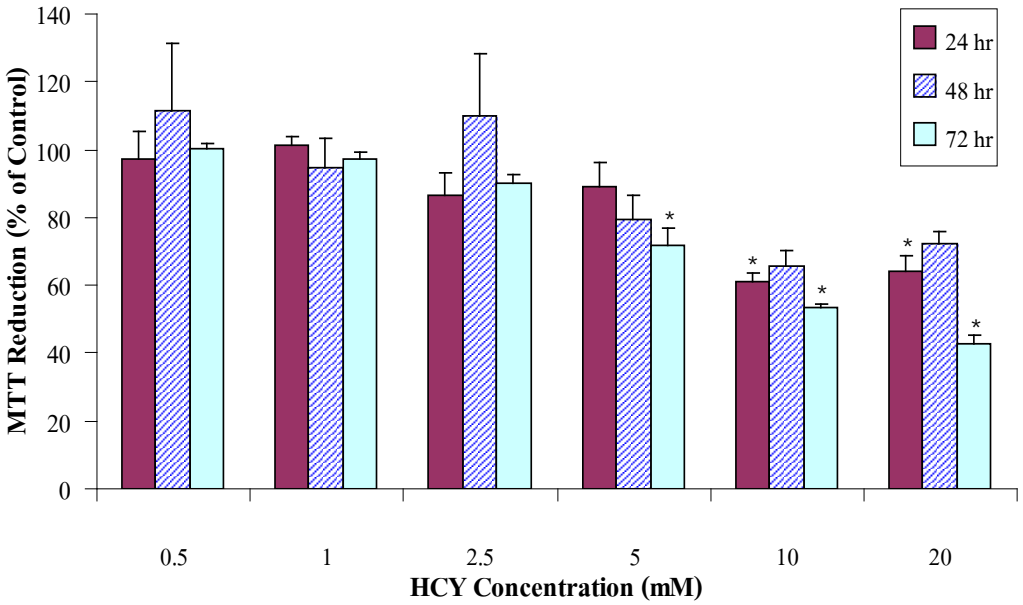


Fig. 4 Inhibitory effects of HCY on cell viability of cultured N1E-115 cell line.

Cultures were exposed to increasing concentrations of HCY for 24, 48 and 72 hr and cell viability was determined by MTT reduction assay. Values are mean ± S.E.M. (N=6; with duplicate experiments). *P<0.05 compared with control cultures (One-way ANOVA followed by Scheffe's test for multiple comparisons).

2. Effects of asiaticoside on mitochondrial activity of N1E-115 neuroblastoma cell line.

Treatment of N1E-115 cultures with a range of asiaticoside concentrations (1-100 μg/ml) for various time intervals showed no cytotoxic effects as considered from cell viability after 24, 48 and 72 hr of incubation (Fig. 5).

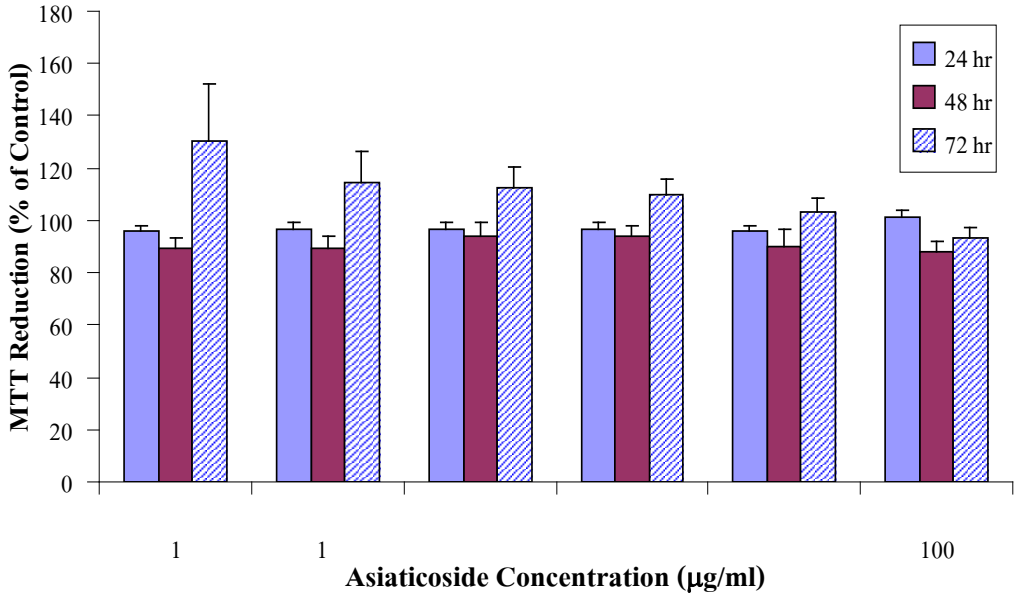


Fig. 5 Effects of asiaticoside on cell viability of cultured N1E-115 cell line.

Cultures were treated with increasing concentrations of asiaticoside. Cell viability was estimated 24, 48 and 72 hr later by using MTT reduction assay. Values are mean ± S.E.M. (N=6; with duplicate experiments). Statistical analysis for differences among means was made by one-way ANOVA.

3. Effects of *Centella asiaticaI* extract on cell viability of N1E-115 neuroblastoma cell line.

Treatment of N1E-115 cultures with a range of *Centella asiatica* extract concentrations (1-100 μg/ml) for various time intervals showed that there were no significant changes in cell viability after 24, 48 and 72 hr of incubation. (Fig. 6).

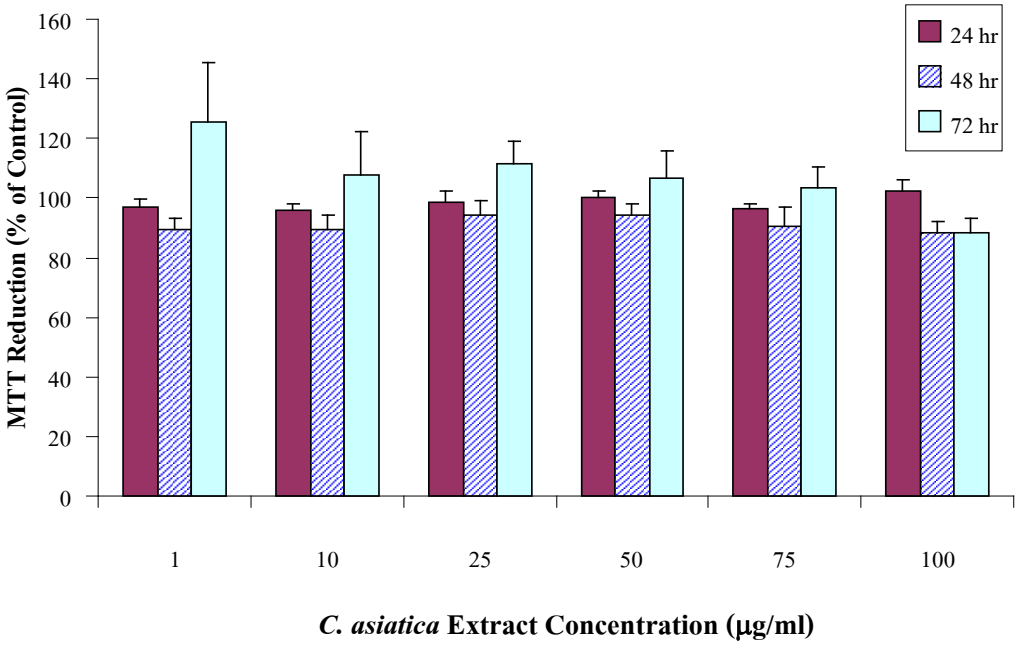


Fig. 6 Effects of *Centella asiatica* extract on cell viability of cultured N1E-115 cell line.

Cultures were treated with increasing concentrations of *Centella asiatica* extract. Cell viability was estimated 24, 48 and 72 hr later by using MTT reduction assay. Values are mean ± S.E.M. (N=6; with duplicate experiments). Statistical analysis for differences among means was made by one-way ANOVA.

4. Effects of pre-treatment on cell viability of homocysteine-exposed N1E-115 neuroblastoma cell line.

Exposure of N1E-115 cultures to homocysteine at a concentration of 10 mM for 72 hr reduced cell viability nearly 50% in comparison with untreated controls. However, the cytotoxicity caused by homocysteine was significantly reduced by pre-exposure to 100 μg/ml of *Centella asiatica* extract and 50-100 μg/ml of asiaticoside in a concentration-related manner. However, other concentrations of *Centella asiatica* extract (1-75 μg/ml) and asiaticoside (1-25 μg/ml) did not attenuate cell injuries caused by homocysteine (Fig. 7).

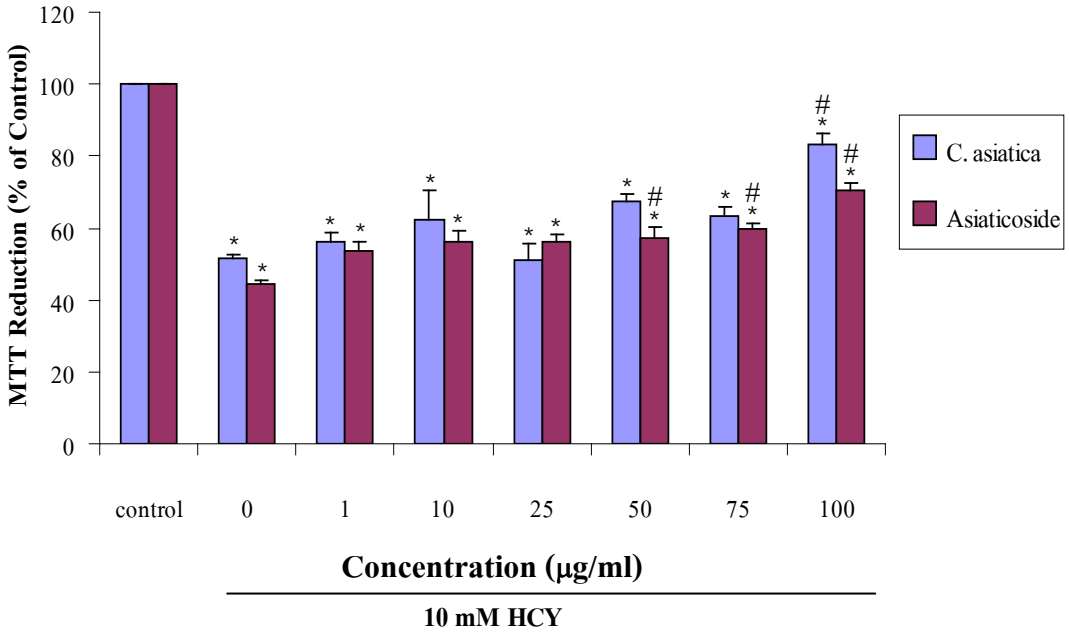


Fig. 7 Effects of pre-treatment on cell viability of cultured N1E-115 cell line exposed to homocysteine.

N1E-115 cultures were incubated with 1-100 μM of asiaticoside or *Centella asiatica* extract for 48 hr and further incubated with fresh medium containing 10 mM homocysteine for 72 hr after which cell viability was determined. Valued are mean ± S.E.M. (N=6). *P<0.05 compared with control cultures, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

5. Effects of pre-treatment on cell death of homocysteine-exposed N1E-115 neuroblastoma cell line.

Exposure of N1E-115 cultures to homocysteine at a concentration of 10 mM for 72 hr induced a marked cell death in comparison with untreated controls. However, the cytotoxicity caused by homocysteine was significantly reduced by pre-exposure to 75-100 μg/ml of *Centella asiatica* extract and 100 μg/ml of asiaticoside in a concentration-related manner. However, other concentrations of *Centella asiatica* extract (1-50 μg/ml) and asiaticoside (1-75 μg/ml) did not attenuate cell death caused by homocysteine (Fig. 8).

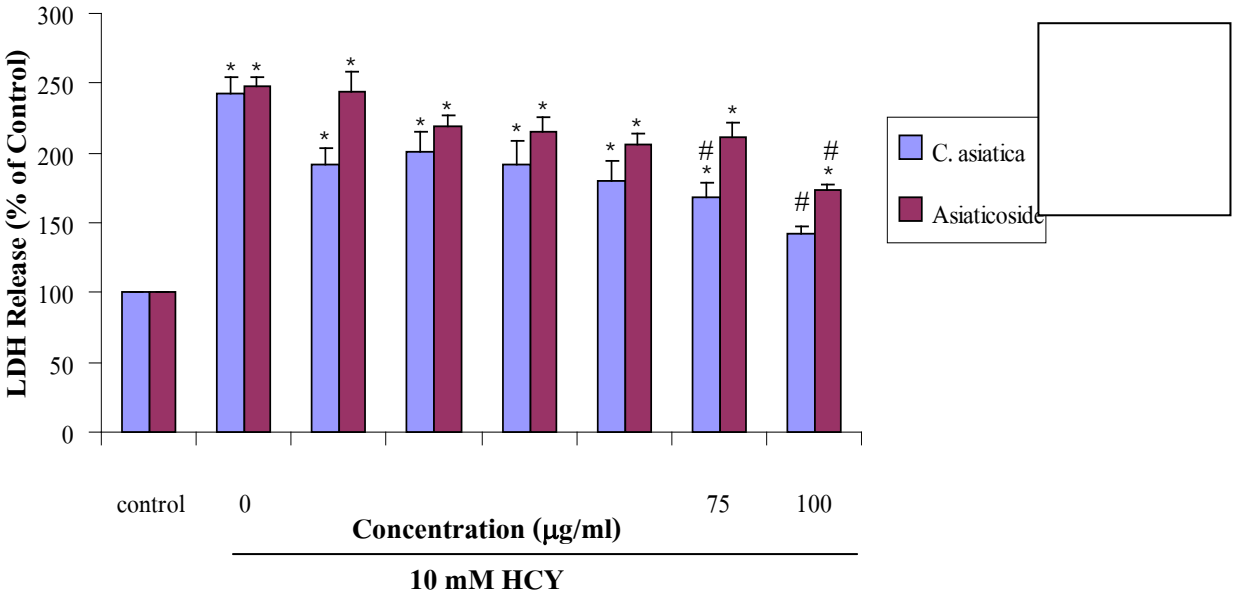


Fig. 8 Effects of pre-treatment on cell death of cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 1-100 μM of asiaticoside or *Centella asiatica* extract for 48 hr and further incubated with fresh medium containing 10 mM homocysteine for 72 hr after which cytoplasmic LDH release was determined. Values are mean ± S.E.M. (N=6). *P<0.05 compared with control cultures, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

6. Effects of co-treatment on cell viability of homocysteine-exposed N1E-115 neuroblastoma cell line.

Exposure of N1E-115 cultures to homocysteine at a concentration of 10 mM for 72 hr reduced cell viability to nearly 50% in comparison with untreated controls. However, the cytotoxicity caused by homocysteine was significantly reduced by co-exposure to 75-100 μg/ml of *Centella asiatica* extract and asiaticoside in a concentration-related manner. However, other concentrations of *Centella asiatica* extract and asiaticoside (1-50 μg/ml) did not attenuate cell viability caused by homocysteine (Fig. 9).

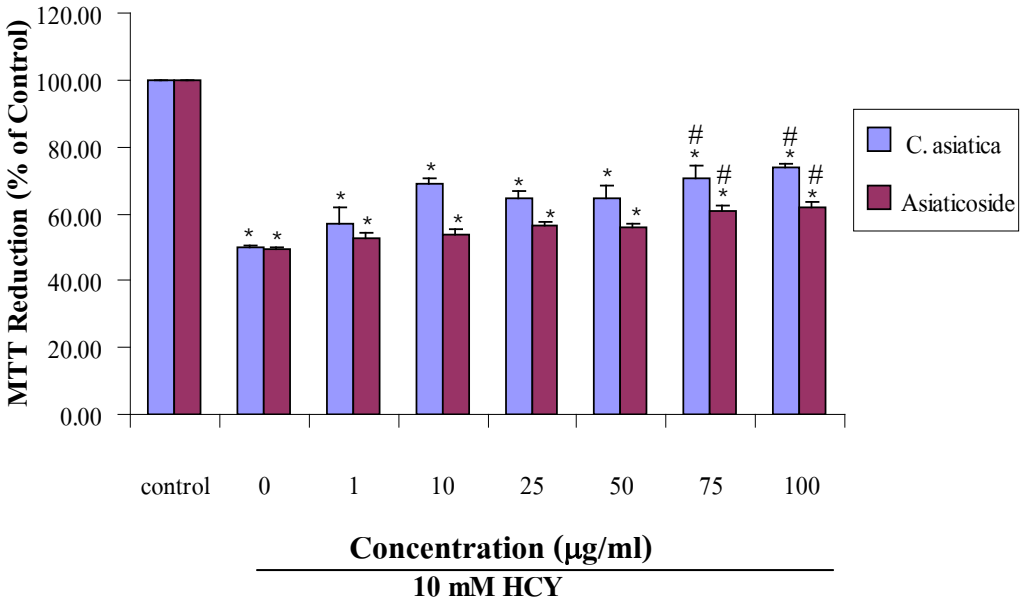


Fig. 9 Effects of co-treatment on cell viability of cultured N1E-115 cell line exposed to homocysteine.

N1E-115 cultures were incubated with 1-100 μM of asiaticoside or *Centella asiatica* extract in combination with 10 mM homocysteine for 72 hr before determination of cellular MTT reduction. Valued are mean ± S.E.M. (N=6). *P<0.05 compared with control cultures, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

7. Effects of co-treatment on cell death of homocysteine-exposed N1E-115 neuroblastoma cell line.

Exposure of N1E-115 cultures to homocysteine at a concentration of 10 mM for 72 hr induced a marked cell death in comparison with untreated controls. However, the cytotoxicity caused by homocysteine was significantly reduced by co-exposure to 100 μg/ml of *Centella asiatica* extract. However, other concentrations of *Centella asiatica* extract (1-75 μg/ml) and all concentrations of asiaticoside (1-100 μg/ml) did not attenuate cell death caused by homocysteine (Fig. 10).

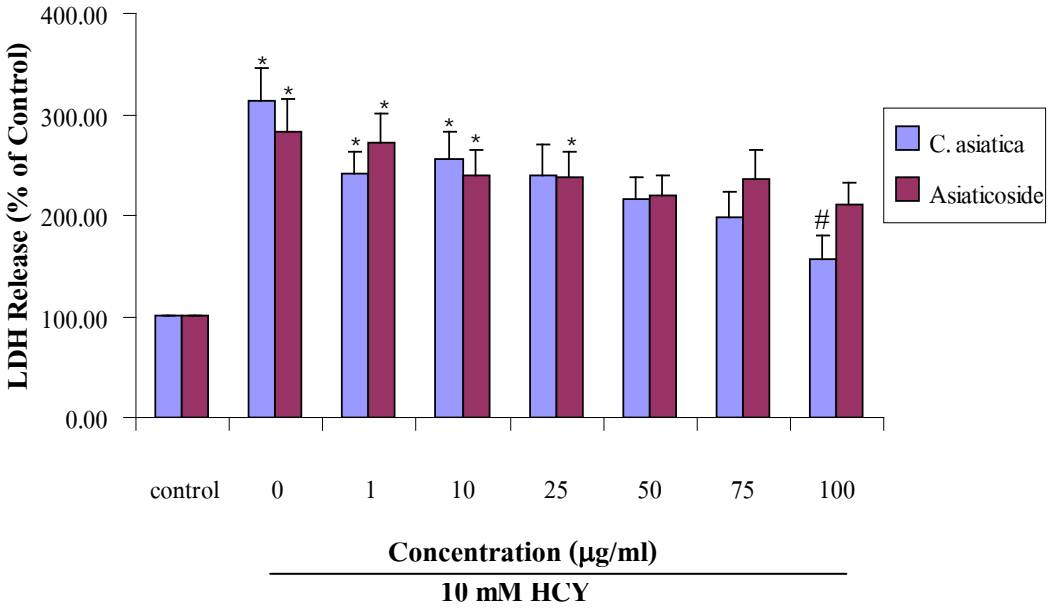


Fig. 10 Effects of co-treatment on cell death of cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 1-100 μM of asiaticoside or *Centella asiatica* extract in combination with 10 mM homocysteine for 72 hr before determination of cytoplasmic LDH release. Valued are mean ± S.E.M. (N=6). *P<0.05 compared with control cultures, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

8. Effects of co-treatment with *Centella asiatica* extract on total glutathione levels of homocysteine exposed N1E-115 neuroblastoma cell line.

Co-treatment with 100 μg/ml of *Centella asiatica* extract and 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 11).

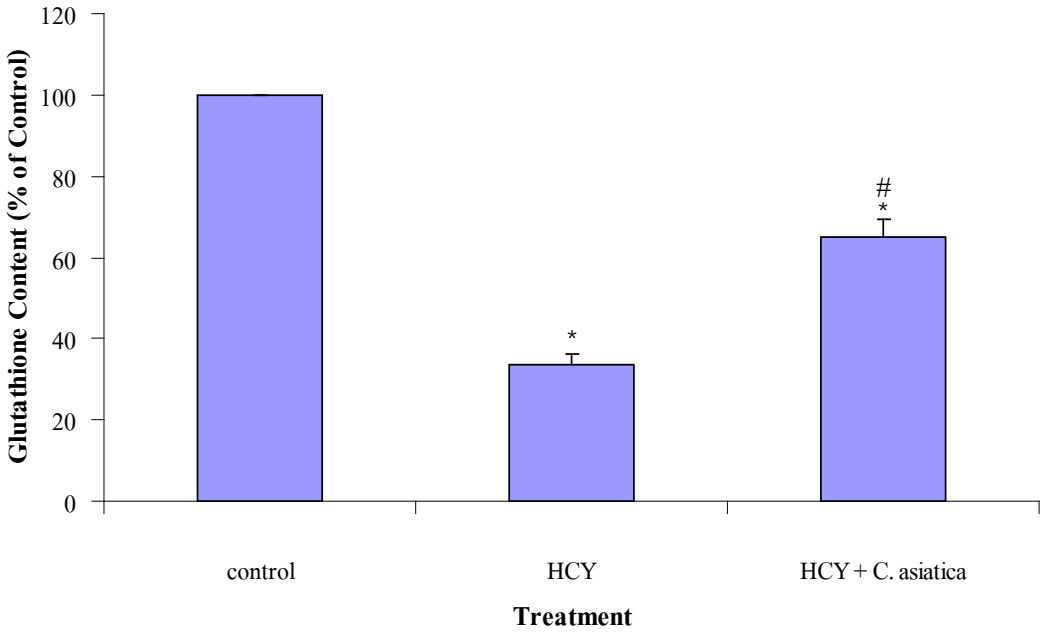


Fig. 11 Effects of co-treatment with *Centella asiatica* extract on glutathione levels of cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 100 μg/ml of *Centella asiatica* extract and 10 mM homocysteine for 72 hr before glutathione content determination. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

9. Effects of co-treatment with asiaticoside on glutathione levels of homocysteine exposed N1E-115 neuroblastoma cell line.

Co-treatment with 100 μg/ml of asiaticoside and 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 12).

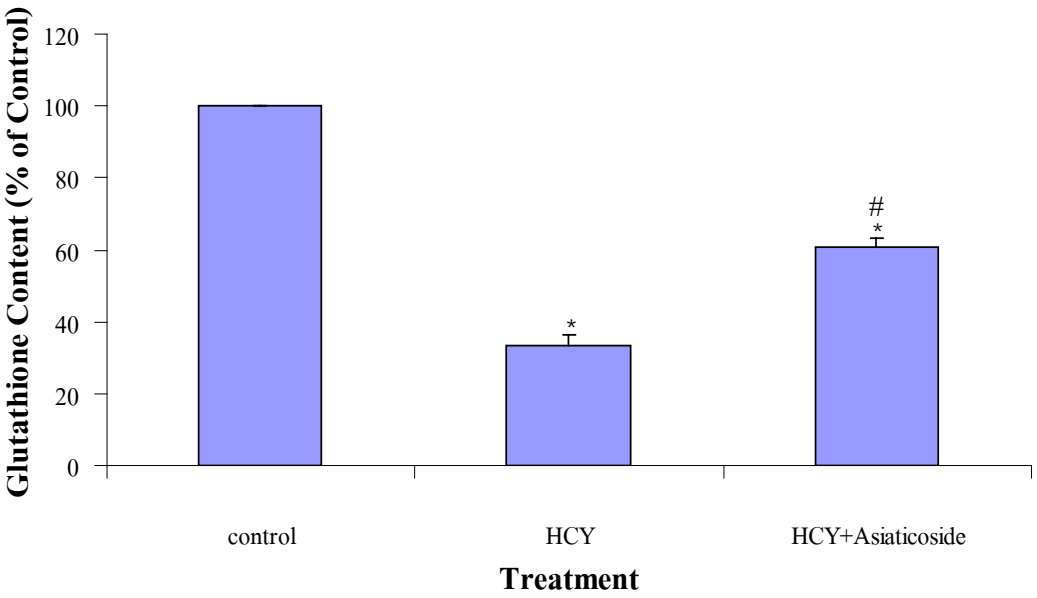


Fig. 12 Effects of co-treatment with asiaticoside on glutathione levels of cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 100 μg/ml of asiaticoside and 10 mM homocysteine for 72 hr before glutathione content determination. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

10. Effects of pre-treatment with *Centella asiatica* extract on glutathione levels of homocysteine exposed N1E-115 neuroblastoma cell line.

Pre-treatment with 100 μg/ml of *Centella asiatica* extract for 48 hr before an exposure to 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 13).

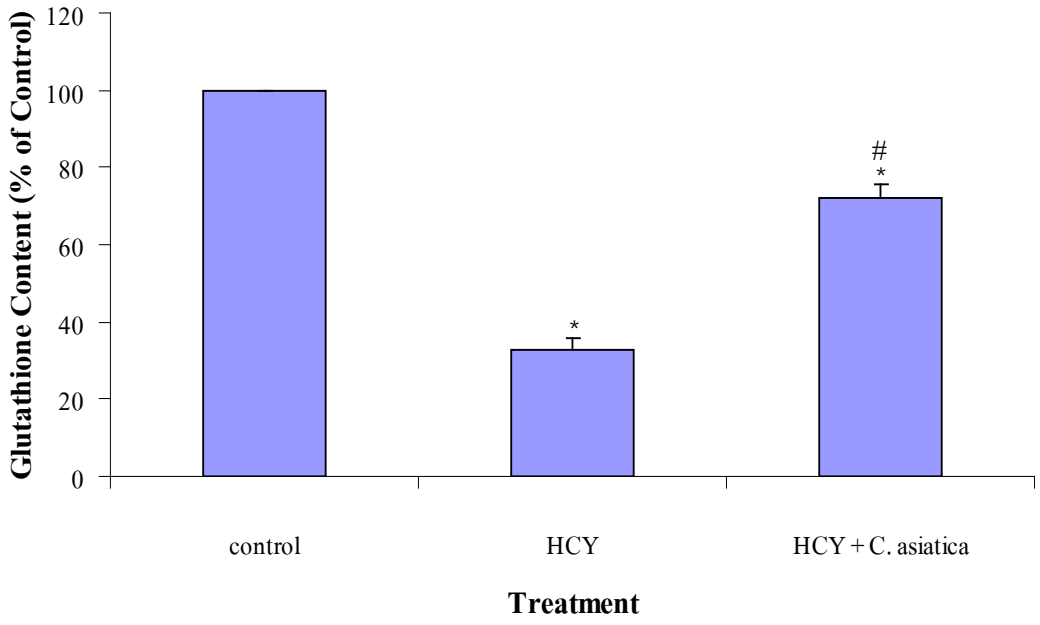


Fig. 13 Effects of pre-treatment with *Centella asiatica* extract on glutathione levels of cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 100 μg/ml of *Centella asiatica* extract for 48 hr and further incubated with 10 mM homocysteine for 72 hr before glutathione content determination. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

11. Effects of pre-treatment with asiaticoside on glutathione levels of homocysteine exposed N1E-115 neuroblastoma cell line.

Pre-treatment with 100 μg/ml of asiaticoside for 48 hr before an exposure to 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 13).

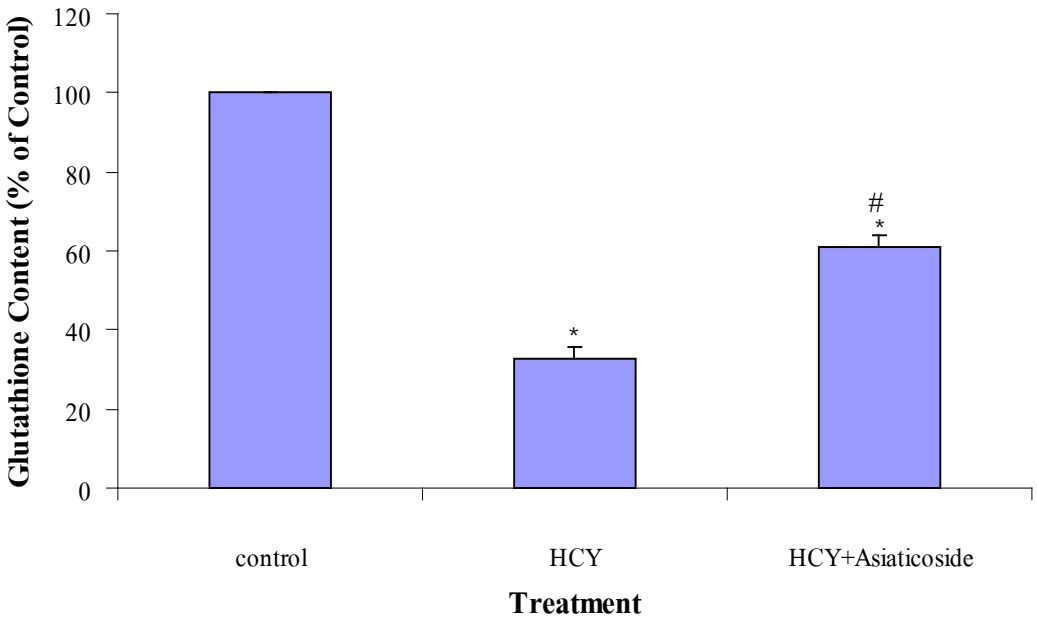


Fig. 14 Effects of pre-treatment with asiaticoside on glutathione levels of cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 100 μg/ml of asiaticoside for 48 hr and further incubated with 10 mM homocysteine for 72 hr before glutathione content determination. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

12. Effects of co-treatment with *Centella asiatica* extract on Hoechst 33342 and propidium iodide (PI) staining in homocysteine-exposed N1E-115 neuroblastoma cell line.

Co-treatment with 100 μg/ml of *Centella asiatica* extract and 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 15).

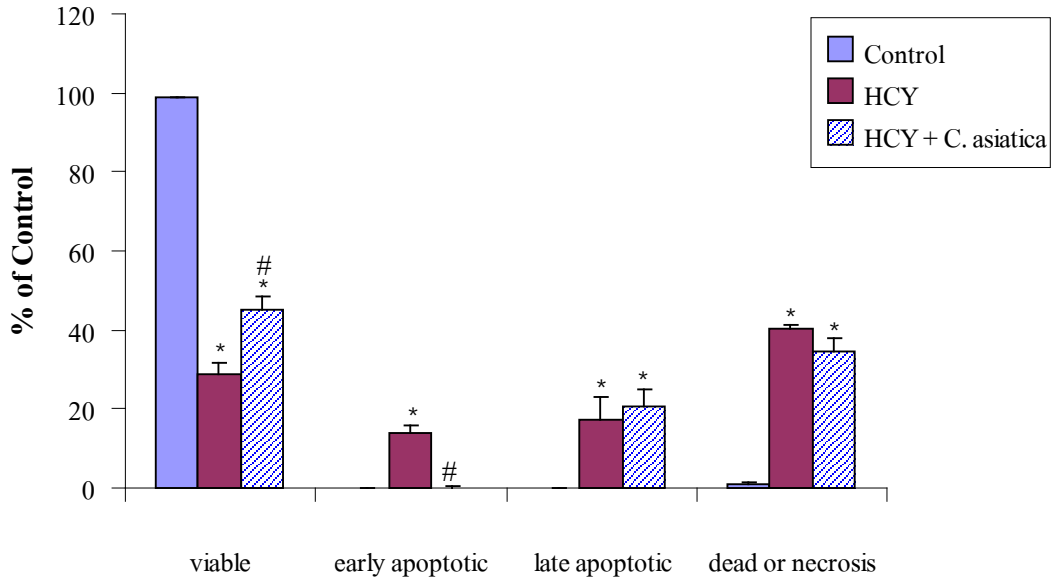


Fig. 15 Effects of co-treatment with *Centella asiatica* extract on Hoechst 33342 and propidium iodide staining in cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 100 μg/ml of *Centella asiatica* extract and 10 mM homocysteine for 72 hr before determining apoptotic cell death by staining with Hoechst 33342 and propidium iodide. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

13. Effects of co-treatment with asiaticoside on Hoechst 33342 and propidium iodide (PI) staining in homocysteine-exposed N1E-115 neuroblastoma cell line.

Co-treatment with 100 μg/ml of asiaticoside and 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 16).

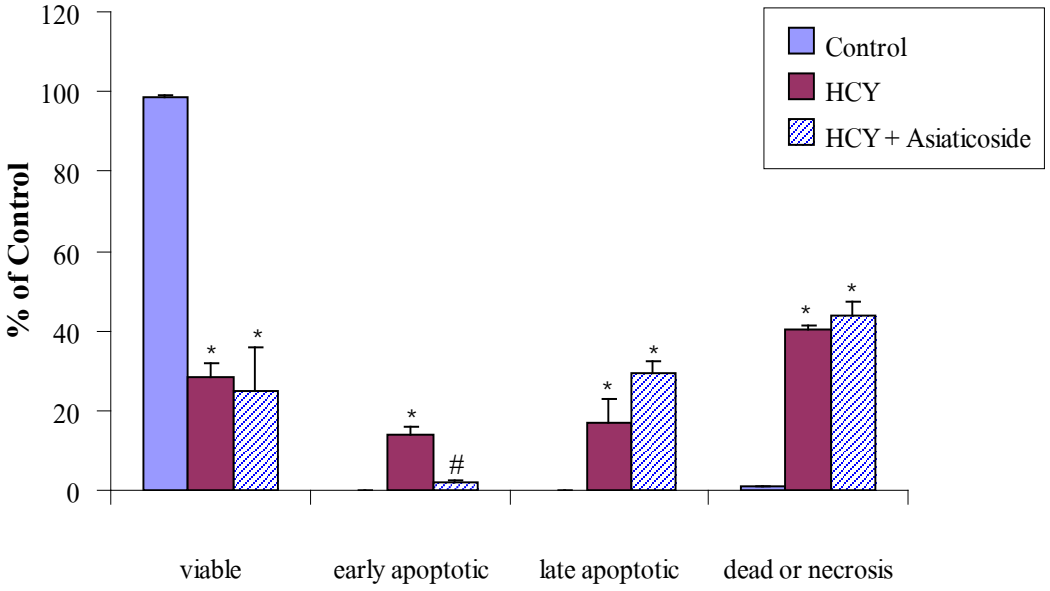


Fig. 16 Effects of co-treatment with asiaticoside on Hoechst 33342 and propidium iodide staining in cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 100 μg/ml of asiaticoside and 10 mM homocysteine for 72 hr before determining apoptotic cell death by staining with Hoechst 33342 and propidium iodide. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

14. Effects of pre-treatment with *Centella asiatica* extract on Hoechst 33342 and propidium iodide (PI) staining in homocysteine-exposed N1E-115 neurobalstoma cell line.

Pre-treatment with 100 μg/ml of *Centella asiatica* extract for 48 hr before an exposure to 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 17).

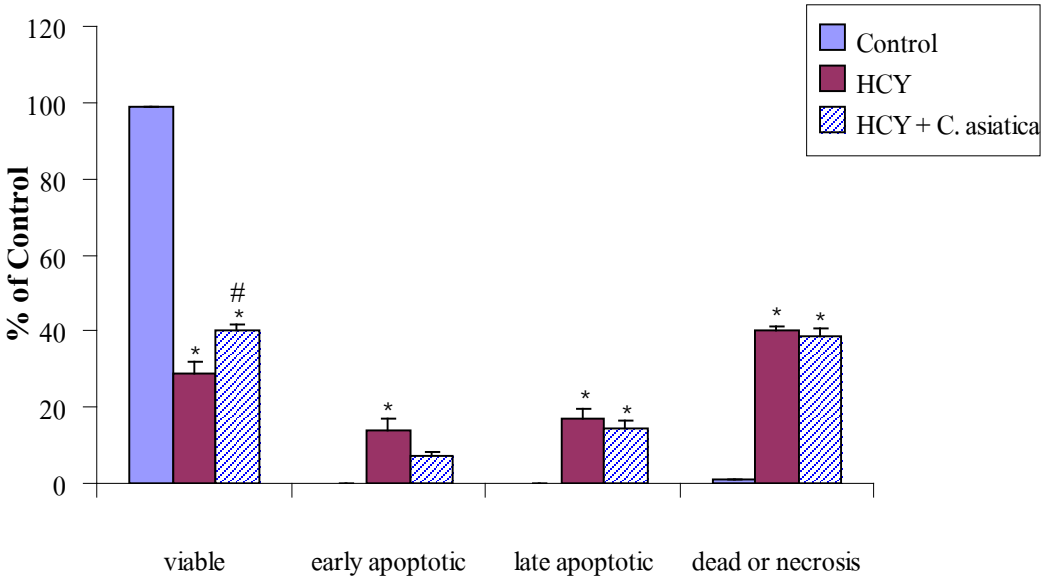


Fig. 17 Effects of pre-treatment with *Centella asiatica* extract on Hoechst 33342 and propidium iodide staining in cultured N1E-115 cell line exposed to homocystine.

Cultures were incubated with 100 μg/ml of *C. asiatica* extract for 48 hr and further incubated with 10 mM homocysteine for 72 hr before determining apoptotic cell death by staining with Hoechst 33342 and propidium iodide. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple compatisons).

15. Effects of pre-treatment with asiaticoside on Hoechst 33342 and propidium iodide (PI) staining in homocysteine-exposed N1E-115 neurobalstoma cell line.

Pre-treatment with 100 μg/ml of asiaticoside for 48 hr before an exposure to 10 mM homocysteine for 72 hr significantly reversed homocysteine-induced glutathione content diminution in cultured N1E-115 cells (Fig. 18).

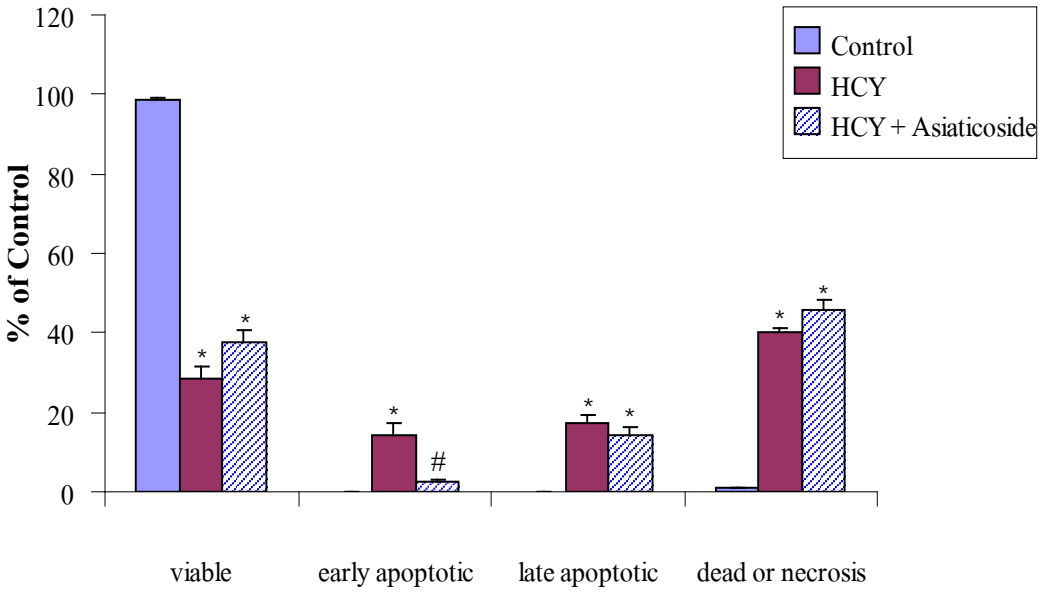


Fig. 18 Effects of pre-treatment with asiaticoside on Hoechst 33342 and propidium iodide staining in cultured N1E-115 cell line exposed to homocysteine.

Cultures were incubated with 100 μg/ml of asiaticoside for 48 hr and further incubated with 10 mM homocysteine for 72 hr before determining apoptotic cell death by staining with Hoechst 33342 and propidium iodide. Values are mean ± S.E.M. (N=6). *P<0.05 vs control group, #P<0.05 compared with homocysteine-exposed group (One-way ANOVA followed by Scheffe's test for multiple comparisons).

CHAPTER V

DISCUSSION AND CONCLUSION

In summary, the present study demonstrated that in neuroblastoma cell cultures, homocysteine induces neuronal injury and death in a concentration-related manner. In addition, it was found that *Centella asiatica* extract and asiaticoside possess a certain degree of neuroprotective effect. However, the mechanism by which this beneficial effect was not fully understood.

In this culture system, exposure of cultured N1E-115 cells to 0.5-20 mM of homocysteine for 24-72 hr induced gradual decreases in cellular MTT reduction in correlation with concentrations and time of exposure. A condition of exposure with 10 mM of homocysteine for 72 hr was adopted as a challenging cytotoxic condition which decreased mitochondrial metabolic activity of the culture to nearly 50% of that of control cultures. This insulting condition resulted in marked cellular injury which was implied by compromised mitochondrial metabolic activity and cell viability, reduced cellular glutathione content, and increased both apoptotic and necrotic cell death.

Homocysteine (HCY) is a sulfur-containing amino acid formed during the intracellular conversion of methionine to cysteine. This toxic intermediate may be remethylated to form methionine or trans-sulfurated to give cysteine. HCY is considered to be neurotoxic and a risk factor for neurodegenerative diseases (Loureiro et al., 2008).

Homocysteic acid (HCA) has been detected in various regions of the central nervous system, particularly in the cerebral cortex and in the hippocampus. The exogenous administration of the sulphur-containing amino acid delivers powerful excitatory effects. When administered systemically, HCA was found to induce seizures both in mature and developing rodents (Langmeier et al., 2003).

Survival of cells was reduced significantly at homocysteine concentrations of 2 mM and above, while no homocysteine toxicity could be observed at concentrations of 1 mM or less, even after incubation periods of up to 96 h (Maler et al., 2003).

Therefore, homocysteine-induced neuronal injuries and death in cultured neuroblastoma cell line should be a convenient and valuable *in vitro* model of neurodegeneration. This model

could be used to investigate the potential neuroprotective activity of test agents from chemical synthesis or natural products.

Centella asiatica extract and asiaticoside have been reported to possess various pharmacological activities. This study primarily investigated effects of *Centella asiatica* extract and asiaticoside in N1E-115 neuroblastoma cell line in term of neuroprotective properties.

Preliminary results revealed that the incubation for 24-72 hr with concentration ranges of 1-100 μg/ml of *Centella asiatica* extract and asiaticoside had no cytotoxic effects on cultured neuroblastoma cells as considered from the mitochondrial metabolic activity. Therefore, this concentration range of *Centella asiatica* extract and asiaticoside was used to investigate the possible neuroprotective effects of these test agents against homocysteine-induced neuronal injuries in neuronal cell line cultures. The results indicated that impairments of mitochondrial metabolic activity induced by homocysteine could be significantly attenuated by pre-treatment and co-treatment with a higher concentration range of *Centella asiatica* extract and asiaticoside (50-100 μg/ml). In addition, increases in cytoplasmic LDH release, an indication of cell death, were also significantly attenuated under similar conditions of effective treatments with *Centella asiatica* extract and asiaticoside which replenished mitochondrial metabolic activity.

The experimental findings suggest that *Centella asiatica* extract and asiaticoside possess a certain degree of cytoprotective activity to cultured neurons. The beneficial neuroprotective activity observed in *Centella asiatica* extract at concentrations of 50-100 μg/ml on neuroblastoma cells is likely due to, at least, asiaticoside content (38.56%). However, previous unpublished observation suggested that asiaticoside might not be an active component responsible for protective effects of *Centella asiatica* extract against NO-induced neurotoxicity. This discrepancy might be due to differences in the mechanism of neurotoxicity between homocysteine and NO.

Despite our present knowledge of some of the cellular pathways that modulate CNS injury, complete therapeutic prevention or reversal of acute or chromic neuronal injury has not been achieved. As a result, identification of novel therapeutic targets for the treatment of neuronal injury would be extremely beneficial to reduce or eliminate disability from CNS disorders. Current studies have begun to focus on pathways of oxidative stress that involve a variety of cellular pathways.

The potential antioxidant therapy includes natural free radicals scavenging enzymes and agents which are capable of augmenting the endogenous antioxidant defenses, e.g., glutathione (Cheeseman and Scater, 1993). Glutathione is an endogenous antioxidant present mainly in the

reduced form within the cells. It reacts with the free radicals and prevents the generation of hydroxyl radicals, the most toxic form of free radicals. During this defensive process, reduced glutathione gets converted to its oxidized form. The decreased levels of reduced glutathione in cultured neurons exposed to homocysteine seen in our study suggested that there was an increased generation of free radicals and the reduced glutathione was depleted during the process of combating oxidative stress. Pre-treatment and co-treatment of cultured neurons with 100 μg/ml of either *Centella asiatica* extract or asiaticoside (an effective neuroprotective concentration as considered from cell viability and cell death) and 10 mM homocysteine significantly attenuated homocysteine-induced glutathione diminution in N1E-115 cell cultures. This beneficial effect might be derived from antioxidant and free radical scavenging properties of *Centella asiatica* extract and asiaticoside.

For determination of apoptotic cell death, a two-color fluorescence staining with two nuclear fluorochromes, Hoechst 33342 (Ho 33342) which labels nuclei of all cells, and propidium iodide (PI) which labels only nuclei of dead cells, was used. By this method it was possible to detect, in the same sample and at the same time, intact cells, cells undergoing apoptosis, and dead cells resulting from apoptotic and/or necrotic processes. In cell culture, unlike *in vivo*, apoptotic cells are not phagocytosed and eventually lyse (postapoptotic or secondary necrosis). This is of practical importance because morphological changes seen in cultures may be due to the transition from apoptosis to necrosis, rather than to the induction of apoptosis itself. With the two-color fluorescence DNA staining, distinguishing between different stages of typical necrosis and apoptosis is generally easy and accurate on the basis of morphological features. Necrotic cells are PI-positive (red), showing generally swolen and round nuclei without any nuclear condensation. Early apoptotic cells are not stained by PI but exhibit classical nuclear alterations (condensation and fragmentation) visualized by Ho 33342 stain (blue). However, during late stage of apoptosis, neurons progressively lost their cell membrane integrity and become PI-positive. These neurons show red-orange apoptotic nuclei due to transition of fluorescence color from blue to red (PI fluorescence plus quenching of the Ho 33342 fluorescence by energy transfer to PI). In late stage of necrosis, cells are progressively disintegrated into small fragments and, similarly, late apoptotic cells or apoptotic bodies undergo postapoptotic necrosis. These small and indiscernible fragments with red-orange fluorescence were not counted. Therefore, the analysis mainly considers relatively early steps of necrosis and is probably only slightly distorted by postapoptotic necrosis. Conversely, necrotic cells probably did not interfere in the count of apoptotic cells.

Therefore, although it is likely that postapoptotic necrosis occurs during the time of the experiments, the possible interference of postapoptotic necrosis in the analysis is probably minor.

Exposure to 10 mM homocysteine for 72 hr caused marked cell death through both apoptotic and necrotic pathways. In general, pre-treatment and co-treatment with 100 μg/ml of *Centella asiatica* extract and asiaticoside significantly reduced number of cells in early apoptosis. Conceivably, *Centella asiatica* extract and asiaticoside might protect cell death by acting directly as an antiapoptotic agent at some points in cellular apoptotic cascade. Alternatively, they might act indirectly through their antioxidant and free radical scavenging properties.

It was notable that both pre-treatment and co-treatment with *Centella asiatica* extract (containing mainly asiaticoside and madecassoside) and asiaticoside displayed a similar profile of neuroprotective efficacy. This interesting finding implies that *Centella asiatica* extract and asiaticoside might have both direct and indirect neuroprotective actions. It also implies that the major site of their neuroprotective actions is preferably intracellular.

In conclusion, experimental results from the present study suggested that *Centella asiatica* extract and asiaticoside may possess the marginal *in vitro* cytoprotective property against homocysteine-induced neuronal damages. On one hand, antioxidant and free radical scavenging activities of *Centella asiatica* extract and asiaticoside may be responsible for this beneficial property by direct action on homocysteine-induced oxidative stress. On the other hand, they may act indirectly through facilitation of enzymatic and non-enzymatic cellular defense mechanisms. It is also plausible that *Centella asiatica* extract and asiaticoside may have a certain degree of antiapoptotic activity. Currently, exact mechanisms of action of *Centella asiatica* extract and asiaticoside are not fully understood. Nevertheless, all of mentioned possibilities could be underlying mechanisms for neuroprotective effects of *Centella asiatica* extract and asiaticoside. Therefore, further detailed investigation on neuroprotective effects of *Centella asiatica* and potential applications in management of neurodegenerative disorders are needed in the future.

References

Abdul-Hamid, A., Zainol, M.K., Yusof, S., and Muse, R. 2003. Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of *Centella asiatica*(L.) Urban. Food Chemistry 81, 575-581.

Abdul-Hamid, A., Hussin, M., Mohamad, S., Saari, N,. Ismail, M., and Bejo, M.H. 2005. Protective effect of *Centella asiatica* extract and powder on oxidative stress in rats. Food Chemistry

Akanji, A.O., Ohaeri, J.U., Al-Shammri, S.A., Fatania, H.R., 2007. Associations of blood homocysteine concentrations in Arab schizophrenic patients. Clinical Biochemistry 40, 1026-1031.

Appa Rao, M.V.R. 1973. Effect of Mandookaparni (*Centella asiatica*) on the general mental ability retarded children. Journal of Research in Indian Medicine 8: 9-16.

Babu, T.D., Kuttan, G., and Padikkala, J. 1995. Cytotoxic and anti-tumor properties of certain taxa of Umbelliferae with special reference to *Centella asiatica*(L.) Urban. Journal of Ethanopharmacology 48, 53-57.

Bailey, E. 1945. Treatment of leprosy. Nature 155: 601.

Boiteau, P., Buzas, A., Lederer, E., and Polonsky, J. 1949. Derivatives of *Centella asiatica* used against leprosy, chemical constitution of asiaticoside. Nature 163: 258-259.

Bonte, F., Dumas, M., Chaudagne, C., and Meybeck, A. 1994. Influence of Asiatic acid, madecassic acid, and asiaticoside on human collagen I synthesis. Planta Medica 60(2): 133-135.

Boose, J.P., Papillon, J., Frenette, G., Dansereau, J., Cadotte, M., and Le lorier, J. 1979. Clinical study of a new antikeloid agent. Annal of Plastic Surgery 3(1): 13-21.

Bradwejn, J., Zhou, Y., Koszycki, D., and Shlik, J. 2000. A double-blind, placebo dontrolled study on the effects of Gotu Kola (*Centella asiatica*) on acoustic startle response in healthy subjects. Journal of Clinical Psychopharmacology 20(6), 680-684.

Brinkhaus, B., et al. Chemical, pharmacological and clinical profile of the East Asian medicinal plant *Centella asiatica*. Phytomedicine 2000; 7: 427-8.

Chen, H.M., Muramoto, K., Yamauchi, F., and Nokihara, K. 1996. Antioxidant activity of design peptides based on antioxidative peptide isolated from digest of a soybean protein. Journal of Agricultural and Food Chemistry 44: 2619-2623.

Chen, Y.J., Dai, Y.S., Chen, B.F., Chang, A., Chen, H.C., Lin, Y.C., Chang, K.H., Lai, Y.L., Chung, C.H., and Lai, Y.J. 1999. The effect of tetrandrine and extracts of *Centella asiatica* on Acute Radiation Dermatitis in Rats. Biological and Pharmaceutical Bulletin 22(7): 703-706.

Cheng, C.L., and Koo, M.W. 2000. Effect of *Centella asiatica* on ethanol induced gastric mucosal lesions in rats. Life sciences 67(21): 2647-2653.

Cheng, C.L., Guo, J.S., Luk, J., and Koo, M.W. 2004. The healing effects of *Centella* extract and asiaticoside on acetic acid induced gastric ulcers in rats. Life sciences 74(18): 2237-2249.

Chopra, R.N., Nayar, S.L., Chopra, I.C. 1956. Glossary of Indian Medicinal Plants New Delhi: CSIR.

Christie, L.A., Riedel, G., Algaidi, S.A., Whalley, L.J., Platt, B., 2005. Enhanced hippocampal long-term potentiation in rats after chronic exposure to homocysteine. Neuroscience Letters 373: 119-124.

Chung, Y.H., Hong, J., Shin, C.M., Joo, K.M., Kim, M.J., Cha, C.I., 2003. Immunohistochemical study on the distribution of homocysteine in the central nervous system of transgenic mice expressing a human Cu/Zn SOD mutation. Brain Research 967: 226-234.

Cox, D.N., Rajasuriya, S., Soysa, P.E., Gladwin, J., and Ashworth, A. 1993. Problems encountered in the community based production of leaf concentrate as a supplement for pre-school children in Sri Lanka. International Journal of Food Science and Nutrition 44: 123-132.

Danese, P., Carnevali, C., and Bertazzoni, M.G. 1994. Allergic contact dermatitis due to *Centella asiatica* extract. Contact Dermatitis 31(3): 201.

De Lucia, R., et al. Pharmacoligical and toxicological studies on *Centella asiatica*. Fitoterapia 1997; 68: 413-16.

Doherty, G.H., 2007. Homocysteine: More than just a matter of life and death. Experimental Neurology 205: 5-8.

Finkelstein, J.D., 1998. The metabolism of homocysteine: pathways and regulation. European Journal of Pediatrics 157 (Suppl. 2): S40– S44.

Goh, S.H., Chuah Mok, J.S.L., and Soepadmo, E. 1995. Malaysian medicinal plants for the treatment of cardiovascular diseases. Petaling Jaya, Pelanduk Publication Sdn. Bhd.

Gonzalo Garijo MA, et al. Allergic contact dermatitis due to *Centella asiatica*: a new case. Allergol Immunopatho. 1996; 24: 132-4.

Grieve, A., Butcher, S.P., Griffiths, R., 1992. Synaptosomal plasma membrane transport of excitatory sulphur amino acid transmitter candidates: kinetic characterization and analysis of carrier specificity. Journal of Neuroscience Research 32: 60– 68.

Grimaldi, R., De Ponti, F., D'Angelo, L., Caravaggi, M., Guidi, G., Lecchini, S., Frigo, G.M., and Crema, A. 1990. Pharmacokinetics of the total triterpenic fraction of *Centella asiatica* after single and multiple administrations to healthy volunteers. A new assay for Asiatic acid. J Ethnopharmacol 28(2): 235-241.

Görtz, P., Hoinkes, A., Fleischer, W., Otto, F., Schwahn, B., Wendel, U., Siebler, M., 2003. Implications for hyperhomocysteinemia: not homocysteine but its oxidized forms strongly inhibit neuronal network activity. Journal of the neurological Sciences 218: 109-114.

Guo, J.S., Cheng, C.L., Koo, M.W., 2004. Inhibitory effects of *Centella asiatica* water extract and asiaticoside on inducible nitric oxide synthase during gastric ulcer healing in rats. Planta Medica 70: 1150–1154.

Gupta, Y.K., Kumar, M.H.V., and Srivastava, A.K. 2003. Effect of *Centella asiatica* on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. Pharamcology, Biochemistry and Behavior 74: 579-585.

Hausen, BM. 1993. *Centella asiatica* (Indian Pennyroyal). An effective therapeutic but a weak sensitizer. Contact Dermatitis 29: 175-9.

Inamdar, P.K., Yeole, A.B., Ghogare, A.B., de Douza, N.J. 1996. Determination of biologically active constituents in *Centella asiatica*. Journal of Chromatography A 742: 127-130.

Jayashree, G., Kurup Muraleedhara, G., Sudarslal, S., and Jacob, V.B. 2003. Antioxidant activity of *Centella asiatica* on lymphoma-bearing mice. Fitoterapia 74(5): 431-434.

Kakkar, K.K. 1990. Mandukparni-medicinal uses and therapeutic efficacy. Probe XXIX, 176-182.

Kan, W.S., 1986. Pharmaceutical botany. P.416. Taiwan: National Research Institute of Chinese Medicine.

Kiesswetter, H., 1964. Treatment of wounds with asiaticoside (Madecassol). Wiener medizinische Wochenschrift 114: 124-126.

Kim, H., Cho, H., Kwon, Y.H., 2008. Synergistic induction of ER stress by homocysteine and β-amyloid in SH-SY5Y cells. Journal of Nutritional Biochemistry 19(11): 754-761.

Kim, J., Cho, S.Y., Lee, J., Jeong, S.M., Yoon, I., Lee, B., Lee, J., Pyo, M.K., Lee, S., Chung, J., Kim, S., Thim, H., Oh, J., Nah, S., 2007. Neuroprotective effects of ginsenoside Rg3 against homocysteine-induced excitotoxicity in rat hippocampus. Brain Research 1136: 196-199.

Kim, W.-K., Pae, Y.-S., 1996. Involvement of N-methyl-D-aspartate receptor and free radical in homocysteine-mediated toxicity on rat cerebellar granule cells in culture. Neuroscience Letters 216: 117–120.

Lipton, S.A., Kim, W.-K., Choi, Y.-B., Kumar, S., D'Emilia, D.M., Rayudu, P.V., Arnelle, D.R., Stamler, J.S., 1997. Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. Proceedings of the National Academy of Sciences of the United States of America 94: 5923–5928.

Loliger, J. 1991. The use of antioxidants in foods. In I. O. Aruoma; and B. Halliwell (eds.), Free radical and food additives P. 121. London: Taylor and Francis.

Loureiro, S.O., Heimfarth, L., Pelaez, P.L., Vanzin, C.S., Viana, L., Wyse, A.T.S., Pessoa-Pureur, R. 2008. Homocysteine activates calcium-mediated cell signaling mechanisms targeting the cytoskeleton in rat hippocampus. International Journal of Developmental Neuroscience 26(5): 447-455.

Maler, J.M., Seifert, W., Huther, G., Wiltfang, J., Ruther, E., Kornhuber, J., Bleich, S., 2003. Homocysteine induces cell death of rat astrocytes in vitro. Neuroscience Letters 347: 85-88.

Maquart, F.X., Bellon, G., Gillery, P., Wegrowski, Y., and Borel, J.P. 1990. Stimulation of collagen synthesis in fibroblast cultures by a triterpene extracted from *Centella asiatica*. Connective Tissue Research 24(2): 107-120.

Martignoni, E., Tassorelli, C., Nappi, G., Zangaglia, R., Pacchetti, C., Blandini, F., 2007. Homocysteine and Parkinson's disease: A dangerous liaison? Journal of the Neurological Sciences 257: 31-37.

Mattson, M.P., Shea, T.B., 2003. Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. Trends in Neurosciences 26: 137–146.

McGuffin M, et al. American Herbal Products Association's Botanical Safety Handbook. Boca Raton, FL: CRC Press, 1997: 26.

Moore, P., El-Sherbeny, A., Roon, P., Schoenlein, P.V., Ganapathy, V., Smith, S.B., 2001. Apoptotic cell death in the mouse retinal ganglion cell layer is induced in vivo by the excitatory amino acid homocysteine. Experimental Eye Research 73: 45-57.

Mukerji, B. 1953. Indian Pharmaceutical Codex New Delhi, India. Pp. 60-61.

Nakajima, H., and Ajiyoshi, K. 1972. Studies on the treatment of keloids and hypertrophic scars with madecassol. Medical Consultation and New Remedy 9: 433-444.

Nalini, K., Arroor, A.R., Karanth, K.S., and Rao, A. 1992. Effect of *Centella asiatica* fresh leaf aqueous extract on leaning and memory and biogenic amine turnover in albino rats. Fitoterapia LXIII (3): 232-237.

Natarajan, S., and Paily, P.P. 1973. Effect of topical Hydrocotyle Asiatica in psoriasis. Indian Journal of Dermatology 18(4): 82-85.

Obeid, R., Hermann, W., 2006. Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. FEBS Letters 580: 2994-3005.

Oldreive, C.E., Doherty, G.H., 2006. Neurotoxic effects of homocysteine on cerebellar Purkinje neurons *in vitro*. Neuroscience Letters 413: 52-57.

Pointel, J.P., Boccallon, H., Clorec, M., Ledvehat, C., and Joubert, M. 1987. Angiology 38: 46.

Punturee, K., Wild, C.P., Kasinrerk, W., and Vinitketkumnuen, U. 2005. Immunomodulatory activities of *Centella asiatica* and *Rhinacanthus nasutus* extracts. Asian Pacific Journal of Cancer Prevention 6(3): 396-400.

Ramswamy, A.s., et al. 1970. Pharmacoligical studies of *Centella asiatica*. Indian Journal of Medical Research 4: 160-175.

Ravokatra, A., Loiseau, A., Ratsimamanga-Urverg, S., Nigeon0Dureuil, M., and Ratsimamanga, A.R. 1974. Action of asiaticoside (Pentacyclic triterpene) extracted from Hydrocotyle madagascariensis on duodenal ulcers induced with mercaptoethylamine in male Wistar rats. Comptes rendus hebdomadaires des séances de l'Académie des sciences. Série D: Sciences naturelles 278(18): 2317-2321.

Robertus, H.C.Bisschops, Yolanda van der Graaf, Williem P.Th. M. Mali, Jeroen van der Ground, 2004. Elevated levels of plasma homocysteine are associated with neurotoxicity. Atherosclerosis 174: 87-92.

Rosen, H., Blumenthal, A., and McCallum, J. 1967. Effect of asiaticoside on wound healing in the rat. Proceedings of the Society for Experimental Biology and Medicine 125(1): 279-180.

Sachdev, P.S., 2005. Homocysteine and brain atrophy. Progress in neuron-psychopharmacology and Biological psychiatry 29: 1152-1161.

Sahu, N.P., Roy, S.K., and Mahato, S.B. 1989. Spectroscopic determination of structure of triterpenoid trisaccharides from *Centella asiatica* Phytochemistry 28 (10): 2852-2854.

Sakina, M.R., and Dandiya, P.C.1990. A psycho-neuropharmacological profile of *Centella asiatica*. Fitoterapia 45(4): 291-296.

Sasaki, S., Shinkai, H., Akashi, Y., and Kishihara, Y. 1972. Studies on the mechanism of action of asiaticoside (Madecassol) on experimental granulation tissue and cultured fibroblasts and its clinical application in systemic scleroderma. Acta Dermato Venereologica 52(2): 141-150.

Selley, M.L., Close, D.R., Stern, S.E., 2001. The effect of increased concentrations of homocysteine on the concentration of (E)-4-hydroxy-2-nonenal in the plasma and cerebrospinal fluid of patients with Alzheimer's disease. Neurobiology of Aging 23: 383-388.

Shobi, V., and Goel, H.C. 2001. Protection against radiation-induced conditioned taste aversion by *Centella asiatica*. Physiology and Behavior. 73(1-2): 19-23.

Shukla, A., Rasil, A.M., and Dhawan, B.N. 1999a. Asiaticoside-induced elevation of antioxidant levels in healing wounds. Phytotherapy Research 13(1): 50-54.

Shula, A., Rasik, A.M., Jain, G.K., Shankar, R., Kulshrestha, D.K., and Dhawan, B.N. 1999b. *In vitro* and *in vivo* wound healing activity of asiaticoside isolated from *Centella asiatica*. J Ethnopharmacol 65(1): 1-11.

Singh, B., and Rastogi, R.P. 1968. Chemical examination of *Centella asiatica* Linn III-constitution of brahmic acid. Phytochemistry 7: 1985.

Subathra, M., Shila, S., Devi, M.A. and Panneerselvam, C. 2005. Emerging role of *Centella asiatica* in improving age-related neurological antioxidant status. Experimental Gerontology 40(8-9): 707-715.

Suguna, L., Sivakumar, P., and Chandrakasan, G. 1996. Effects of *Centella asiatica* extract on dermal wound healing in rats. Indian Journal of Experimental Biology 34(12): 1208-1211.

Tallat, H.A. and Abbas, M.E. 1971. The effect of titrated extract of *Centella asiatica* on abnormal scar formation. Journal of Egyptian Surgery Society 6: 408-419.

Troen, A.M., 2005. The central nervous system in animal models of hyperhomocysteinemia. Progress in neuron-psychopharmacology and Biological psychiatry 29: 1140-1151.

Tsurumi, K., Hiramatsu, Y., Hayashi, M., and Fujimura, H. 1973. Oyo Yakuri 7: 833.

Vaidyaratnam, P.S.V. 1994. Indian Medicinal Plants: A Compendium of 500 Species, vol. 2. India: Orient Longman Ltd.

Veechai, A.D., Senmi, J., Gassan, G., and Mohinaro, M. 1984. Effect of *Centella asiatica* on the biosynthetic activity of fibroblast in culture. Farmacie Edition 39: 355-364.

Velasco, M. and Romero, E. 1976. Drug interaction between asiaticoside and some anti-inflammatory drugs in wound healing of the rat. Current therapeutic research, clinical and experimental 19: 121.

Viala, A., Cano, J.P., Duranid, A., Paulin, R., Roux, F., Placidi, M., Pinhas, H., and Lefournier, C. 1977. Study in animals of transcutaneous penetration of labeled active principles of the titrated extract of *Centella asiatica* applied on the skin in two topical forms. Therapie 32: 573-584.

Veerendra Kumar, M.H., and Gupta, Y.K. 2002. Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats. Journal of ethnopharmacology 79(2): 253-260.

Vogel, H.G., De Souza, N.J., and D'Sa, A. 1990. Acta Therapeutica 16: 285.

Wijeweera, P., Arnason, J.T., Koszycki, D., and Merali, Z. 2006. Evaluatoin of anxiolytic properties of Gotukola – (*Centella asiatica*) extracts and asiaticoside in rat behavioral models. Phytomedicine 13: 668-676.

Yen, G.C., Chen, H.Y., and Peng, H.H. 2001. Eveluation of the cytotoxicity, mutagenicity and antimutagenicity of emerging edible plants, Food and chemical toxicology 39(11): 1045-1053.

Yoshinori, A., Reiko, M., and Tsunematsu, T. 1982. Mono and sesquiterpenoids from Hydrocotyle and *Centella* species. Phytochemistry 21: 2590-2592.

VITAE

Miss Pakawadee Kidchanapanish was born on May 12, 1980, in Bangkok, Thailand. She had graduated the bachelor degree in Pharmacy from Chulalongkorn University since 2002. She started to work as a pharmacist in Nonsuwan Hospitol, Burirum for one year and four months. Consequently, she moved to HRH Princess Maha Chakri Sirindhorn Medical Center, Nakhon Nayok. She has enrolled for the Master's Degree in Pharmacology at the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University since June 2005.