กลไกปกป้องเซลล์ประสาทของสารสกัดและสารออกฤทธิ์ทางชีวภาพจากผลมะเกี๋ยงต่อภาวะเครียดเอ็นโดพลาสมิกเรติคิวลัมในเซลล์ประสาทส่วนฮิปโปแคมปัสของหนูชนิด HT22

นางสาวมลฤดี สุขประสารทรัพย์

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

NEUROPROTECTIVE MECHANISMS OF *CLEISTOCALYX NERVOSUM* VAR. *PANIALA* FRUIT EXTRACT AND ITS BIOACTIVE COMPOUND ON ENDOPLASMIC RETICULUM STRESS IN MOUSE HIPPOCAMPAL NEURONAL HT22 CELL LINE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Clinical Biochemistry and Molecular Medicine
Department of Clinical Chemistry
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Thesis Title	NEUROPROTECTIVE MECHANISMS OF *CLEISTOCALYX NERVOSUM* VAR. *PANIALA* FRUIT EXTRACT AND ITS BIOACTIVE COMPOUND ON ENDOPLASMIC RETICULUM STRESS IN MOUSE HIPPOCAMPAL NEURONAL HT22 CELL LINE
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มลฤดี สุขประสารทรัพย์ : กลไกปกป้องเซลล์ประสาทของสารสกัดและสารออกฤทธิ์ทางชีวภาพจากผลมะเกี๋ยงต่อภาวะเครียดเอ็นโดพลาสมิกเรติคิวลัมในเซลล์ประสาทส่วนฮิปโปแคมปัสของหนูชนิด HT22 (NEUROPROTECTIVE MECHANISMS OF *CLEISTOCALYX NERVOSUM* VAR. *PANIALA* FRUIT EXTRACT AND ITS BIOACTIVE COMPOUND ON ENDOPLASMIC RETICULUM STRESS IN MOUSE HIPPOCAMPAL NEURONAL HT22 CELL LINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. เทวิน เทนคำเนาว์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. ปิติ จันทร์วรโชติ, 131 หน้า.

สารกลูตาเมตที่มากเกินความจำเป็นทำให้มีอนุมูลอิสระเพิ่มขึ้นเหนี่ยวนำให้เกิดภาวะเครียดออกซิเดชั่นที่สัมพันธ์กับภาวะเครียดเอ็นโดพลาสมิกเรติคิวลัมเป็นสาเหตุสำคัญนำไปสู่การตายของเซลล์ประสาท ซึ่งเกี่ยวข้องกับกลุ่มโรคที่เกิดความเสื่อมทางระบบประสาท มะเกี๋ยง เป็นผลไม้กลุ่มเบอร์รี่พบในท้องถิ่นทางภาคเหนือของประเทศไทย มะเกี๋ยงมีองค์ประกอบของสารออกฤทธ์ทางชีวภาพที่สำคัญคือ กลุ่มแอนโทไซยานิน ซึ่งมีคุณสมบัติต้านอนุมูลอิสระได้อย่างมีประสิทธิภาพ วัตถุประสงค์ของงานวิจัยนี้ เพื่อศึกษาผลของสารสกัดผลมะเกี๋ยงและสารออกฤทธ์ทางชีวภาพแอนโทไซยานินในการป้องกันเซลล์ประสาทต่อภาวะเครียดเอ็นโดพลาสมิกเรติคิวลัมที่ถูกเหนี่ยวนำด้วยกลูตาเมตในเซลล์ประสาทส่วนฮิปโปแคมปัสของหนูชนิด HT22 จากการทดลองพบว่าสารสกัดผลมะเกี๋ยงมีความสามารถในการจับและออกฤทธิ์ต้านอนุมูลอิสระสูง มีปริมาณสารประกอบฟีนอลิกรวมและสารแอนโทไซยานินในปริมาณสูง โดยพบปริมาณสารไซยานิดิน-3-กลูโคไซด์ ในสารสกัดสูง ซึ่งเป็นสารออกฤทธิ์สำคัญทางชีวภาพกลุ่มแอนโทไซยานิน ผลวิจัยแสดงว่า สารสกัดผลมะเกี๋ยง และสารบริสุทธิ์ไซยานิดิน-3-กลูโคไซด์ สามารถป้องกันการตายของเซลล์ประสาทชนิด HT22 จากการเหนี่ยวนำด้วยสารกลูตาเมต ซึ่งการเหนี่ยวนำดังกล่าวนี้ทำให้เกิดอนุมูลอิสระส่งผลต่อการเกิดภาวะเครียดเอ็นโดพลาสมิกเรติคิวลัม นำไปสู่วิถีการตายแบบอะพอพโตซิสของเซลล์ประสาท โดยทั้งสารสกัดผลมะเกี๋ยงและสารไซยานิดิน-3-กลูโคไซด์ สามารถยับยั้งการเกิดอนุมูลอิสระภายในเซลล์ได้ และยังพบว่าสามารถลดระดับการแสดงออกของโปรตีนสำคัญที่เกี่ยวข้องกับภาวะเครียดเอ็นโดพลาสมิกเรติคิวลัมดังกล่าว ได้แก่ calpain caspases-12 และ CHOP อย่างมีนัยสำคัญทางสถิติ ซึ่งกลไกการออกฤทธิ์ปกป้องการตายของเซลล์ประสาท HT22 โดยสารสกัดผลมะเกี๋ยงและสารไซยานิดิน-3-กลูโคไซด์ นี้ มีความสัมพันธ์กับกระบวนการกำจัดหรือต้านอนุมูลอิสระ โดยพบว่า สารสกัดผลมะเกี๋ยงและสารไซยานิดิน-3-กลูโคไซด์ มีผลต่อการแสดงออกที่เพิ่มขึ้นของโปรตีน ERK และ Nrf2 ซึ่งเป็นโปรตีนที่ควบคุมและเกี่ยวข้องกับกลไกการอยู่รอดของเซลล์ ผ่านระบบการทำงานของเอนไซม์ต่างๆ ที่ทำหน้าที่กำจัดหรือลดอนุมูลอิสระ ยิ่งกว่านั้นเมื่อทำการทดสอบสารทั้งสองดังกล่าวต่อเซลล์ประสาท HT22 พบว่าสามารถเพิ่มการแสดงออกของยีน SOD CAT GPx และ GST ซึ่งเป็นยีนเอนไซม์ที่ทำหน้าที่ในการกำจัดและลดอนุมูลอิสระที่มากเกินภายในเซลล์ได้ จากผลการศึกษานี้แสดงให้เห็นถึงข้อมูลทางวิทยาศาสตร์ที่เป็นประโยชน์เกี่ยวข้องกับกลไกการออกฤทธิ์ปกป้องการตายของเซลล์ประสาทของสารสกัดผลมะเกี๋ยงซึ่งเป็นผลไม้ตระกูลเบอร์รี่พื้นเมืองของไทย และสารไซยานิดิน-3-กลูโคไซด์ที่เป็นสารออกฤทธิ์สำคัญทางชีวภาพที่พบมากในผลไม้นี้ ด้วยการกระตุ้นกระบวนการมีชีวิตรอดของเซลล์ผ่านวิถี ERK/Nrf2 ในเซลล์ และอาจเป็นแนวทางในการนำมาประยุกต์ใช้เป็นสาร neuroprotectant ต่อไป

ภาควิชา	เคมีคลินิก	ลายมือชื่อนิสิต	.....
สาขาวิชา	ชีวเคมีคลินิกและอณูทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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5576954137 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE
KEYWORDS: CLEISTOCALYX NERVOSUM VAR. PANIALA / ANTHOCYANIN / CYANIDIN-3-GLUCOSIDE / GLUTAMATE / ROS / ER STRESS / NEUROPROTECTION / APOPTOSIS

MONRUEDEE SUKPRASANSAP: NEUROPROTECTIVE MECHANISMS OF *CLEISTOCALYX NERVOSUM* VAR. *PANIALA* FRUIT EXTRACT AND ITS BIOACTIVE COMPOUND ON ENDOPLASMIC RETICULUM STRESS IN MOUSE HIPPOCAMPAL NEURONAL HT22 CELL LINE. ADVISOR: ASST. PROF. DR. TEWIN TENCOMNAO, CO-ADVISOR: ASSOC. PROF. DR. PITHI CHANVORACHOTE, 131 pp.

The excessive glutamate-induced oxidative and endoplasmic reticulum (ER) stress is the one of the leading causes of neuronal cell death which associates with many neurodegenerative diseases. *Cleistocalyx nervosum* var. *paniala* (CNP) is an indigenous berry fruit found in the North of Thailand. It has many bioactive compounds, especially anthocyanin which has been shown to possess powerful antioxidant properties. The aim of this research was to investigate the neuroprotective effect of CNP extract and its major bioactive compound; cyanidin-3-glucoside (C3G), against ROS-mediated ER stress induced by glutamate in HT22 mouse hippocampal neuronal cells. CNP extract was clarified for its radical scavenging activities, total phenolic and anthocyanin contents. The key anthocyanin-C3G used as a marker to standardize the extract in the study. We found that CNP extract and C3G prevented glutamate-induced apoptosis cell death. For mechanistic approach, glutamate-induced cell death through reactive oxygen species (ROS) - mediated ER stress pathways, indicating by the increase of ROS and ER stress signatures including calpain, caspases-12 and C/EBP homologous proteins (CHOP). CNP extract and C3G suppressed ROS and ER stress in glutamate-treated cells by up-regulating the survival proteins, including extracellular regulated protein kinase (ERK) and nuclear factor E2-related factor2 (Nrf2). Moreover, the gene expressions of endogenous antioxidant enzymes; superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidase (GPx)), and phase II enzymes (glutathione-S-transferases (GSTs)) were significantly increased in CNP extract and C3G treated cells. Taken together, our results provide the information and molecular mechanism of CNP extract and C3G as a promising neuroprotectant and antioxidant via activation of ERK/Nrf2 survival pathway.

Department:	Clinical Chemistry	Student's Signature .....
Field of Study:	Clinical Biochemistry and Molecular Medicine	Advisor's Signature .....
		Co-Advisor's Signature .....
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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
APPH	((2,2'-azobis(2-methylpropionamidine) dihydrochloride))
CAT	Catalase
CHOP	C/EBP homologous proteins
CNP	*Cleistocalyx nervosum* var. *paniala*
C3G	Cyanidin-3-glucoside
$DCFH_2$-DA	2',7'- dichlorofluorescein diacetate
DM	Deferoxamine mesylate
DMEM	Dulbecco's modified Eagle medium
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ER	Endoplasmic reticulum
ERK	Extracellular-regulated kinase
FBS	Fetal Bovine Serum
FPAP	Ferric reducing antioxidant power
Glu	Glutamate
GPx	Glutathione peroxidase

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GSH	Glutathione
GST	Glutathione-S-transferase
HPLC	High performance liquid chromatography
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MnTBAP	Mn(III)tetrakis (4-benzoic acid) porphyrin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetyl-L-cysteine
Nrf2	Nuclear factor E2-related factor2
ORAC	Oxygen radical absorbance capacity
PI	Propidium iodide
ROS	Reactive oxygen species
SOD	Superoxide dismutase
UPR	Unfolded protein response

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CHAPTER I

INTRODUCTION

Background and rationale

Brain aging has become an area of interest for recent research and a subject of much speculation fueled largely from the widely recognized fact that age is the biggest risk factor in most neurodegenerative diseases (Markesbery & Carney, 1999; Weiner et al., 2012). It is well known that oxidative stress increases in the brain during aging process (Behl & Moosmann, 2002; Jin et al., 2014). Supporting evidences from a variety of *in vitro* and *in vivo* studies suggest an imbalance in the antioxidant and oxidant systems in age-associated disease, implying that the oxidative stress mechanisms are imported in the neurodegenerative pathogenesis and/or progression of dementia especially, Alzheimer's disease (AD) (Maher & Schubert, 2000; Pratico, 2008). Regarding pathological mechanisms, the neurotransmitter glutamate has been recognized as one key initiating factor for neuronal damages in many diseases (Jia et al., 2013). High or accumulated contents of glutamate can cause cellular oxidative stress leading to cell apoptosis (Cheng et al., 2011; Greenwood & Connolly, 2007; Jin et al., 2014), or contributing to acute and chronic neurodegenerative disorders (Weiner et al., 2012). Several lines of evidence have shown that glutamate-induced cell death involves the competitive inhibition of cystine uptake through the glutamate/cystine antiporter,

resulting in the depletion of cellular antioxidant glutathione and increase of reactive oxygen species (ROS) (Ha & Park, 2006; Kritis et al., 2015; Yang et al., 2013). Excessive free radicals and ROS could damage cells by several ways, for example, interacting with membrane and inducing lipid peroxidation, modifying signal and structural proteins, and oxidizing RNA/DNA to interrupt transcription (Li et al., 2013; Maher & Schubert, 2000; Valko et al., 2007).

Among many means of ROS-mediated cell damage, the oxidative-endoplasmic reticulum (ER) stress pathway has garnered the most attention as it was recognized as an important pathway causing neurodegeneration (Choi et al., 2010; Doyle et al., 2011; Jin et al., 2014; Yang et al., 2013). ER stress is a key factor in the progression of this disease (Bernales, Soto, & McCullagh, 2012b; Chaudhari et al., 2014b; Lee et al., 2010; Liu, Zhang, & Yin, 2013). Two main mechanisms of ER stress mediated cell apoptosis have been proposed. The first one involves the activation of calpain, a calcium-dependent neutral protease, leading to the initiation of caspase-12 proteolytic activity (Lamkanfi, Kalai, & Vandenabeele, 2004; Momoi, 2004; Nakagawa et al., 2000). Both calpain and caspase-12 have been recognized as specific markers of ER stress and unfolded protein response (UPR) (Bernales, Soto, & McCullagh, 2012b; Lamkanfi, Kalai, & Vandenabeele, 2004; Lee et al., 2010). In addition, CHOP (C/EBP-homologous protein); also known as GADD153 was found to be up-regulated in the cell undergoing

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ER stress-induced apoptosis by suppressing Bcl-2 activation (Chaudhari et al., 2014b; Choi et al., 2010; Gross, McDonnell, & Korsmeyer, 1999).

Accumulating evidence in experimental models of different disorders suggests that nuclear factor E2-related factor2 (Nrf2) pathway activation represents a promising therapeutic approach to restore the systemic and neuronal redox balance by reducing ROS-mediated neuronal damage (Slemmer & Weber, 2014). It has been reported that Nrf2 signaling promotes a survival in response to ER stress in neurons (Chaudhari et al., 2014b; Liu, Zhang, & Yin, 2013). Nrf2 has the function as a key endogenous antioxidants and phase II detoxification enzymes systems for increasing cellular defense against oxidative stress (Behl & Moosmann, 2002; Essa et al., 2012). However, only a few Nrf2-activating compounds have been tested in a clinical setting. Additionally, it has been demonstrated that Nrf2 is activated by the mitogen-activated protein kinases (MAPKs) signaling under conditions of oxidative stress (Jin et al., 2014; Leong et al., 2011; Sun, Huang, & Zhang, 2009a). With respect to the MAPKs such as extracellular-regulated kinases (ERKs), they have been originally shown to be also involved in the stress response and an important for cell survival in role of anti-apoptotic signals in neuronal cells (Chang & Karin, 2001; Doyle et al., 2011; Li et al., 2013; Stanciu & DeFranco, 2002).

Cleistocalyx nervosum var. *paniala* (CNP) family Myrtaceae, is an indigenous Thai berry fruit in Northern Thailand. The taste of ripe CNP fruit is sweet and sour with a good flavor; therefore, it is used as a material for local beverages and marmalades

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(Patthamakanokporn et al., 2008). CNP contains many bioactive compounds, high amounts of phenolic compounds, and anthocyanins (Charoensin et al., 2012; Jansom, Bhamarapravati, & Itharat, 2008; Patthamakanokporn et al., 2008). Previous study has found that CNP extract significantly stimulated human lymphocyte proliferative responses and significantly enhanced natural killer cells activity (Sriwanthana et al., 2007). Its seeds also presented antibacterial activity against *Propionibacterium acnes* and *Staphylococcus aureus*. In addition, the seed extract of CNP had no acute or subacute toxic effects both *in vitro* and *in vivo* (Inboot et al., 2012), and significantly enhanced activity of heme oxygenase-1 and reduced oxidative stress in rat liver (Taya et al., 2009). Interestingly, recent studies have reported that the major anthocyanin from ripe berry fruit of CNP was cyanidin-3-glucoside (C3G) (Charoensin et al., 2012; Jansom, Bhamarapravati, & Itharat, 2008). Anthocyanins produce the red, purple and blue pigments seen in many edible plants, particularly rich in berry fruits. Additionally, anthocyanin in berry fruit is shown to protect against oxidative damage (Slemmer & Weber, 2014). C3G has been reported to produce health benefits through a wide range of biological effects including antioxidant, anti-inflammatory, anti-ischemic, and anti-cancer properties (Fang, 2014; Marczylo et al., 2009; P. H. Shih, Yeh, & Yen, 2005; Slemmer & Weber, 2014; Xu et al., 2010). C3G and other anthocyanins were found in the cerebellum, cortex, hippocampus or striatum of the blueberry supplementation rats, but not the controls (Andres-Lacueva et al., 2005). Importantly, C3G can also cross the blood brain barrier and localize in various brain regions important for learning and

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memory (Andres-Lacueva et al., 2005; Milbury & Kalt, 2010). Previous studies have found that C3G blocked ethanol-mediated intracellular accumulation of ROS and also inhibited ER stress-induced neuronal cell death. In addition, it reversed ethanol-induced inhibition of neurite outgrowth (Chen et al., 2009; Chen et al., 2008). Some supporting evidence demonstrated that anthocyanin in the form of C3G increased glutamate-cysteine ligase expression, which in turn mediated the reduction in ROS levels (W. Zhu et al., 2012). In addition, C3G induced Nrf2 and activated the cellular antioxidant pathway by suppressing the oxidative stress in human umbilical vein endothelial cells (Fratantonio et al., 2015).

Although much research has focused on CNP and/or C3G for which protective mechanisms, is still poorly understood. However, there was a few studies concerning effects of CNP extract and its major bioactive compound (anthocyanin-C3G) on glutamate-induced neurodegenerative disorder through ER stress in HT22 mouse hippocampal neuronal cells. Thus, it was very interesting to study the neuroprotective effects of CNP extract and C3G on glutamate-induced ROS and ER stress mediated apoptosis cell death. We purposed to investigate the effect and underlying mechanism by which CNP extract and C3G protect against glutamate-mediated neurotoxicity via oxidative-ER stress pathway and examined the function of endogenous antioxidant system through ERK/Nrf2 signaling pathway. Our results would contribute to the understanding of the molecular mechanisms of antioxidant protective effects of CNP-

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Thai berry fruit and its C3G-anthocyanin might exert a neuroprotectant against glutamate-induced oxidative stress toxicity by reducing the ER stress-mediated cell death signaling and enhancing the function of endogenous antioxidants system activity via ERK/Nrf2 signaling pathway.

Review of related literature

To support background and rationale of this research, related articles are categorized and reviewed as follows:

1. Aging and neurodegeneration

Aging is a time-dependent progressive functional impairment process that leads to mortality. The most prominent characteristics of aging are a progressive decrease in physiological capacity, a reduced ability to respond adaptively to environmental stimuli, an increased susceptibility to diseases, and increased mortality. Many theories have been advanced to explain aging, but the biological mechanisms that underlie aging are still unknown. Major hypotheses of aging include altered proteins; DNA damage and less efficient DNA repair; inappropriate cross-linking of proteins, DNA, and other structural molecules; UPR or protein misfolding and aggregation processes; a failure of neuroendocrine secretion; cellular senescence in the cell culture system; an increase in free radical-mediated oxidative stress; and changes in the order of gene expression (Allen & Tresini, 2000; Andersen, 2004; Bishop, Lu, & Yankner, 2010; Brown,

Lockwood, & Sonawane, 2005; Calabrese et al., 2004; Farooqui & Farooqui, 2009; Granholm, Boger, & Emborg, 2008; Hui, 2015; Prior & Wu, 2006; Shetty et al., 2011). Aging is an important factor for the pathogenesis of neurodegenerative diseases. It is not only makes patients more prone to neurodegeneration, but also impairs their abilities of self-repair (Butterfield, Perluigi, & Sultana, 2006; Hui, 2015; Li et al., 2013; Markesbery & Carney, 1999). In a prospective cohort study, AD rates rose from 2.8 per 1000 person years in the age group of 65-69 years to 56.1 per 1000 person-years in the older than 90 years age group (Shetty et al., 2011). An understanding of the relation of the neuropathologies of the common causes of dementia with longitudinal rates of cognitive decline among persons without dementia has implications for efforts to reduce the public health burden posed by cognitive decline in old age (Farooqui & Farooqui, 2009; Shetty et al., 2011). With advances in molecular biology, the knowledge of aging and cognitive decline is accumulating. Many signaling pathways involved in the regulation of aging and lifespan have been identified, including oxidative stress signaling, insulin/IGF-1 signaling, target of rapamycin signaling, sirtuins, and caloric restriction (Behl & Moosmann, 2002; Bishop, Lu, & Yankner, 2010; Maher & Schubert, 2000). Recent studies have implicated the involvement of these signaling pathways in age-related cognitive decline (Bishop, Lu, & Yankner, 2010; Farooqui & Farooqui, 2009; Granholm, Boger, & Emborg, 2008; Hung et al., 2010). Thus, alteration of the molecular mechanisms of aging may contribute to the pathogenesis of neurodegenerative diseases.

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What are some of the physiological processes leading to these age-related brain dysfunctions? It is well known from animal and human studies that movement disorders and memory loss associated with aging have some of the same pathological hallmarks, but starting in different brain regions and involving different neuronal circuits. Among the best described are: protein aggregation, oxidative stress, microglial activation and specific neurotransmitter loss. In Figure 1, age-related neurodegenerative complex pathway can be caused by a number of different factors, isolated or in combination. While aging itself is the most common denominator, oxidative stress, inflammation and protein aggregation, for example, are all part of the common pathology seen in AD and Parkinson's disease (PD) patients (Brown, Lockwood, & Sonawane, 2005; Granholm, Boger, & Emborg, 2008). Studies on movement disorders or memory loss with aging should include at least minimal research on the other modalities described as well, to reach further understanding of this common co-morbidity in the aged population. There are also primary factors that can affect the aging brain and have severe implications for normal everyday function, such as sleep disturbances, sedentary lifestyle, high blood pressure, metabolic syndrome, obesity, and diabetes. Studies have shown that all of the above can be considered risk factors for both AD and PD (Allen & Tresini, 2000; Andersen, 2004; Brown, Lockwood, & Sonawane, 2005; Shetty et al., 2011).

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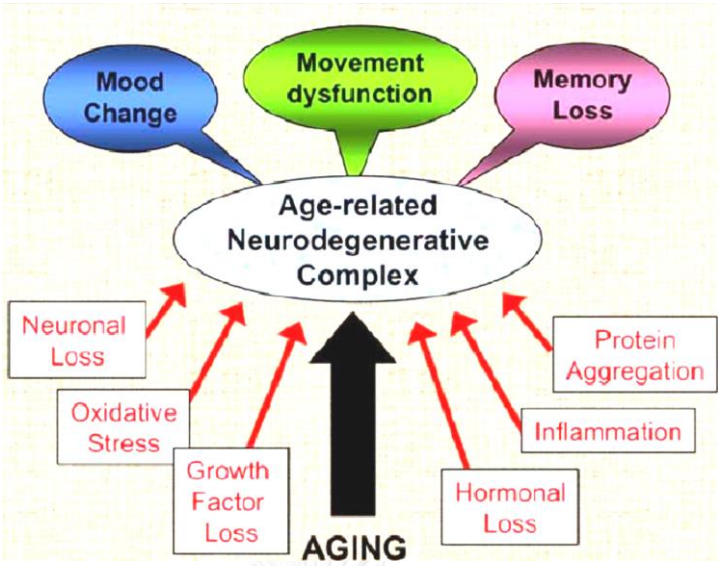

Figure 1. Age-related neurodegenerative complex pathway (Granholm, Boger, & Emborg, 2008).

All of these are unfortunate side effects of modern society, undoubtedly leading to a surge in these diseases in future aged generations. However, the relationship of oxidative stress and aging brain may be the key factors of the earliest biochemical alterations of neurodegenerative disorders.

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2. Oxidative stress and age-associated neurodegeneration

Oxidative stress is a biochemical state of imbalance in the production of ROS and/or reactive nitrogen species and antioxidant defenses. It refers to cytotoxic consequences caused by oxygen free radicals generated in a cell by processes that utilize molecular oxygen. Oxidative damage is inflicted by ROS, implicated in the cause of certain diseases, and has an impact on the body's aging process. ROS is a collective term that

includes oxygen radicals and non-radical oxidizing agents that can be converted into radicals. At low levels, ROS function as signaling intermediates for the modulation of fundamental cell activities such as growth and adaptation responses, but at higher concentrations, ROS contribute to neuronal membrane damage. Almost every gene that has been implicated in the response to stress has been shown to be affected by altered ROS levels (Allen & Tresini, 2000; Andersen, 2004; Li et al., 2013). ROS formation causes oxidative damage to membranes, proteins, lipids and genes, where this can be controlled by the body's defense mechanisms. ROS that are particularly responsible in oxidative stress include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$). Furthermore, the RNS include nitric oxide (NO) and peroxynitrite ($ONOO^-$). These oxidative stresses are essential for many physiological functions at low concentrations and killing of invading microorganisms (Calabrese et al., 2004; Hung et al., 2010; Leonarduzzi, Sottero, & Poli, 2010; Li et al., 2013). However, several lines of evidence have suggested that the pathogenesis of human diseases is attributed to increased oxidative stress (Allen & Tresini, 2000; Hui, 2015; Valko et al., 2007).

Many neurodegenerative diseases share oxidative stress as common terminal process (Andersen, 2004; Brown, Lockwood, & Sonawane, 2005). According to free radical theory of aging, an elevation in ROS damages neural membranes and induces oxidative stress. The increase in oxidative stress is accompanied by the concomitant decline in cognitive and motor performance in the elderly population. Markedly increased rate

of oxidative stress is the major factors associated with the pathogenesis of neurodegenerative diseases. Therefore, ROS-mediated oxidative damage has been implicated in normal aging and various neurodegenerative diseases (Bishop, Lu, & Yankner, 2010; Farooqui & Farooqui, 2009; Shetty et al., 2011). It is involved in the physiopathology of degenerative and chronic neuronal disorders, such as AD (Hui, 2015; Hung et al., 2010). One of pathogenesis of AD has been associated with the deregulation of the neuronal glutamatergic system, which can lead to glutamate-induced neuronal cell death (Ha & Park, 2006; Jin et al., 2014; Yang et al., 2013). Glutamate is an endogenous excitatory neurotransmitter, and it has been estimated that this neurotransmitter is utilized by as much as one-third of the synapses in the central nervous system (Breyer et al., 2007; Fukui et al., 2009). At high concentrations, glutamate is neurotoxic, and glutamate-induced neuronal cell death is believed to be an important contributing factor in the development of various neurodegenerative diseases, including AD (Choi et al., 2010; Greenwood & Connolly, 2007; Ha & Park, 2006).

Glutamate can induce cytotoxicity via two pathways. One of the pathways is mediated by the ionotropic glutamate receptors (receptor-initiated excitotoxicity) (Breyer et al., 2007; Kritis et al., 2015; Shah et al., 2014), which trigger Ca^{2+} fluxes, and leads to cell death by alteration of calcium homeostasis and subsequent accumulation of ROS. The other pathway is non-receptor-mediated oxidative stress. It involves the inhibition by extracellular glutamate (when present at high concentrations) of cystine uptake

mediated by the glutamate/cystine antiporter, which subsequently results in low intracellular glutathione (GSH) levels and culminates in oxidative stress and neuronal cell death, as shown in Figure 2 (Cheng et al., 2011; Chhunchha et al., 2013; Choi et al., 2010; Fukui et al., 2009; Kritis et al., 2015). AD results from the combination of genetic factors with different epigenetic events. Among them, oxidatively metabolic reactions and their by- products have been consistently implicated in AD pathogenesis and represent the biological basis for the oxidative stress and AD. Numerous studies demonstrate that different biomarkers of oxidative stress-mediated events are elevated in the AD brain (Butterfield, Perluigi, & Sultana, 2006; Li et al., 2013; Markesbery & Carney, 1999; Pratico, 2008).

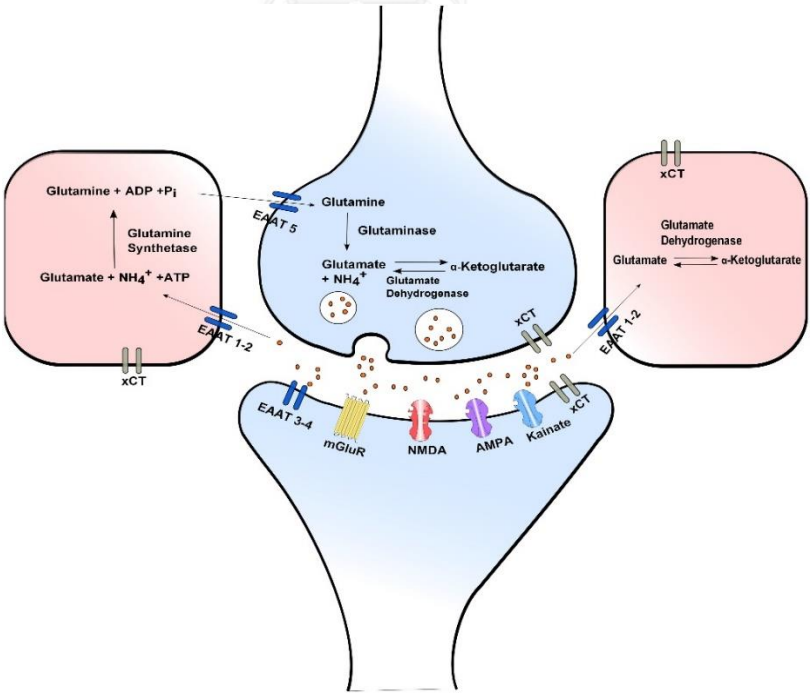

Figure 2. Glutamate release and uptake, the glutamate/cystine (Xc^- antiporter and the glutamate/glutamine cycle. In glial cells re-uptaken glutamate is converted to glutamine

by glutamine synthetase. Glial glutamine is taken up into the presynaptic neuron via Na^+-dependent glutamine uptake systems, where it is converted to glutamate by glutaminase. Extra- and intra-cellular glutamate concentrations are modulated through the Xc^- antiporter. Neurotransmission is ended by efficient glutamate reuptake via Na^+-dependent high affinity glutamate membrane excitatory amino acid transporters (Kritis et al., 2015).

Studies in animal models of the disease with antioxidants report significant improvements of their AD-like phenotype (Behl & Moosmann, 2002; Cheng et al., 2011; Shetty et al., 2011; Slemmer & Weber, 2014; Wang et al., 2006). Epidemiologic studies show that dietary intake of antioxidants reduces the risk of AD (Miller & Shukitt-Hale, 2012; Pratico, 2008). The involvement of oxidative stress in the pathogenesis of hippocampal neuronal cell death in AD, for example, age-related hippocampal changes in Bcl-2:Bax ratio, oxidative stress, redox-active iron and apoptosis associated with aluminum-induced neurodegeneration: increased susceptibility with aging (Farooqui & Farooqui, 2009; Hung et al., 2010; Kim et al., 2015). In addition, hippocampal neurogenesis in response to partial hippocampal deafferentation is lost in old rats (Shetty et al., 2011).

ROS can be generated from various sites in a cell, the ROS formations as described above. The cell protects its own damage from excessive ROS by suppression of ROS levels with antioxidant mechanism system. In brain tissue, it contains specific antioxidant enzymes to deal with ROS in the cytoplasm, as well as in neural

membranes, where enzymatic antioxidant, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) detoxify ROS (Ansari & Khodagholi, 2013; Jia et al., 2012; Li et al., 2013; Valko et al., 2007) (Figure 3). SOD converts the superoxide anion radical into hydrogen peroxide, which can readily diffuse through neural membranes (Behl & Moosmann, 2002; Calabrese et al., 2004). Hydrogen peroxide itself is not a free radical, but a major source for the generation of hydroxyl radical that is formed in the Fenton reaction catalyzed by iron and copper. Hydrogen peroxide is removed by GPx and CAT (Valko et al., 2007). In addition to the enzymatic defense systems, the human body also uses non-enzymatic antioxidants to limit over-accumulation of ROS. These include, but are not limited to, ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), and polyphenols such as flavonoids (Li et al., 2013).

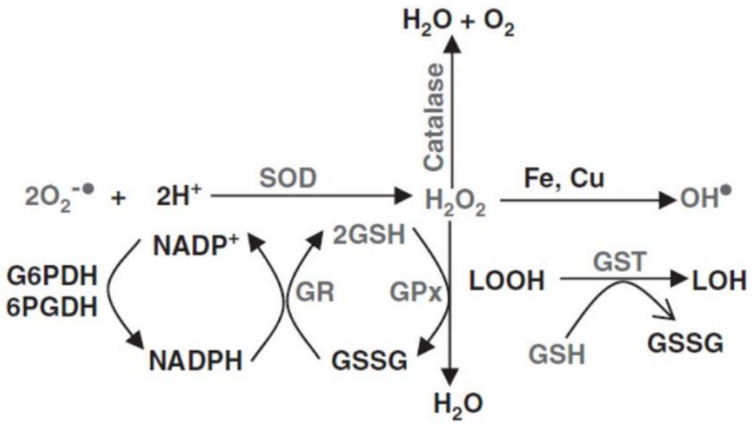

Figure 3. ROS and their detoxification by cellular antioxidants (Jia et al., 2012). GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized form of glutathione; GST, glutathione S-transferase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH,

6-phosphogluconate dehydrogenase; LOH, lipid alcohol; LOOH, lipid hydroperoxide; SOD, superoxide dismutase.

Collectively, these studies suggest that the oxidative process crosslinking of neural membrane proteins and oxidation of neural cell DNA are significant chemical events that are associated with oxidative stress and the disruption of ion homeostasis during lipid peroxidation (Farooqui & Farooqui, 2009). All these processes are related to the decline of antioxidant defense and repair mechanisms which results in accumulating oxidative stress-mediated neurodegenerative disorders. In addition, there are interactions among oxidative stress and other molecular mechanisms which cause neurodegeneration, such as protein misfolding-related ER stress, proteasomal malfunction, glial cell activation, mitochondrial dysfunction and programmed cell death (Allen & Tresini, 2000; Andersen, 2004; Butterfield, Perluigi, & Sultana, 2006; Chaudhari et al., 2014b; Doyle et al., 2011; Emerit, Edeas, & Bricaire, 2004; Liu, Zhang, & Yin, 2013).

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2.1 Oxidative stress based-ER stress-induced neuronal cell death: a role in neurodegeneration

The oxidative stress-induced ER stress pathway has garnered the most attention as it was recognized as an important pathway causing neurodegeneration (Doyle et al., 2011; Jin et al., 2014; Kim et al., 2015; Li et al., 2013; Mota et al., 2015). The ER is a

major organelle that crucially controls for protein synthesis, folding and trafficking, in addition to calcium and redox homeostasis. Protein folding is fulfilled in the lumen of the ER where related proteins and enzymes are located, including immunoglobulin binding protein (BIP), protein disulfide isomerase (PDI), calnexin, calreticulin, etc. (Chaudhari et al., 2014b; Li et al., 2013; Liu, Zhang, & Yin, 2013).

Only properly folded proteins can export to the Golgi apparatus for further modification. In contrast, misfolded or incompletely folded proteins are retained in the ER, leading to a cell adaptive response, named the ER stress response or UPR (Brown & Naidoo, 2012).

Recently, it has been revealed that ER stress-induced cell death plays critical roles in the pathogenesis of neurodegenerative diseases, such as AD (Choi et al., 2010; Doyle et al., 2011; Jin et al., 2014). However, if ER stress is prolonged or excessive in a disturbance, such as oxidative stress, the cells will elicit apoptotic processes to remove over-stressed cells via what is known as ER stress induced apoptosis. Three ER stress sensors have been reported initiating UPR (Figure 4); these include inositol requiring enzyme-1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6). ATF6 promotes folding of protein and removal of misfolded protein by up-regulation of chaperones, foldases, and components of the ER-associated degradation (ERAD) machinery, while IRE1 and PERK are functionally involved in both reducing ER protein load and triggering apoptosis (Bernales, Soto, & McCullagh, 2012b;

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Duran-Aniotz, Martínez, & Hetz, 2014; Liu, Zhang, & Yin, 2013). Prolonged ER stress directly induces neuronal apoptosis by activating CHOP, caspase-12, c-Jun N-terminal kinase (JNK), and glycogen synthase kinase-3 beta (GSK3β) pathway (Chhunchha et al., 2013; Lee et al., 2010; Oyadomari & Mori, 2004). The prolong increase and activation of CHOP down-regulate the expression of anti-apoptotic Bcl2 family proteins and activates the transcription of pro-apoptotic Bcl2 family members leading to apoptosis cell death, which functions as an integrator and amplifier of the cell death pathway (Doyle et al., 2011; Ghosh et al., 2012; Oyadomari & Mori, 2004; Yang et al., 2013). As well, CHOP induces death receptor 5 (DR5), which further sensitizes cells to apoptotic stimulation by a variety of conditions that cause ER stress. Recent evidence also suggests that mitochondrial dysfunction provides a significant contributing factor to ER stress-induced apoptosis (Bernales, Soto, & McCullagh, 2012b; Shiraishi et al., 2006).

In case of severe and sustained ER stress, a number of pro-apoptotic events begin to dominate and lead to apoptosis. Transcription factors ATF4 and ATF6-p50 stimulate CHOP expression. On one hand, CHOP stimulates expression of growth arrest and DNA damage inducible protein 34 (GADD34), which associates with protein phosphatase type 1 (PP1), resulting in dephosphorylation of eukaryotic initiation factor 2 alpha (eIF2), thus reactivating global cellular protein synthesis.

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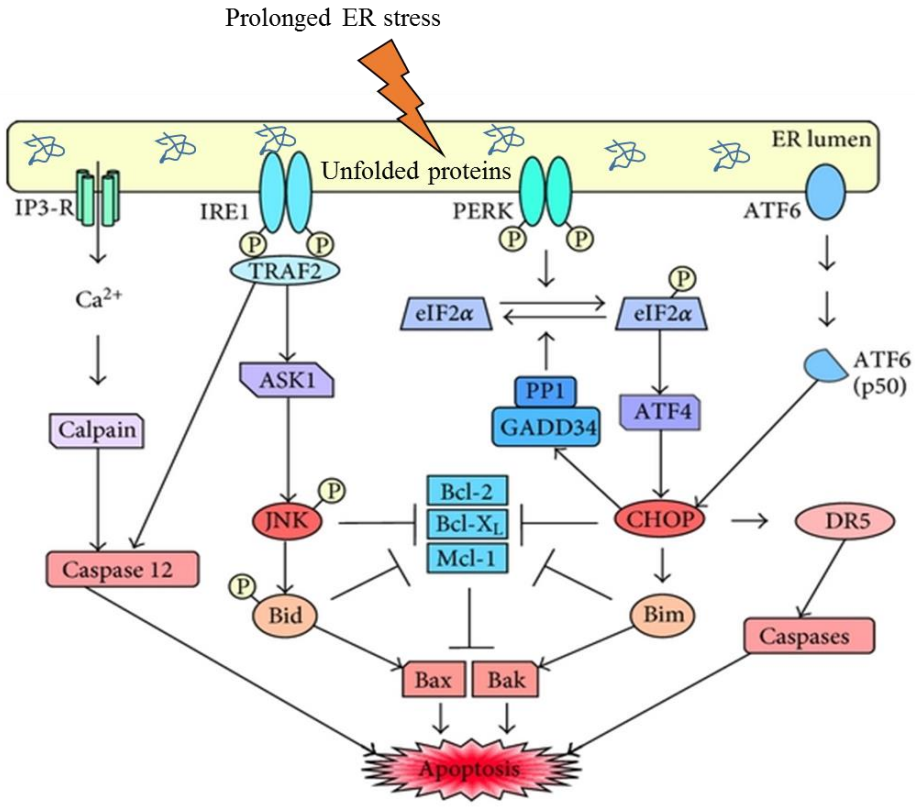

Figure 4. Overview of cell death signaling pathways involved in ER stress response/UPR (Schonthal, 2012).

On the other hand, CHOP inhibits anti-apoptotic proteins of the Bcl-2 family and stimulates pro-apoptotic Bim, altogether leading to heterodimerization and activation of pro-apoptotic Bax and Bak. CHOP also stimulates expression of cell surface death receptor DR5, which sensitizes cells to pro-apoptotic stimuli, presumably via calibrating the extrinsic apoptotic pathway involving caspase 12. Similarly, activated JNK complements the pro-apoptotic efforts of CHOP. JNK becomes phosphorylated and activated by protein kinase apoptosis signal-regulated kinase 1 (ASK1) upon association of tumor-necrosis- factor-receptor-(TNFR-) associated factor 2 (TRAF2) with activated

IRE1. Association of TRAF2 with activated IRE1 also leads to activation of caspase 12. Calcium release from the ER via inositol 1,4,5-trisphosphate (IP3) receptors can activate calpains, which further stimulate caspase 12 activation via proteolytic cleavage of its inactive pro-caspase precursor (Bernales, Soto, & McCullagh, 2012b; Doyle et al., 2011; Galehdar et al., 2010; Ghosh et al., 2012; Liu, Zhang, & Yin, 2013; Schonthal, 2012; Shiraishi et al., 2006; Q. Zhang et al., 2016).

3. Antioxidant defense mechanisms: a role in neuroprotection

Oxidative stress has been implied to be a common underlying mechanism of several health conditions, including brain injury and aging. Supporting lines evidences found that the importance of pro-survival pathways are PERK-Nrf2 and MEK1/ERK which involved in ER stress signaling pathway (Cullinan & Diehl, 2006; Doyle et al., 2011; Mota et al., 2015). Nrf2 is regarded as an important regulator in cytoprotection against oxidative injury. It regulates the expression of many antioxidant proteins and phase II detoxifying enzyme genes, including SOD, GPx, GST, CAT, GSH, peroxiredoxins, heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase-1 (NQO-1), etc (Jin et al., 2014; Leong et al., 2011; Liu, Zhang, & Yin, 2013; Sun, Huang, & Zhang, 2009a) (Figure 5). Accumulating evidence in experimental models of different disorders suggests that Nrf2 pathway activation represents a promising therapeutic approach to restore the systemic and neuronal redox balance by reducing ROS-mediated neuronal damage.

However, only a few Nrf2-activating compounds have been tested in a clinical setting. The pathogenesis of many neurodegenerative diseases is thought to be associated with oxidative stress due to the accumulation of ROS. Previous works demonstrated that compounds that undergo redox cycling to form ROS as well as oxidants such as H_2O_2 activate the Nrf2-ARE transcriptional pathway (Leong et al., 2011; Liu, Zhang, & Yin, 2013; Niture, Khatri, & Jaiswal, 2014; Slemmer & Weber, 2014; Sun, Huang, & Zhang, 2009a). Work from other laboratories has demonstrated that activation of the Nrf2 pathway provides protection from glutamate- and H_2O_2-induced cell death (Kraft, Johnson, & Johnson, 2004; Shih et al., 2003). Furthermore, astrocytes expressing Nrf2 protect neurons from oxidative stress (Kraft, Johnson, & Johnson, 2004).

Moreover, it has been demonstrated that Nrf2 is activated by MAPK-ERK signaling under conditions of oxidative stress (Jin et al., 2014; Leong et al., 2011; Sun, Huang, & Zhang, 2009a; Tan et al., 2014; Wang et al., 2015). The MAPK signaling system responds to diverse stimuli, including oxidative stress, has been implicated in Nrf2 induction by many previous reports (Chang & Karin, 2001; McCubrey, Lahair, & Franklin, 2006; Sun, Huang, & Zhang, 2009a; Wada & Penninger, 2004). ERK is a member of the MAPK family, required for memory formation, aging and neurodegeneration (Kraft, Johnson, & Johnson, 2004; Shih et al., 2003). ERK activation is typically associated with cell survival, proliferation and differentiation given their activation by mitogens and some cell survival factors (Leong et al., 2011; Tan et al., 2014; Wada & Penninger, 2004).

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Supporting evidences have demonstrated that the activation of ERK/Nrf2 signaling pathway is likely to contribute to the neuroprotection of lipoxin A4 methyl ester against chronic cerebral hypoperfusion injury (Jin et al., 2014). Cytoprotective effects of lindenenyl acetate isolated from the roots of *Lindera strychnifolia*, it increases cellular resistance to glutamate-induced oxidative injury in mouse hippocampal HT22 cells, presumably through the ERK pathway-Nrf2/ARE-dependent heme oxygenase-1 expression (Li et al., 2009). Recently, Wang and colleagues (2015) investigated dimethyl fumarate protected neural stem/progenitor cells and neurons from H_2O_2-induced oxidative damage through Nrf2-ERK1/2 MAPK pathway. Interestingly, berry fruits and their bioactive compounds protected against oxidative stress-induced cell death in neurons both *in vitro* and *in vivo*, which they enhanced beneficial Nrf2 signaling in the brain (Chen & Luo, 2010; Di Giacomo et al., 2012; Essa et al., 2012; Heo & Lee, 2005; Kraft, Johnson, & Johnson, 2004; Kropat et al., 2013; J. Lee et al., 2014; Miller & Shukitt-Hale, 2012; Prior & Wu, 2006; Slemmer & Weber, 2014; Sun, Huang, & Zhang, 2009a; Talavera et al., 2005; Tan et al., 2014; Wang et al., 2006; Zhang et al., 2013) Several works have been reported the natural compound products such as polyphenols act as antioxidants via Nrf2 gene expression mechanisms signaling in neurons, in this regard, these compounds indeed provide a neuroprotection against oxidative stress damage (Ansari & Khodagholi, 2013; Behl & Moosmann, 2002; DeGracia et al., 2002; Fratantonio et al., 2015; Kraft, Johnson, & Johnson, 2004; Kropat et al., 2013; Leong et al., 2011; Zhang et al., 2013).

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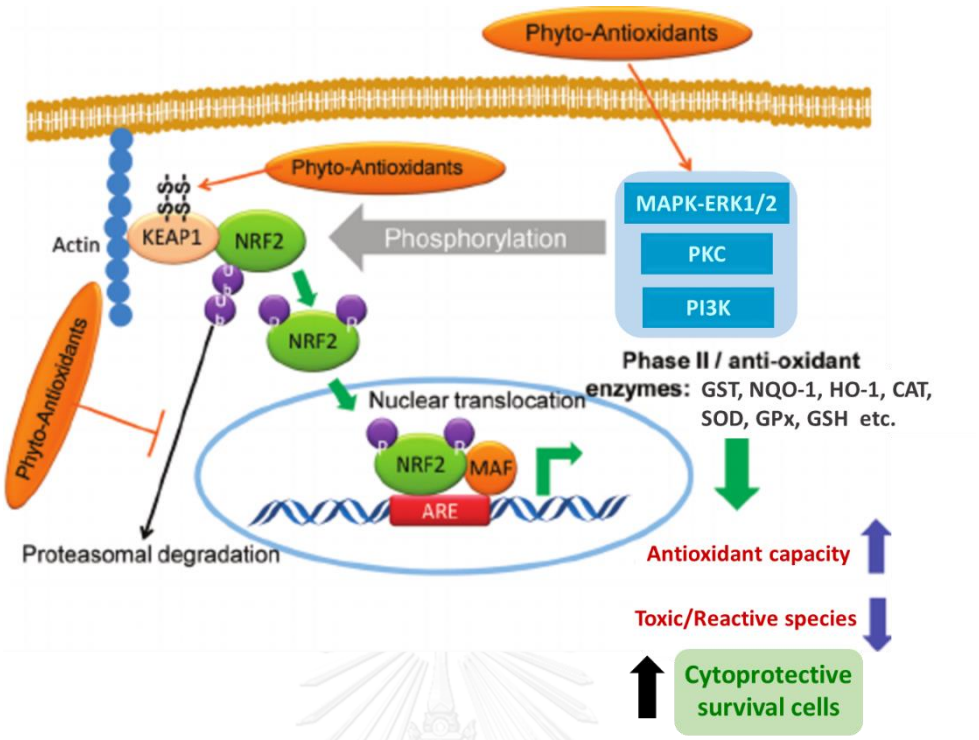

Figure 5. Antioxidant activity mediated through gene expression (Slight modified from Finley and colleague (2011)). The promoter region of select genes allows for coordinated up-regulation of antioxidant and detoxifying proteins in response to dietary constituents (phyto-antioxidants). This up-regulation is mediated through Nrf2 that may be activated directly or induced by series of protein kinases. Phosphorylation of Nrf2 at serine and threonine residues by kinases such as phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) results in release of Nrf2 from Keap1 and subsequent nuclear translocation of Nrf2. In the cell nucleus translocated Nrf2 interacts with small MAF protein, forming a heterodimer that binds to the ARE sequence in the promoter region and up-regulates transcription of many genes encoding detoxifying enzymes (Finley et al., 2011).

4. Thai berry fruit (*Cleistocalyx nervosum* var. *paniala*) and its bioactive anthocyanin compound (cyanidin-3-glucoside)

The potential of various berries to protect the brain from aging and neurodegenerative disorders has gained increased attention in recent years, in large part due to their high polyphenol content and antioxidant capacity. A growing body of preclinical and clinical research has identified neurological benefits associated with the consumption of fruits, such as berry fruits. In addition to their well-known antioxidant effects, dietary supplementation with berry fruits also has direct effects on the brain. Intake of these fruits may help to prevent age-related neurodegeneration and resulting changes in cognitive and motor function. In cell and animal models, berry fruits mediate signaling pathways involved in inflammation and cell survival in addition to enhancing neuroplasticity, neurotransmission, and calcium buffering, all of which lead to attenuation of age- and pathology-related deficits in behavior. Recent clinical trials have extended these antioxidant, anti-inflammatory, and cognitive sparing effects to humans (Mazza et al., 2002; Miller & Shukitt-Hale, 2012).

In Thailand, one of berry fruits is *C. nervosum* var. *paniala* (CNP) belonging to the Myrtaceae family. The local name is Makiang, it is an indigenous fruit in the North. The ripe fruit taste of CNP is sour and slightly astringent with scent smell that is commonly consumed either as fresh fruit or its fruit products. The present popular and available fruit products in the market are functional health foods, cosmetic ingredients and

healthy drinks. This ripe fruit contains many bioactive compounds, high amounts of phenolic compounds and flavonoids; moreover, the rich purplish red in the ripe fruit of CNP is characterized by an anthocyanin profile (Jansom, Bhamarapravati, & Itharat, 2008; Patthamakanokporn et al., 2008; Taya et al., 2009). Several bioactive compounds are powerful antioxidants in berry fruits, especially anthocyanin can neutralize the damage of oxidation which previous studies have shown this activity in Thai berry fruit like CNP. Interestingly, cyanidin-3-glucoside (C3G) have been reported as the major anthocyanin from ripe berry fruit of CNP (Charoensin et al., 2012; Jansom, Bhamarapravati, & Itharat, 2008). There are a few data concerning the biological activity of CNP fruit and its bioactive compound *in vitro* or in *vivo*, with neuroprotective mechanisms. However, some report revealed that the extracts from fruit of CNP significantly stimulate human lymphocyte proliferative responses and enhance natural killer cell activity which could be clinically used for the modulating immune functions (Sriwanthana et al., 2007). Recently, Charoensin et al (2012) found that anthocyanins isolated from the CNP fruits were not only safe in acute toxicity test, but also displayed antimutagenicity *in vitro*. Furthermore, the methanol extract of CNP seed has no genotoxic effect on the bacterial or animal systems studied. It demonstrated antigenotoxicity against some environmental mutagens in the bacterial mutation assay (Inboot et al., 2012). In addition, the aqueous extract of CNP ripe fruits at low dose exhibited the pro-oxidant effect but at high dose, it reduced oxidative stress in rat liver (Taya et al., 2009). Recent evidence has revealed CNP extract was able to protect

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against the progression of cadmium nephrotoxicity, mostly via its antioxidant power (Poontawee, Natakankitkul, & Wongmekiat, 2016).

Specific bioactive compound “anthocyanin” is the major and abundant in ripe CNP fruits. It is a class of flavonoids in subclass of polyphenols that produce the bright red, purple, and blue pigments seen in many berries. They are potent antioxidant properties which have been reported to produce health benefits through a wide range of biological effects in anti-inflammatory, anticancer, reduction of the risk of cardiovascular diseases and prevention of neurodegenerative disorders (Behl & Moosmann, 2002; Essa et al., 2012; Jansom, Bhamarapravati, & Itharat, 2008; Mattson, Chan, & Duan, 2002; Prior & Wu, 2006; Slemmer & Weber, 2014; Talavera et al., 2005; Tan et al., 2014; W. Zhu et al., 2012). Some research has demonstrated that once consumed, anthocyanins from berry fruits are bioavailable and increase serum antioxidant capacity in human subjects (Mazza et al., 2002; Slemmer & Weber, 2014). Several anthocyanins were found in the cerebellum, cortex, hippocampus or striatum of the blueberry supplementation rats, but not the controls (Andres-Lacueva et al., 2005). Essentially, anthocyanin in form of C3G are able to cross the blood brain barrier and localize in various brain regions important for learning and memory, which are neuroavailable, as well, with anthocyanins residing in tissue longer than in plasma (Andres-Lacueva et al., 2005; Mazza et al., 2002; Milbury & Kalt, 2010; Miller & Shukitt-Hale, 2012; Prior & Wu, 2006; Slemmer & Weber, 2014; Talavera et al., 2005). Some

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supporting evidence demonstrated that C3G increased glutamate-cysteine ligase expression, which in turn mediated the reduction in ROS levels (W. Zhu et al., 2012). Moreover, Tan and colleague (2014) found that anthocyanin in form of cyanidin-3-galactoside and blueberry extracts also increased SOD activity and reduced oxidative stress in brain tissues and plasma, and increased hippocampal phosphorylated ERK expression in SAMP8 mice. Recently, Fratantonio et al (2015) discovered that C3G protected human umbilical vein endothelial cells from oxidative stress by inducing Nrf2 nuclear localization and activating the cellular antioxidant pathway. Some previous research suggested modulation Nrf2-dependent gene transcription by bilberry anthocyanins *in vivo* (Kropat et al., 2013). Although much research has focused on anthocyanins, berry fruits contain a wide array of other polyphenolic compounds for which neuroavailability and the relative contribution of polyphenolics to neuroprotective mechanisms, compared to their metabolites, is still poorly understood.

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5. Mouse hippocampal neuronal cells (HT22) model

HT22 cell line, a subline derived from parent HT4 cells that were originally immortalized from primary mouse hippocampal neuronal culture, this cell line previously shown to be sensitive to glutamate via the oxidative pathway (Greenwood & Connolly, 2007; Maher & Davis, 1996). The cells are particularly sensitive to glutamate

because these cells lack ionotropic glutamate receptors and thus effectively exclude the compounding cell death pathway mediated by the ionotropic glutamate receptor. Therefore, HT22 cell line is widely used to research the non-receptor mediated oxidative glutamate toxicity (Fukui et al., 2009; Kritis et al., 2015; Maher & Davis, 1996). An immortalized mouse hippocampal cell line has been a valuable tool in understanding molecular and cellular processes relevant to the given tissues. If used properly, they can provide an inexpensive, quick, and simple way to identify and test molecular and cellular mechanisms that would be more complex, difficult, and sometimes even impracticable, *in vivo*. HT22 cells have become an extensively used *in vitro* model in recent years for studying oxidative stress-induced neuronal cell death especially, there are used as a successful model to research the glutamate-induced oxidative neurotoxicity (Breyer et al., 2007; Brimson et al., 2012; Brimson & Tencomnao, 2011; Choi et al., 2010; Fukui et al., 2009; Greenwood & Connolly, 2007; Ha & Park, 2006; Jeong et al., 2010; Jin et al., 2014; Maher & Davis, 1996; Yang et al., 2013). Figure 6 shows the mechanism of oxidative glutamate toxicity in HT22 cells model, high concentrations of extracellular glutamate cause intracellular GSH depletion and increase ROS production, resulting in cell damage and death. This toxicity is exerted by decreasing in GSH generation through the glutamate/cysteine antiporter (Breyer et al., 2007; Chhunchha et al., 2013; Choi et al., 2010; Fukui et al., 2009; Ha & Park, 2006; Jeong et al., 2010; Kritis et al., 2015; Li et al., 2009; Maher & Davis, 1996; Yang et al., 2013).

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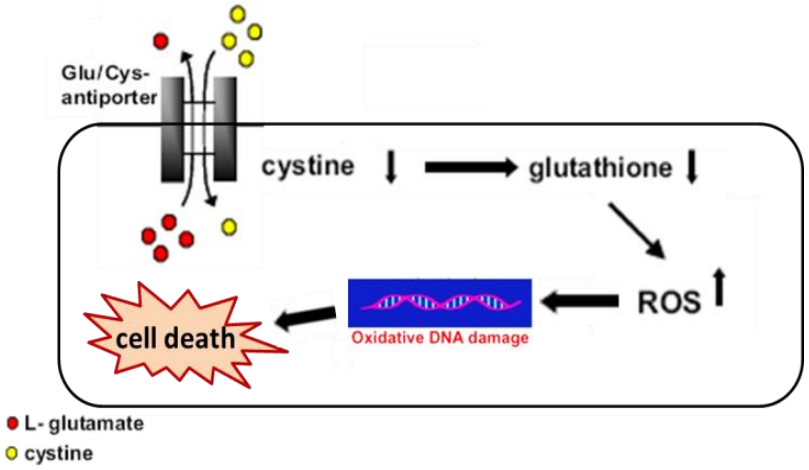

Figure 6. Mechanism of oxidative glutamate toxicity in HT22 cells model (Slightly modified from Breyer and colleague (2007)). High extracellular glutamate concentrations via interaction with the glutamate/cystine-antiporter result in an inhibition of cysteine uptake. This leads to decreased internal glutathione causing an accumulation of ROS, finally causing the cell death cascade (Breyer et al., 2007).

Research questions

1. Can CNP extract and its major bioactive compound (anthocyanin: C3G) decrease glutamate-induced oxidative damage based-ER stress-mediated cell death in HT22 mouse hippocampal neuronal cells?

2. Can CNP extract and its major bioactive compound (anthocyanin: C3G) up-regulate the cellular antioxidant enzymes system through activation of ERK/Nrf2 signal pathways in HT22 mouse hippocampal neuronal cells?

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Hypotheses

- CNP extract and its major bioactive compound (anthocyanin: C3G) could attenuate glutamate-induced oxidative and ER stress-associated cell death signaling, and enhance/restore the survival mechanisms of HT22 mouse hippocampal neuronal cells via ERK/Nrf2 pathway.

Objectives

General objectives of the study

To find out the Thai berry fruit-CNP and its major bioactive anthocyanin-C3G on the neuroprotective effects and underlying mechanisms of them against glutamate-induced oxidative/ER stress cell death in mouse hippocampal neuronal HT22 cells by enhancing the survival mechanisms of the extract and C3G via ERK/Nrf2 antioxidant system pathway.

Specific objectives of the study

1. To assess the antioxidant capacity of CNP extract.

2. To evaluate the toxicity of CNP extract and C3G on mouse hippocampal neuronal HT22 cells.

3. To investigate the protective effects of CNP extract and C3G against ROS-mediated ER stress induced cell death by glutamate in HT22 cells.

4. To examine the effects of CNP extract and C3G on enhancing the endogenous antioxidants and phase II enzymes expression in HT22 cells.

5. To determine the effects of CNP extract and C3G on up-regulating the survival mechanisms in HT22 cells.

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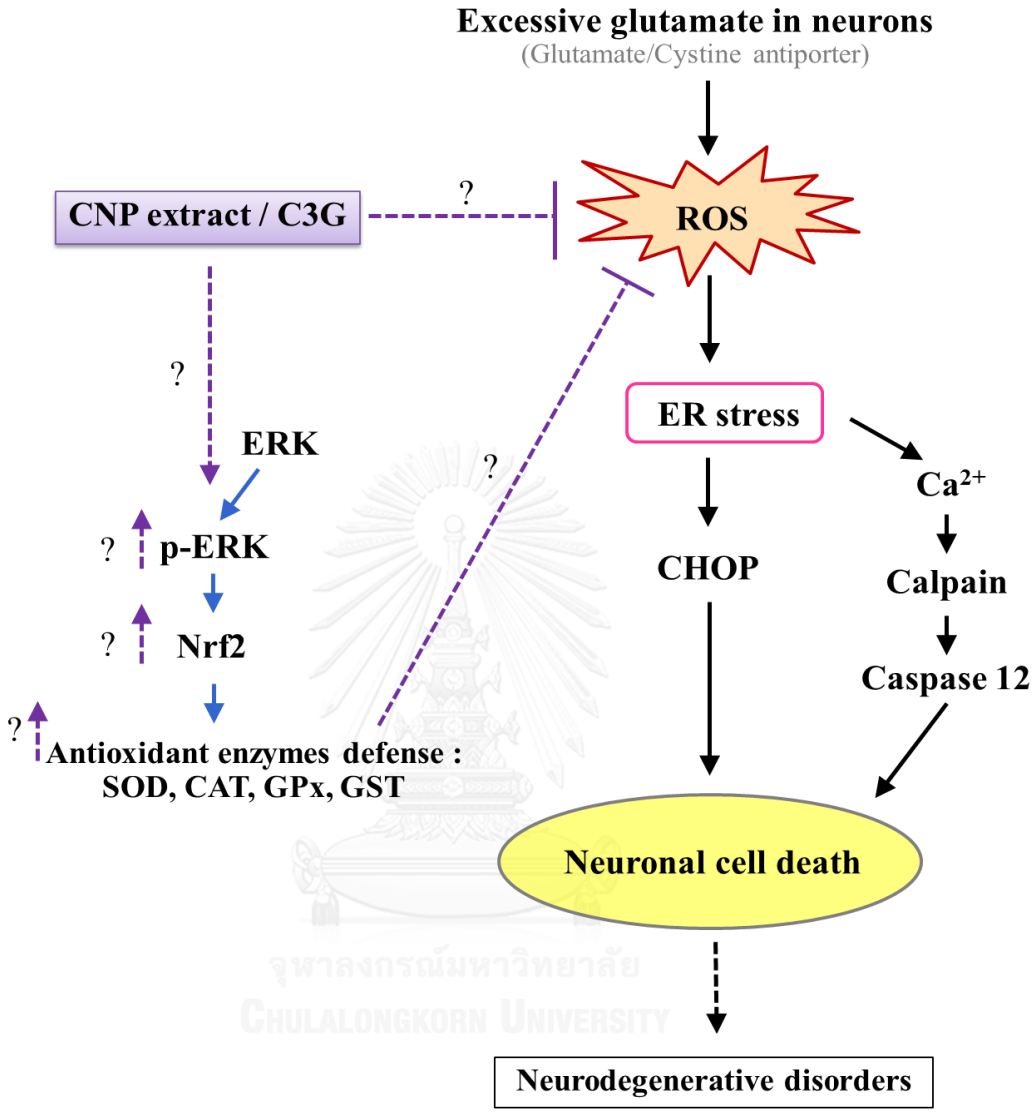
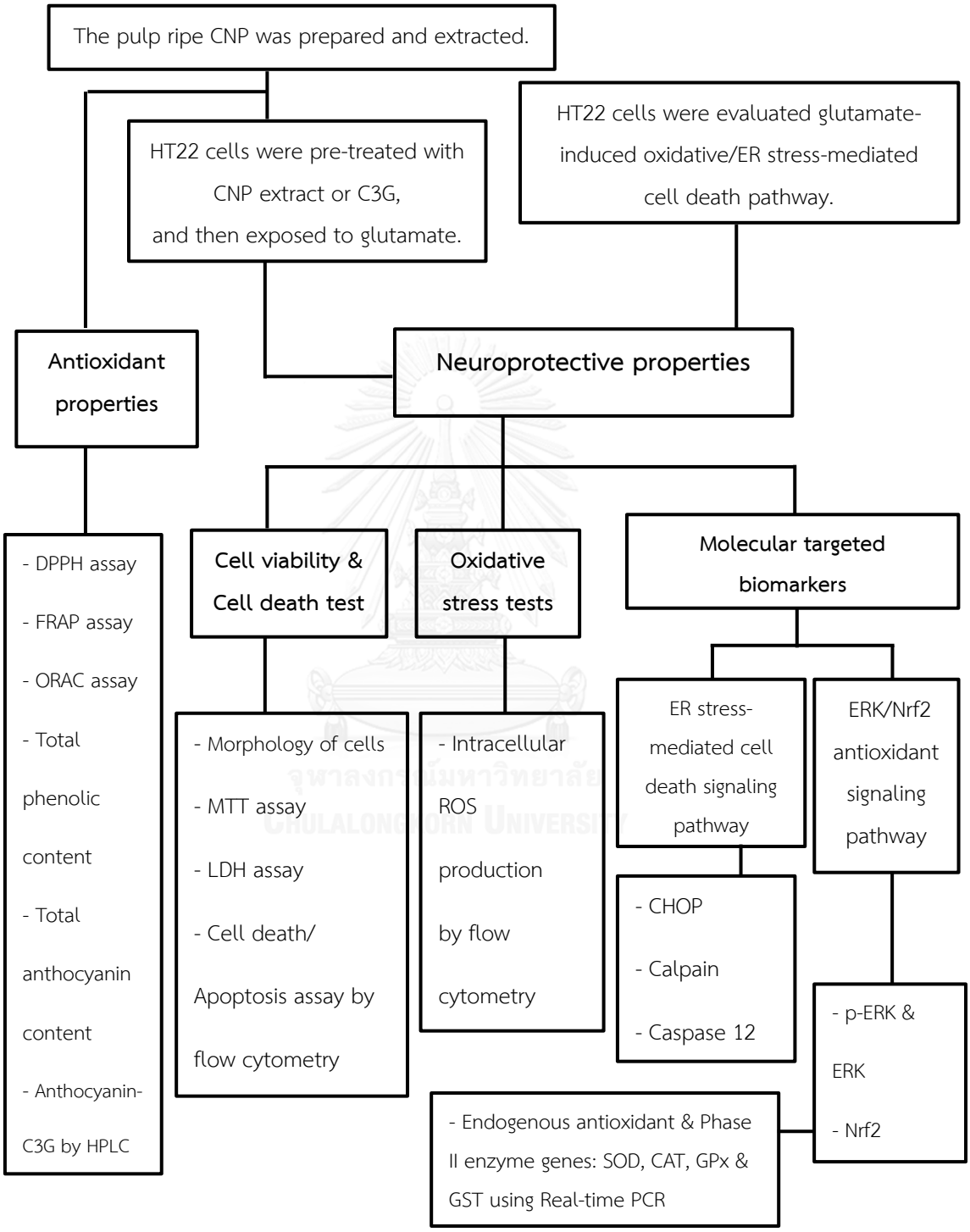
Conceptual framework



HT22 cells model

C. nervosum var. *paniala* fruit (CNP)
Cyanidin-3-Glucoside (C3G)

Experimental designs



CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

In this study, all reagents used molecular and analytical grade.

Chemicals	Companies, Countries
Acetic acid	Sigma-Aldrich, USA
30% Acrylamide and bis-acrylamide solution	Bio-Rad Laboratories, USA
Ammonium persulfate	Omnipur, Germany
Annexin-V/FITC and propidium iodide kit	Biolegend, USA
Agarose gel	Research Organics, USA
2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)	Sigma-Aldrich, USA
Blotting-grade blocker	BIO-RAD Laboratories, USA
Bovine Serum Albumin (BSA)	GE Healthcare Bio-sciences, UK
Catalase (CAT)	Sigma-Aldrich, USA
Cell lysis buffer	Cell Signaling Technology, USA

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Chemiluminescence reagent	GE Healthcare Bio-sciences, Austria
Chloroform	Sigma-Aldrich, USA
Coomassie Brilliant Blue G	Sigma-Aldrich, USA
Cyanidin-3-glucoside	Sigma-Aldrich, USA
2'-7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA)	Life technology, USA
Deferoxamine mesylate (DM)	Sigma-Aldrich, USA
Developer-GBX	KODAK, USA
Dimethyl Sulfoxide (DMSO)	Merck, Germany
3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)	Bio basic, Canada
DNA Ladder 100 bps	Fermentas, Lithuania
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Merck, Germany
Dulbecco's modified Eagle medium (DMEM)	Sigma-Aldrich, USA
Fetal Bovine Serum (FBS)	Sigma-Aldrich, USA
Fixer solution	KODAK, USA
Folin-Ciocalteu's phenol reagent	Sigma-Aldrich, USA

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Hydrochloric acid	Merck, Germany
Hydrogen peroxide 30% W/V	Merck, Germany
Gallic acid	Merck, Germany
Glycine	Vivantis Inc., USA
Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$)	Sigma-Aldrich, USA
Isopropanol	Merck, Germany
L-glutamic acid	Sigma-Aldrich, USA
L-glutathione reduced	Sigma-Aldrich, USA
Methanol	Merck, Germany
Mouse monoclonal anti-β-actin (13E5) antibody	Cell Signaling, USA
Mouse monoclonal anti-calpain (μ-type) antibody	Cell Signaling, USA
Mouse monoclonal anti-caspase 12 antibody	Cell Signaling, USA
Mouse monoclonal anti-CHOP (D46F1) antibody	Cell Signaling, USA
Mouse monoclonal anti-phospho ERK1/2 (D13144E) (Thr202/Tyr204) antibody	Cell Signaling, USA
Mouse monoclonal anti-ERK1/2 (137F5) antibody	Cell Signaling, USA
Mouse polyclonal anti-Nrf2 (C-20) antibody	Santa Cruz Biotechnology, USA

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Mouse monoclonal anti-rabbit IgG, HRP-linked antibody	Cell Signaling, USA
MnTBAP	Calbiochem, EMD Chemicals, USA
Oligo-dT primers	Bioneer, South Korea
Penicillin-streptomycin solution	Corning Inc., USA
Potassium persulfate	Merck, Germany
Phosphate Buffered Saline (PBS)	Hyclone, USA
Potassium chloride	Bio Basic, Canada
Primers	Bioneer, South Korea
Protease inhibitor cocktails	Roche Diagnostics GmbH, Germany
Protein ladder	ThermoScientific, USA
RNase inhibitor	Hyclone, USA
Sodium chloride	Merck, Germany
Sodium lauryl sulphate (SDS)	Bio basic Inc., Canada
N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)	Omnipur, Germany
2, 4, 6-tripyridyls-triazine (TPTZ)	Sigma-Aldrich, USA

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Tris-base	Vivantis, USA
Tris-hydrochloride	Vivantis, US
Trypsin	Hyclone, USA
Trypan Blue Stain	Invitrogen, USA
Tween-20	Vivantis, USA

2.1.2 Devices and Tools

Names	**Companies, Countries**
6, 12, 96 well culture plate flat bottom with lid	Corning Inc., USA
96 well plate medium binding	Corning Inc., USA
25, 75 cm^2 cell culture flask	Corning Inc., USA
15, 50 ml centrifuge tube	Corning Inc., USA
2 ml cryovial tube	Corning Inc., USA
5, 10, 25 ml disposable serological pipette	ProSourceScientific, Canada
Adhesive optical sealing film	Bioneer, South Korea
Analytical balances	MettlerToledo, Switzerland
Autoclave	Hirayama, Japan

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Auto pipette	Gilson, France
Block heater	Wealtec Corp, USA
Centrifuge	Beckman Coulter, USA
CO_2 incubator	Thermo Scientific, USA
Electrophoresis power supply	Bio-Rad laboratories, USA
Exicycler real-time quantitative thermal block	Bioneer, South Korea
Evaporator	Genevac, USA
-20°C freezer	Sanyo Electric, Japan
-80°C freezer	Liofreeze, USA
Flow cytometer	BD Biosciences, USA
Gel documentation (gel doc) systems	Syngene, UK
Gel electrophoresis apparatus	Bio-Rad laboratories, USA
Glassware	Pyrax, USA
HPLC	Shimadzu Scientific Instruments, Japan
Incubator	Memmert, Germany
Inverted microscope	Olympus Optical, Japan

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Laminar flow cabinet	Haier, China
Laminar flow clean bench	Esco, Singapore
Light microscope	Olympus Optical, Japan
Liquid nitrogen tank	Taylor Wharton, USA
Magnetic stirrer	DAIHANScientific, South Korea
Microcentrifuge machine	Beckman Coulter, USA
1.5 ml microcentrifuge tube	Greiner Bio-One, Austria
Micro high speed refrigerated centrifuge	Vision Scientific, South Korea
Multichannel pipette	Gilson, France
0.2 ml PCR tube for real-time PCR	Bioneer, South Korea
pH meter	MettlerToledo, Switzerland
Phase contrast microscope	Zeiss, Germany
Pipette controller	Jencons Scientific, UK
Pipette tips 10, 20 µl	Sorenson, USA
Pipette tips 100, 200 µl	Gilson, France
Pipette tips 1000 µl	Hycon, USA
Polyvinyldifluoride membrane	GE Healthcare Bio-sciences, UK

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4°C refrigerator	Sharp, Japan
Rotary evaporator	Heidolph Instruments, Germany
UV-visible spectrophotometer	BioTek, USA
Vortex mixer	FINEPCR, South Korea
Water bath	Memmert, Germany

2.2 Plant sample

Cleistocalyx nervosum var. *paniala* family Myrtaceae was used in this study as shown in Figure 7. Its local name is Makiang, an indigenous berry fruit in the Northern Thailand. This ripe CNP fruits were collected during July-August, from Maekue, Doi Saket, Chiang Mai, Thailand and identified the scientific name by the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand and their voucher specimen was A013732 (BCU).

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Figure 7. The ripe *C. nervosum* var. *paniala* fruits

2.3 Cell culture model

The immortalized mouse hippocampal neuronal HT22 cell line (Figure 8), served as *in vitro* model for glutamate-induced oxidative stress neurotoxicity, was a generous gift from Prof. David Schubert (The Salk Institute, San Diego, CA, USA). The cells were cultured with DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin). Cells were incubated under 5% CO_2 in humidified atmosphere at 37ºC. The cells were collected and carried out by de-adherence from the culture flask by trypsinization. They were counted using a Neubauer haemocytometer and seeded in a culture multiwell plate. The HT22 culture plate was then incubated in the CO_2 incubator, maintained at a temperature of 37°C in a humidified atmosphere at 5% CO_2 overnight with medium supplemented with 5% FBS. The adhered cells were grown to 70-80% confluence.

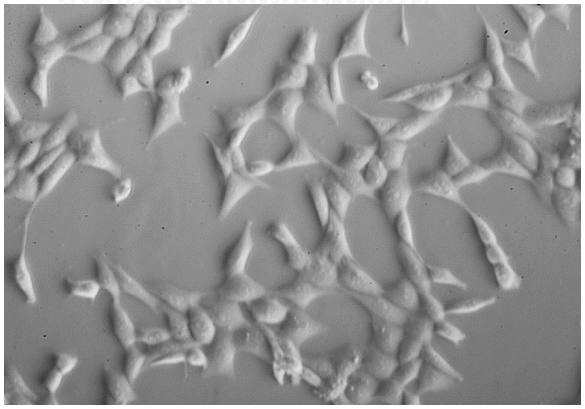

Figure 8. Morphology of immortalized mouse hippocampal neuronal HT22 cell line by phase contrast microscope (5X).

2.4 Experiment procedure

2.4.1 Preparation and extraction of CNP fruit

The ripe fruits were washed with clean water, followed by rinsing with deionized water. The pulp was manually separated from seed and weighed. They were freeze-dried immediately to protect the rotten ripe and the degradation of their antioxidants and other phytochemicals. Freeze-dried sample was kept with vacuum-packed in laminated aluminium foil bags and stored at -20ºC until use. Extraction process was conducted according to slightly modified from Charoensin and colleague (2012). Briefly, CNP pulp was extracted with distilled water and homogenized using food blender. The mixture was centrifuged at 3000 g for 15 min and filtered through a filter paper. The supernatants were collected and lyophilized. The aqueous crude extract was dissolved in dimethyl sulfoxide (DMSO), and then sterilized through syringe filter with 0.2 µm pore and used as stock solution (100 mg/ml) that kept in dark at -20°C until further investigation.

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2.4.2 Determination of antioxidant properties

2.4.2.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH test is a common assay for antioxidant studies and offers a rapid technique for screening the radical scavenging activity of pure synthetic compounds, crude plant extracts and foods. It is also important to note that the DPPH assay only recognizes free radical scavenging effects and not pro-oxidant activity (Fukumoto & Mazza, 2000).

This assay base on the hydrogen donor of antioxidant. DPPH is a stable free radical (DPPH•) which accepts hydrogen from an antioxidant. The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from the antioxidant. The procedure of DPPH method was slightly modified from Fukumoto & Mazza (2000). Briefly, twenty-two microliters of sample extract were added in a 96-well plate and then 200 µl of 150 µM DPPH reagent in 80% methanol. The plate was incubated in the dark at room temperature. After 30 min, the plate was read in microplate reader using at 520 nm. Trolox solution was used as a standard antioxidant 0.01-0.64 µM. Data expressed as the percentage of scavenging activity. It was calculated according to the following formula:

$$\% \text{ Scavenging activity} = \frac{[\text{Abs(control)} - (\text{Abs(sample)} - \text{Abs(blank of sample)})]}{\text{Abs(control)}} \text{ x } 100$$

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2.4.2.2 Oxygen radical absorbance capacity (ORAC) assay

A well-established and reliable method to determine the antioxidant capacity of a substance is the ORAC assay. It is based on the inhibition of oxyradical-induced oxidation of AAPH by hydrogen atom transfer mechanism with substance of antioxidant capacities (Cao, Alessio, & Cutler, 1993; Kohri et al., 2009). Peroxyl radicals produced in a time-dependent manner during the thermal decomposition of AAPH will quench the fluorescence signal. Antioxidants present in the assay work to block the peroxyl radical oxidation of the fluorescent probe until the antioxidant activity in the sample

is depleted. The remaining peroxyl radicals destroy the fluorescence of the fluorescent probe. This assay continues until completion, which means both the antioxidant's inhibition time and inhibition percentage of free radical damage is a single value. This method was described by Ou and colleague (2002) with some modification. Briefly, 25 µl of sample extract was mixed with 150 µl of fluorescein solution and then added 25 µl of AAPH solution. The plate was incubated for 30 min at 37ºC. Fluorescence was recorded using a spectrofluorometer (an excitation wavelength of 493 nm and an emission wavelength of 515 nm) every 15 sec over 60 min. Trolox solution was used as a standard antioxidant (3.125-100 µM). The results were calculated using the differences of areas under the curve between samples or standard, trolox, and blank. ORAC values expressed as µmol TE/g dry weight (DW).

2.4.2.3 Ferric reducing antioxidant power (FPAP) assay

FRAP method is a simple and inexpensive procedure that measures the total antioxidant levels in a sample. It utilizes the reducing potential of the antioxidants to react with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex (yellow) and produce a colored ferrous tripyridyltriazine (Fe^{2+}-TPTZ) form (blue) (Benzie & Strain, 1996; Ou et al., 2002). This assay was determined from the modified procedure of Benzie & Strain (1996). Briefly, 150 µl FRAP reagent, consisting of ferric chloride and TPTZ in acetate buffer (pH 3.6, were added to 20 µl the extract of sample in a 96-well plate. The mixture allowed standing in the dark for 8 min. Trolox solution was used as a standard

antioxidant (7.813- 250 μM). Absorbance of the sample and trolox were read at 600 nm. The results were presented as μmol TE/g DW.

2.4.2.4 Total phenolic content

This method measures the total concentration of phenolic hydroxyl groups in the plant extract. Polyphenols in plant extracts react with specific redox reagents (Folin-Ciocalteu reagent) to form a blue complex that can be quantified by visible-light spectrophotometry. The total phenolic contents was determined according to the method described by Amarowicz and colleague (2004). Briefly, 10 μl of the extract was transferred into a 96-well microplate containing 160 μl of distilled water. After mixing, 10 μl of Folin–Ciocalteu reagent and 20 μl of a saturated sodium carbonate solution were added. The solution was mixed well and the absorbance measured at 750 nm after 30 min incubation in dark using a microplate reader. Gallic acid was served as standard antioxidant (10-200 μg/ml). Total phenolic contents expressed as mg gallic acid equivalent (GAE)/100g DW.

2.4.2.5 Total anthocyanin content

Anthocyanins constitute a large family of polyphenols in plants and are responsible for many of the fruit which anthocyanin pigments have demonstrated ability to protect against oxidative stress of human diseases (Fang, 2014). Anthocyanins were extracted with acidified methanol (methanol and 1 M HCl, 85:15, v/v) with a solvent to sample ratio of 1:10 for 30 min on a magnetic stirrer and then separated by centrifugation. The

supernatants were collected and kept in the dark and cold until procedure. The residues were twice re-extracted under the same conditions, and supernatants of all three cycles were combined. Total anthocyanin content calculated using a calibration curve of cyanidin-3-glucoside (C3G) as reference. Extracts are measured at 525 nm by spectrophotometer. Contents are expressed as mg C3G equivalent/100g DW (Abdel-Aal & Hucl, 1999)(Abdel-Aal & Hucl, 1999).

2.4.2.6 Identification of C3G in CNP fruit extract by HPLC analysis

The analysis of bioactive compounds present in the plant extracts involving the applications of common phytochemical screening assays, chromatographic techniques such as high performance liquid chromatography-HPLC widely used technique for the isolation of natural products (Sasidharan et al., 2011). This chromatography is a technique to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition. This involves a stationary phase and a mobile phase. The mobile phase flows through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. This difference in rates cause the separation of various components.

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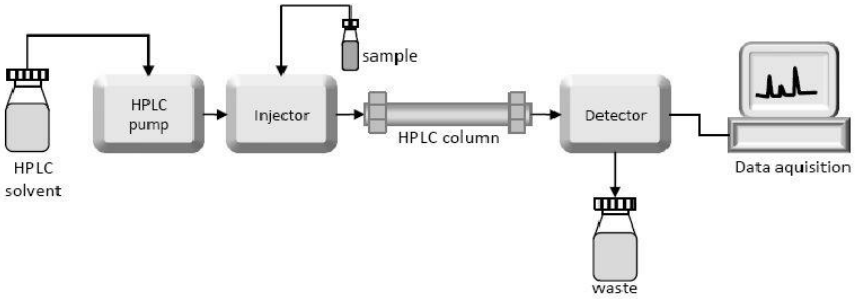

Figure 9. The schematic diagram of HPLC instrumentation

(http://laboratoryinfo.com/hplc/#prettyPhoto)

Sample was determined in triplicate by HPLC with diode array detection (HPLC-DAD) using the SHIMADZU LC-10 series (Shimadzu Scientific Instruments, Japan). Zorbax Eclipse XDB-C18 column (4.6 × 150 mm) was used for analytical separation. A gradient elution program consisted of two mobile phases: A (2% acetic acid in water) and B (absolute methanol). Elution was performed at a flow rate of 1.0 ml/min and the solvent gradient was as follows: from 0 to 40 min, 90% A and 10% B, from 40 to 45 min, 50% A and 50% B, and from 45 to 60 min, 90% A and 10% B. The injection volume was 20 µl and the column was thermostated at 35 °C. The C3G was performed at 520 nm via UV-DAD. Peak identity was confirmed by comparing their retention times and absorption spectra with pure C3G (≥99%, Sigma Chemical Co., St. Louis, MO, USA) commercially available standard. Quantification was carried out by external standard calibration curves. The amount of the identified compound was expressed in mg/100g DW.

2.4.3 Evaluation of cell viability and cell death

2.4.3.1 The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cytoxicity of sample and of glutamate treatments were assessed using a microculture MTT-based colorimetric assay. The method used to evaluate cell decrease by detecting metabolic activity decrease. The principle of this assay is that MTT is changed from yellow of MTT to purple of formazan by mitochondrial dehydrogenase of viable cells. In dead cells, there is no mitochondrial dehydrogenase changing MTT to formazan. Briefly, cells were seeded onto 96-well plates at the density of 5,000 cells/well and were treated with specific condition treatments. Cells were incubated with 0.5 mg/ml of MTT for 4 hours at 37 °C. After that the supernatant removed, and the formazan crystals produced in viable cells were solubilized in DMSO. The absorbance of each well was read at 550 nm using a microplate reader. The percentage of viable cells was calculated relative to control cells, according to the following formula:

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$$\% \text{ Cell viability} = \frac{\text{Abs(sample} - \text{Abs (blank of sample)}}{\text{Abs(control)} - \text{Abs (blank of sample)}} \text{ x } 100$$

2.4.3.2 Lactate dehydrogenase (LDH) assay

Cytotoxicity was evaluated by the quantification of plasma membrane damage using LDH assay. LDH is a cytosolic enzyme that is an indicator of cellular toxicity. The LDH-cytotoxicity assay provides a fast and simple method for quantitating cytotoxicity based on the measurement of activity of LDH released from damaged cells. Unlike many other cytoplasmic enzymes which exist in many cells either in low amount or unstable such as alkaline and acid phosphatase, LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity can be determined by a coupled enzymatic reaction. The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA) is a colorimetric cytotoxicity assay kit. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay, which results from LDH oxidizes lactate to pyruvate which then convert a tetrazolium salt (iodonitrotetrazolium violet; INT) into a red formazan product. The amount of color formed by formazan produced in culture supernatant is proportional to the number of lysed cells.

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Briefly, the cells were seeded in 96 well plates. After the specified treatment and time the plates spun at 1,500 g before transferred 50μl aliquots from all test and control wells to a new microtiter plate. Then, the 50μl of substrate mix reagent was added. To obtain the maximum LDH release for the cells a set of control cells were lysed 30 min prior to the assay and the supernatant treated in the same is at the test wells.

The microtiter plate was incubated in the dark for 30 min at room temperature before adding 50 μl of stop solution. The absorbance at 490 nm was measured using a microplate reader. Data were calculated as percentage of maximum LDH release activity of cells that were exposed to inducer alone, which was defined as 100%, according to the following formula:

$$\% \text{ LDH release} = \frac{\text{Abs(sample LDH release)} - \text{Abs (blank of sample)}}{\text{Abs(maximum LDH release} - \text{Abs (blank of maximum LDH release)}} \times 100$$

2.4.3.3 Apoptosis assay

Apoptosis is the process that regulates cell death. In live cells, cytoplasmic surface of cellular membrane is composed of phosphatidyl serine. It is translocated to outer surface of cellular membrane in apoptotic process. The cells were performed using the fluorescein isothiocyanate (FITC)-annexin V apoptosis detection kit with propidium iodide to evaluate apoptosis and necrosis, respectively, according to the manufacturer's instructions. Annexin V is the protein that acts as the anticoagulant by competing with prothrombin to bind PS. Annexin V is conjugated to fluorophore which expresses green fluorescence by binding outer PS of apoptotic cells. In addition to, propidium iodide is red fluorescent dye which tightly stains nucleic acid in dead cells but stains live and apoptotic cells non-permanent. Briefly, the treatment cells were incubated with Annexin V-FITC reagent and then propidium iodide solution for 15 min at room temperature in the dark. After 15 min of incubation, the binding buffer was

added to mark the final volumes. The cells were determined by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

2.4.4 Determination of intracellular ROS production

The accumulation and increase in ROS production or a decrease in ROS-scavenging capacity due to exogenous stimuli or endogenous metabolic alterations can disrupt redox homeostasis, leading to an overall increase of intracellular ROS levels, or oxidative stress. Increased oxidative stress plays a crucial role in a variety of pathologic conditions including aging and neurodegenerative diseases (Trachootham et al., 2008). Measuring the effect of antioxidant therapies and ROS activity intracellularly is crucial to suppressing or treating oxidative stress inducers. DCFH-DA assay is a cell-based assay providing a convenient and sensitive means to monitor oxidative activity in living cells. The assay employs the cell-permeable fluorogenic probe 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA). DCFH-DA has been widely used as a marker for oxidative stress, and has been suggested to be a good indicator of the overall oxidative status of the cell (Chen et al., 2008). This hydrophobic non-fluorescent molecule, DCFH-DA, penetrates rapidly into the cell and is hydrolysed by intracellular esterases to give the DCFH molecule which can be oxidised to its fluorescent 2-electron product 2′,7′dichlorofluorescein (DCF) by ROS. The fluorescence intensity is proportional to the ROS levels within the cell cytosol. Thus, intracellular ROS was detected using $DCFH_2$-DA as a specific ROS probe in this study. Briefly, the

cells were seeded in 12-well plate, and then pre-treated with the extract in different concentrations for 24 h. This specific treatment cells were exposed to glutamate for 18 h, followed by 10 μM $DCFH_2$-DA for 45 min at 37°C. After the completed incubation, the cells were washed, trypsinized and resuspended in buffer. Immediately, fluorescence intensity was measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA), using excitation and emission wavelengths of 488 and 525 nm, respectively. Results were expressed as the relative ROS level compared with the group of control cells.

2.4.5 Measurement of protein concentration by Bradford assay

Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) is the method to measure protein concentration in solution. The principle of this assay is the binding Coomassie Brilliant Blue G-250 dye to proteins. This dye consists of 3 forms: cationic, neutral and anionic. In acidic condition, it is in a red cationic form and is converted to a blue anionic form when binding to proteins. The anionic form has maximum absorbance at 595 nm. Briefly, in order to measure protein concentration, 1X Bradford working buffer was added to protein samples in microtiter plate, incubated at room temperature for 10 min and measured at 595 nm using spectrophotometer. BSA was used as a standard protein (0-1 mg/ml). Protein concentration was calculated from a standard curve.

2.4.6 Western blot analysis

Western blotting is an important technique used in cell and molecular biology. It also known as immunoblotting, is widely used technique for the detection and analysis of proteins. The method is based on building an antibody: protein complex via specific binding of antibodies to proteins immobilized on a membrane and detecting the bound antibody with one of several detection methods. The technique uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize (Mahmood & Yang, 2012).

Briefly, the specific cell treatments were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadade, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride and commercial protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) for 45 min on ice. The cell lysates were collected and the protein concentration was determined using the Bradford protein assay, as mentioned above. Equal amount of protein samples (40 µg) were denatured by heating in laemmli loading buffer at 95°C for 5 min and subsequently loaded onto a 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories). Transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, and 0.05% Tween 20) and incubated with specific ER stress apoptotic proteins primary antibodies against calpain, caspase12, CHOP, ERK, p-ERK (Thr202/Tyr204) and β-actin (Cell Signaling,

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Danvers, MA, USA), Nrf2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 4°C overnight. Membranes were washed three times with TBST for 15 min and incubated with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. β-actin was used for loading control. Subsequently, the bands were visualized using a X-ray film exposure with the chemiluminescence detection system and quantified using Image J software.

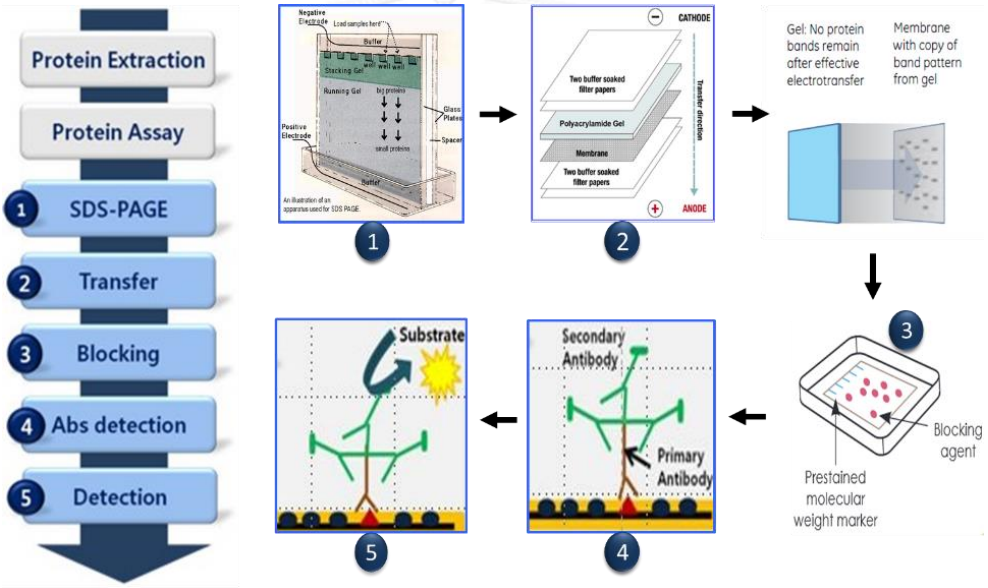

Figure 10. The schematic diagram of Western blotting workflow

2.4.7 Gene expression analysis by real-time PCR analysis

Real-time PCR using fluorescence dyes is currently the most sensitive and precise method for investigation of RNA level and has long been widely used for absolute and

relative quantification of mRNA in the cell. This highly sensitive method allows measurement of different type RNA level in the cell based on the kinetics of the corresponding double-stranded cDNA amplification. Upon its binding to the minor groove of double-stranded DNA, the yield increasing fluorescent signal in direct proportion to the number of PCR product molecules generated. Data collected in the exponential phase of the reaction yield quantitative information on the starting quantity of the amplification target. During the real-time RT-PCR procedure the level of amplified DNA is measured after every cycle of amplification, which permits to perform quantification at the cycles when amplification curve has not yet reached the period range and corresponds to the range of exponential increase in DNA amount (Nikitina et al., 2003).

The cellular antioxidant enzyme expressions were evaluated by a real-time polymerase chain reaction (real-time PCR) analysis to determine mRNA expression between untreated control cells and treated group in this study. The process started with reverse transcribing RNA of interest into its DNA complement through the use of reverse transcriptase. Subsequently, the newly synthesized specific cDNA was amplified using real-time polymerase chain reaction with primers. Briefly, total RNA was isolated from specific treatment cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of total RNA using Accupower RT Premix (Bioneer, Daejeon, South Korea) with oligo-dT primers following the manufacturer's

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protocol. Real-time PCR was carried out using the Exicycler Real Time Quantitative Thermal Block (Bioneer) and SYBR Green was used for the detection of double-stranded DNA. Quantitative real-time PCR for specific genes were performed by using Green Star PCR Master Mix (Bioneer). The PCR for all genes were performed and fluorescent signals were measured in real time under the following conditions: 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 15 sec and primer annealing/extension at 55°C for 30 sec. Melting curve analysis was performed to determine primer specificity. The relative expression of each gene was normalized against the internal control gene (β-actin). The antioxidant gene-specific sequence of primers, namely SOD1, SOD2, CAT, GPx, GSTo1, GSTa2 and β-actin (Panee et al., 2007). The primers sequences are shown in Table 1.

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Table 1. The specific sequence of primers

Genes	Sequence of primer	Product size (bp)
SOD1 forward	5'-CAGGACCTCATTTTAATCCTCAC-3'	76
SOD1 reverse	5'-CCCAGGTCTCCAACATGC-3'	
SOD2 forward	5'-CTGGACAAACCTGAGCCCTA-3'	62
SOD2 reverse	5'-TGATAGCCTCCAGCAACTCTC-3'	
CAT forward	5'-CAGCGACCAGATGAAGCA-3'	68
CAT reverse	5'-CTCCGGTGGTCAGGACAT-3'	
GPx forward	5'-ACAGTCCACCGTGTATGCCTTC-3'	238
GPx reverse	5'-CTCTTCATTCTTGCCATTCTCCTG-3'	
GSTo1 forward	5'-CAGCGATGTCGGGAGAAT-3'	102
GSTo1 reverse	5'-GGCAGAACCTCATGCTGTAGA-3'	
GSTa2 forward	5'-TCTGACCCCTTTCCCTCTG-3'	85
GSTa2 reverse	5'-GCTGCCAGGATGTAGGAACT-3'	
β-actin forward	5'-GGCTGTATTCCCCTCCATCG-3'	154
β-actin reverse	5'-CCAGTTGGTAACAATGCCATGT-3'	

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2.5 Statistical analysis

All experiments were performed independently at least three times. Data are expressed as the mean ± standard deviation (S.D). Statistical analysis was determined using one-way analysis of variance-ANOVA followed by Dunnett's post hoc test at a significance level of $p < 0.05$ using SPSS version 22 for Windows.

CHAPTER III

RESULTS

3.1 Antioxidant properties and anthocyanin content in CNP extract

Certain contents and properties of the CNP extract including radical scavenging activities, total phenolic and anthocyanin contents were first clarified prior to subject to all cell-based evaluations. The aqueous extract had a purplish-red and the yield of the extract was 11.42% as calculated from dried raw material. The result revealed that CNP extract had relatively potent ability to scavenge the DPPH radical and AAPH radical (ORAC assay) (Table 2). However, CNP extract exerted lesser reducing power evaluated by FRAP assay. For the constituent determinations, CNP extract was found to have the total phenolic content of 535.91 $\pm$ 0.09 mg GAE/100g DW and total anthocyanin content of 50.49 $\pm$ 0.64 mg G3G/100g DW (Table 2). In addition, the key anthocyanin-C3G was used as a marker to standardize the extract used in the study; therefore, the extract has also identified the bioactive anthocyanin marker. We discovered that the ripe CNP fruit extract was a rich source of C3G (24.06 $\pm$ 0.29 mg/100g DW, with 1.18 % RSD) by HPLC analysis (Figure 11).

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Table 2. Antioxidant properties of CNP extract

DPPH (%Scavenging activity)	ORAC (μmole TE/g DW)	FRAP (μmole TE/g DW)	Total phenolic content (mg GAE/100g DW)	Total Anthocyanin content (mg C3G/100g DW)
93.49 ± 0.24	91.06 ± 3.35	42.89 ± 0.85	535.91 ± 0.09	50.49 ± 0.64

Values are mean ± SD of three independent experiments.

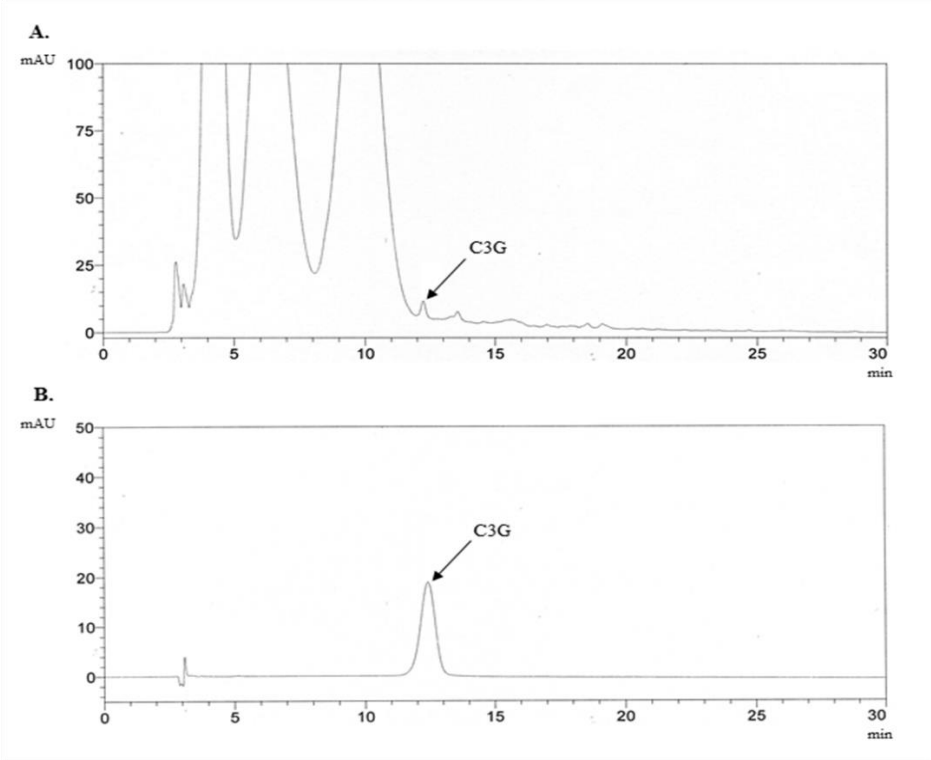

Figure 11. Representative HPLC chromatogram of C3G in CNP extract. (A) peak identification of CNP extract, (B) peak identification of standard. Peak identification was performed by matching retention time and UV spectra against pure commercially available standard.

3.2. Characterization of cytotoxicity in response to CNP extract and C3G, and glutamate in mouse hippocampal neuronal cells

In order to evaluate the optimal concentration of glutamate induced death in HT22 mouse hippocampal neuronal cells. The MTT assay was used to identify the degree of cell death after treatment with glutamate at different concentration. Cells were cultured in the presence or absence of glutamate (0-10 mM) for 18 h. After treatment with glutamate, the results indicated that cell viability of HT22 cells decreased in a concentration-dependent manner, as shown in Figure 12. The significant decrease in cell viability was detected in response to glutamate treatment at the concentration of 5 mM lead to about 50% cell death (Figure 12). Based on this data, glutamate at 5 mM was then chosen for the subsequent experiments.

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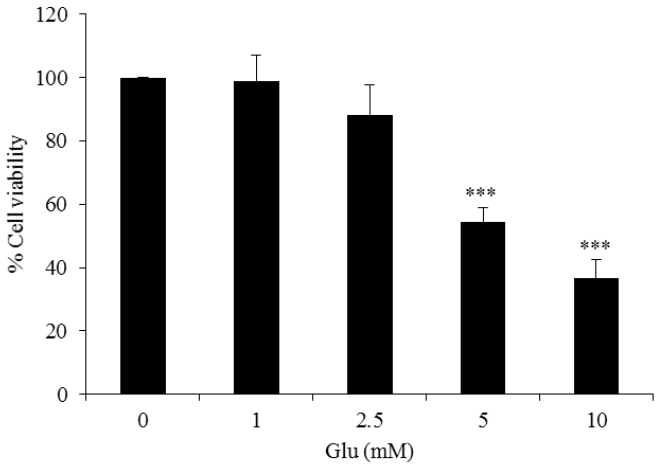

Figure 12. Cytotoxic effect of glutamate-induced neuron death in HT22 cells. After seeded cells for 24 h, and then exposed to various glutamate concentrations for 18 h, cell viability was evaluated using MTT assay. Values are expressed as mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 versus non-treated control.

Next, to examine whether CNP extract and C3G might protect cell death induced by glutamate, we first needed to determine the non-cytotoxic concentration of the extract using MTT assay. Treatment of the cells with the extract at different concentrations (0-100 μg/ml) and C3G (0-100 μM) for 24 h caused no significant change in cell viability compared with the non-treated control cells. These results indicated that the CNP extract (Figure 13A) and C3G (Figure 14A) was relatively non-cytotoxic at the tested doses for HT22 cells. Then, cells pretreated with CNP extract (0-100 μg/ml) and C3G (0-100 μM) for 24 h, exposed to 5 mM glutamate for further 18 h, and cell viability was determined. In the absence of CNP extract or C3G, glutamate significantly

reduced viability of the cells approximately 50 %. Treatment of the cells with CNP (0.05-1 μg/ml) and C3G (0.05-1 μM) could attenuate the glutamate-induced toxicity in a concentration-dependent manner with the maximum protective effect at 1 μg/ml the extract (Figure 13B) and/or 1 μM C3G (Figure 14B. The results were confirmed by cell death LDH assay. Figure 13C and 14C show that glutamate caused approximately 59% damaged cells and the pretreatment if the cells with CNP extract (0.05-1 μg/ml) and C3G (0.05-1 μM) significantly suppressed such cell deaths.

We further clarify the mode of cell death from cytotoxic results above. Apoptosis and necrosis were evaluated by Annexin V-FITC and propidium iodide (PI) stains, respectively. The HT22 cells were pretreated with different concentrations of CNP extract (0-1 μg/ml) and C3G (0-1 μM) for 24 h and exposed to glutamate for 18 h. A flow cytometric analysis was used to quantify the percentage of cell apoptosis by Annexin V-FITC/PI double staining. As shown in Figure 13D-E and Figure 14D-E, treatment of the cells with glutamate caused approximately 44% and 2% of apoptosis and necrosis, respectively. While the PI-positive necrotic cells were not detectable in both of CNP extract and C3G-treated cells, addition of CNP extract and C3G clearly protected against glutamate-induced apoptosis with the highest protective effect could be observed at the concentration of 1 μg/ml or 1 μM, respectively. These apoptosis data consisted with the morphology of cells detected by a phase contrast microscopy (Figure 13F and 14F). The glutamate-treated cells exhibited round, shrink

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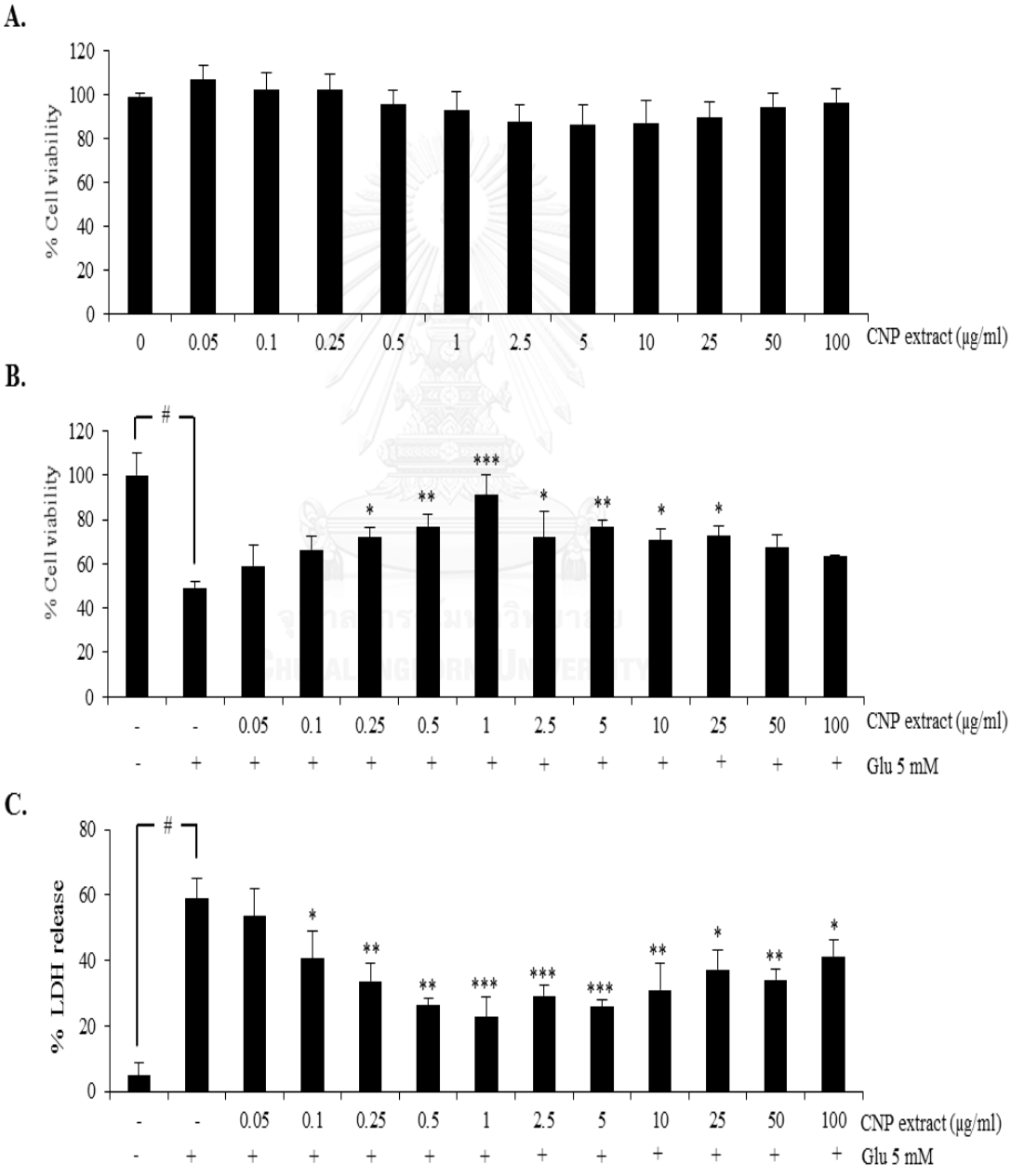
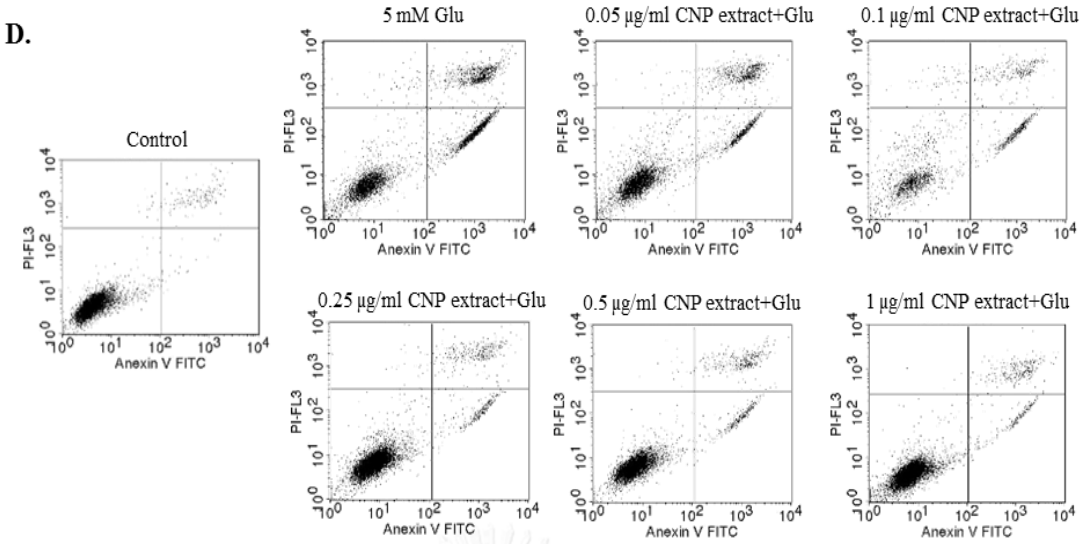
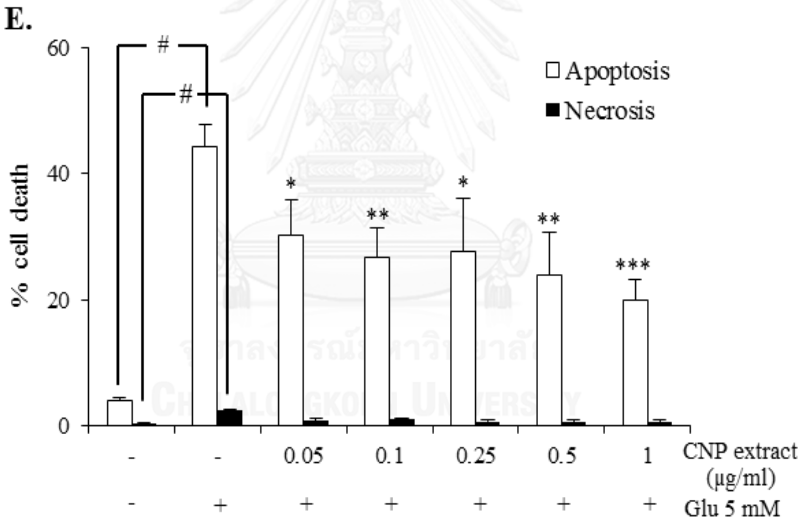
and lost elongated neuron shape. The pretreatment with CNP extract and C3G restored the native morphology as observed in the non-treated control. These results have demonstrated the neuroprotective effect of CNP extract and C3G in this system.




D.
Control
5 mM Glu
0.05 µg/ml CNP extract+Glu
0.1 µg/ml CNP extract+Glu
0.25 µg/ml CNP extract+Glu
0.5 µg/ml CNP extract+Glu
1 µg/ml CNP extract+Glu
PI-FL3
Anexin V FITC


E.
% cell death
Apoptosis
Necrosis
CNP extract (µg/ml)
Glu 5 mM

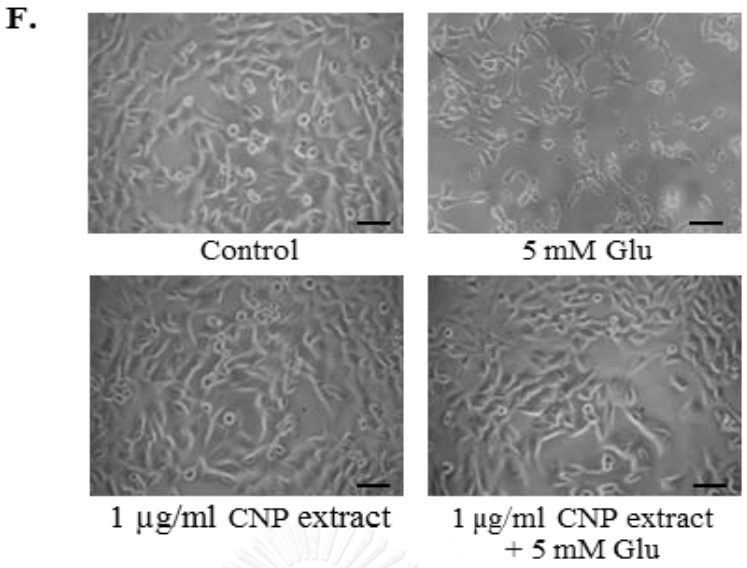
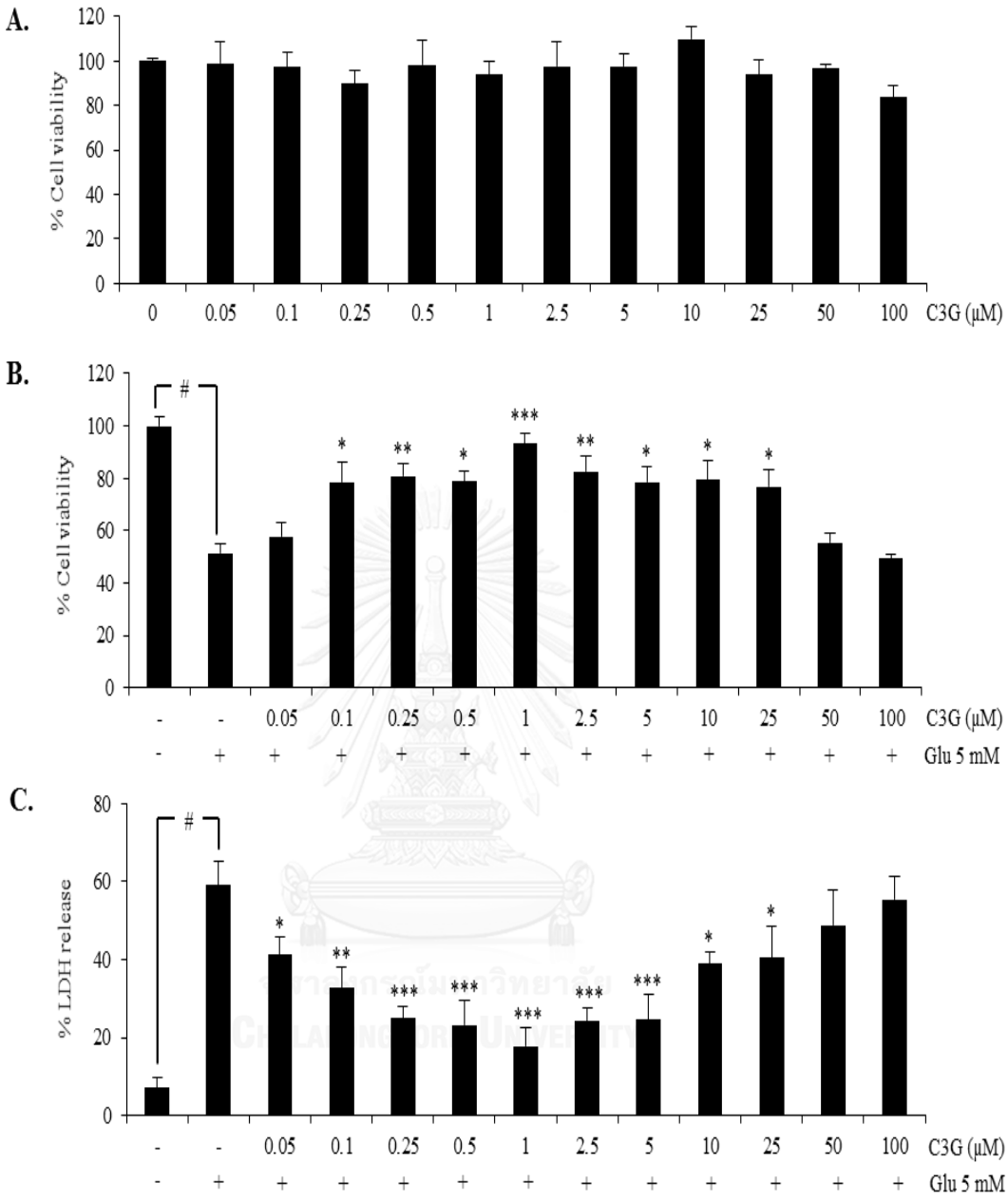
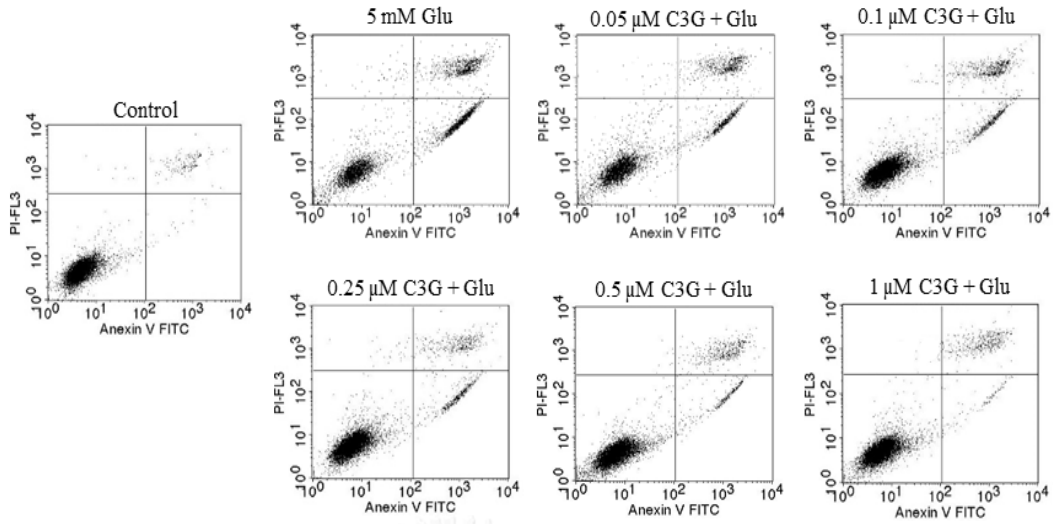


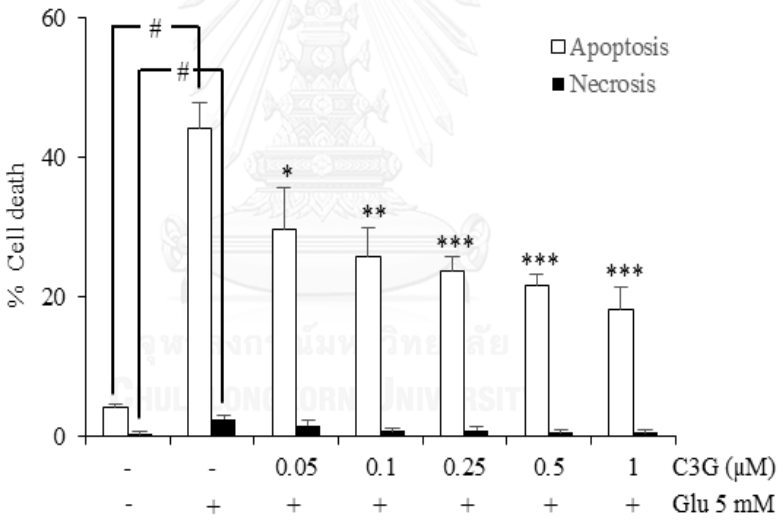
Figure 13. Effect of CNP extract on cell viability and apoptosis in HT22 cells. (A) Cells were treated with CNP extract (0-100 µg/ml) for 24 h. Cell viability was evaluated by MTT assay. (B) After indicated treatment for 24 h, followed by 5 mM glutamate for 18 h, cell viability was detected using the (C) MTT assay and (D) cell death was measured using LDH assay. (E) Cells were pretreated CNP extract (0-1 µg/ml) and followed by 5 mM glutamate, mode of cell death was examined by Annexin V-FITC/PI double staining, and analyzed using flow cytometry. (F) After indicated treatment, morphology of HT22 cells was observed using phase contrast microscope (scale bar is 50µm). Values are expressed as mean ± SD (n=4). $^{\#}p<0.05$ versus non-treated control, *p<0.05, **p<0.01, ***p<0.001 versus 5 mM glutamate-treated cells.


A.
% Cell viability
120
100
80
60
40
20
0
0 0.05 0.1 0.25 0.5 1 2.5 5 10 25 50 100 C3G (μM)
B.
% Cell viability
#
*
**
***
- - 0.05 0.1 0.25 0.5 1 2.5 5 10 25 50 100 C3G (μM)
- + + + + + + + + + + + + Glu 5 mM
C.
% LDH release
#
*
**
***
- - 0.05 0.1 0.25 0.5 1 2.5 5 10 25 50 100 C3G (μM)
- + + + + + + + + + + + + Glu 5 mM

D.


Control
5 mM Glu
0.05 µM C3G + Glu
0.1 µM C3G + Glu
0.25 µM C3G + Glu
0.5 µM C3G + Glu
1 µM C3G + Glu
PI-FL3
Anexin V FITC

E.


Apoptosis
Necrosis
% Cell death
60
40
20
0
#
#
*
**
***
***
***
- - 0.05 0.1 0.25 0.5 1 C3G (µM)
- + + + + + + Glu 5 mM

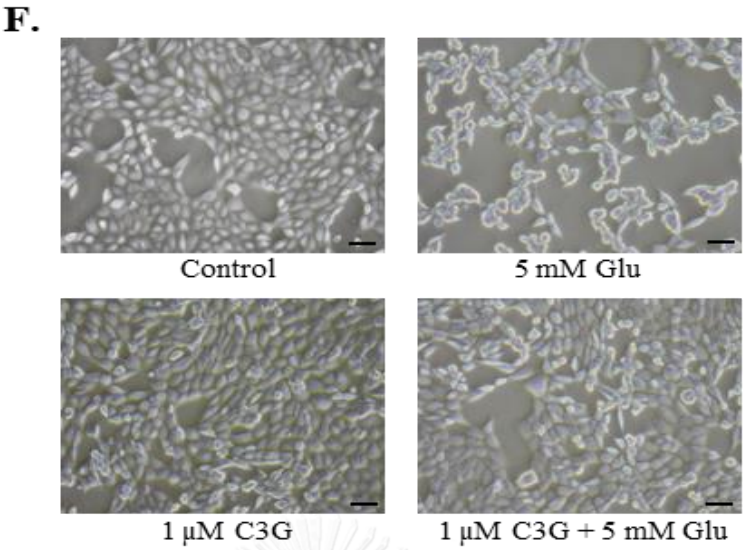


Figure 14. Effect of C3G on cell viability and apoptosis in HT22 cells. (A) Cells were treated with C3G (0-100 μM) for 24 h. Cell viability was evaluated by MTT assay. (B) After indicated treatment for 24 h, followed by 5 mM glutamate for 18 h, cell viability was detected using the (C) MTT assay and (D) cell death was measured using LDH assay. (E) Cells were pretreated C3G (0-1 μM) and followed by 5 mM glutamate, mode of cell death was examined by Annexin V-FITC/PI double staining, and analyzed using flow cytometry. (F) After indicated treatment, morphology of HT22 cells was observed using phase contrast microscope (scale bar is 50μm). The data are expressed as mean ± SD (n=4). $^{\#}p<0.05$ versus non-treated control, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus 5 mM glutamate-treated cells.

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3.3 Effect of CNP extract and C3G on glutamate-induced intracellular ROS production in HT22 cells

It has been well known that glutamate induced neuron cell death via oxidative stress-dependent mechanism. Several studies have demonstrated the roles of ROS on neurodegenerative disorders (Andersen, 2004; Duran-Aniotz, Martínez, & Hetz, 2014; Emerit, Edeas, & Bricaire, 2004; Jin et al., 2014; Kim et al., 2015). To elucidate the effects of CPN extract and C3G on glutamate-induced oxidative stress, the levels of ROS in the cells were measured using the fluorescence probe $DCFH_2$-DA. HT22 cells were pretreated with the different concentrations of CNP extract and C3G for 24 h before being exposed to glutamate for 18 h. Results showed that glutamate treatment induced approximately 5-fold increase in cellular ROS level compared to the non-treated control group CNP extract (Figure 15) and C3G (Figure 16). Pretreatment of the cells with CNP significantly attenuated the elevated levels of ROS induced by glutamate as shown in Figure 15, as well as pretreated with C3G (Figure 16). Additionally, we demonstrated the antioxidant activity of C3G using DPPH radical scavenging assay. The result showed that C3G had relatively potent ability to scavenge the DPPH radical in a dose-dependent manner, especially at dose of 1μM showed the highest scavenging activity (Figure 16C).

We further confirmed HT22 cells generate ROS by glutamate treatment, the ROS-inducing effect of glutamate-induced oxidative toxicity can be inhibited by specific ROS

inhibitors namely, 5 mM N-acetyl-L-cysteine (NAC); total radical inhibitor, 5 mM glutathione (GSH) and 7500 Unit/mg catalase (CAT); hydrogen peroxide inhibitors, 50 μM Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP); superoxide anion inhibitor, and 1 mM deferoxamine mesylate (DM); hydroxyl radical inhibitor. Each inhibitor was pretreatment with cells for 30 min and then stimulated to glutamate, after that they were demonstrated using MTT assay. Results indicated that treatment with the glutamate caused the all of radical species up-regulation in the HT22 cells and such event could be abolished by the addition of these specific inhibitors, confirming that the all of ROS formation species can be induced by glutamate treatment in our system (Figure 17).

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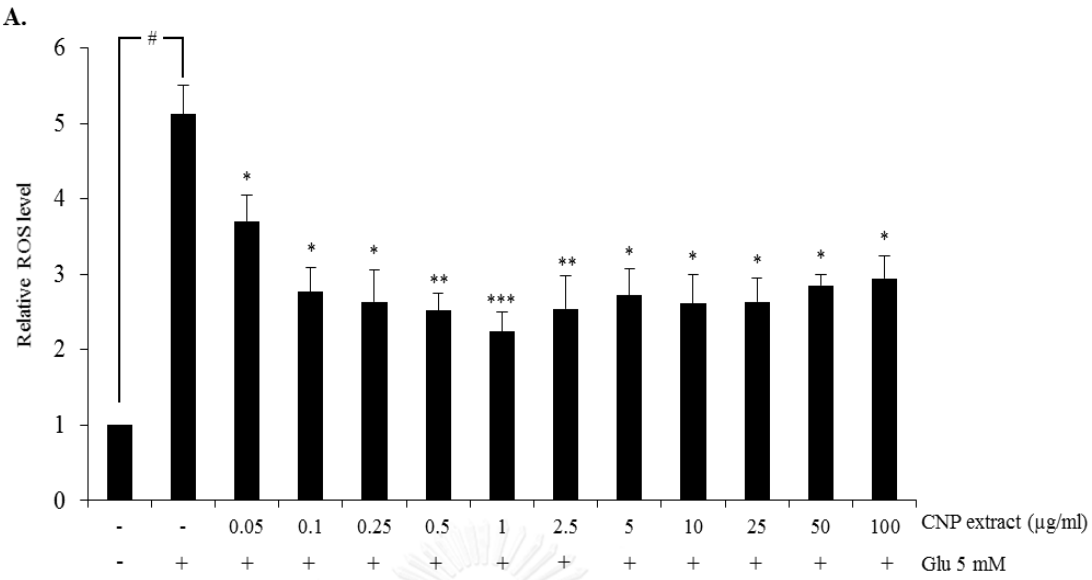
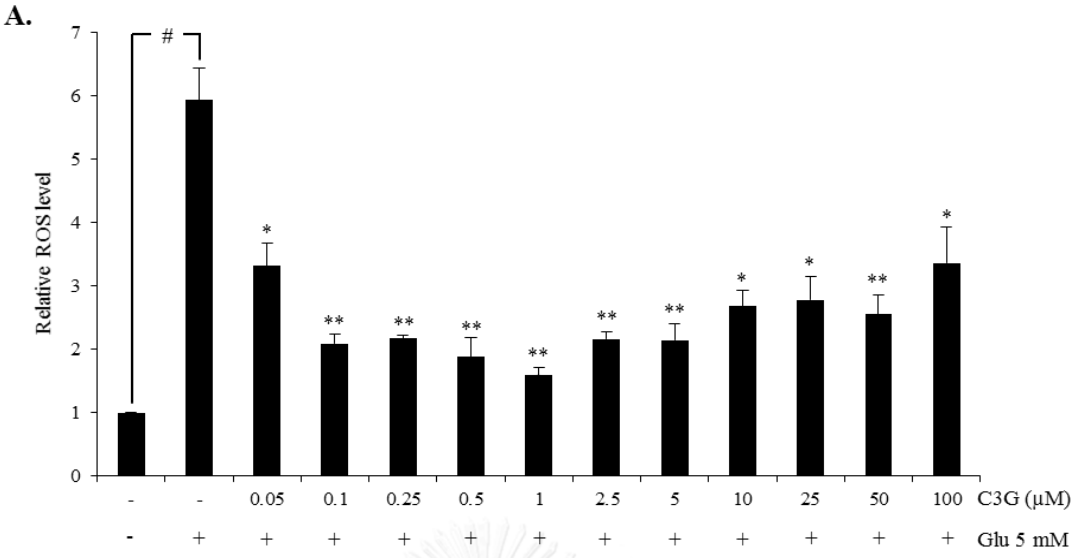
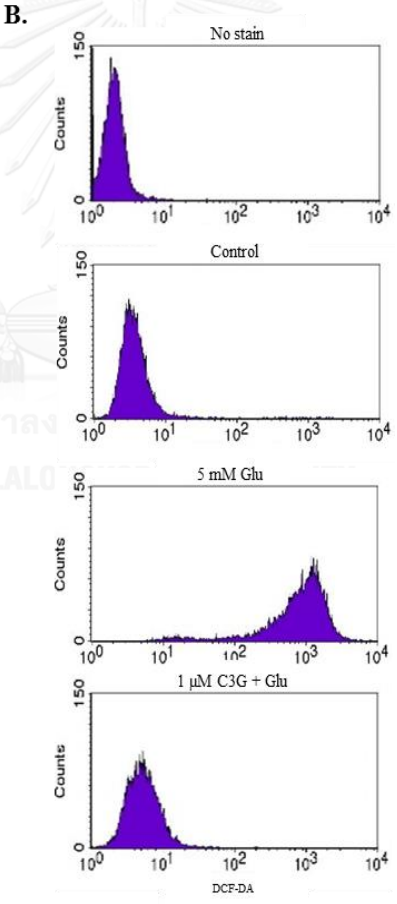


Figure 15. Effect of CNP extract on glutamate-induced production of intracellular ROS in HT22 cells. The pretreatment of cells with CNP extract (0-100 μg/ml) for 24 h, followed by 5 mM glutamate for 18 h. After that the cells were incubated with 10 μM $DCFH_2$-DA for 45 min at 37°C. The fluorescence intensity was measured by flow cytometry. Data were expressed as a relative ROS level of non-treated control. Values are the mean ± SD (n=4). #p<0.05 versus non-treated control, *p<0.05, **p<0.01, ***p<0.001 versus 5 mM glutamate-treated cells.


A.
Relative ROS level
0
1
2
3
4
5
6
7
#
*
**
-
-
0.05
0.1
0.25
0.5
1
2.5
5
10
25
50
100
C3G (µM)
-
+
+
+
+
+
+
+
+
+
+
+
+
Glu 5 mM


B.
No stain
Control
5 mM Glu
1 µM C3G + Glu
Counts
0
150
10^0
10^1
10^2
10^3
10^4
DCF-DA

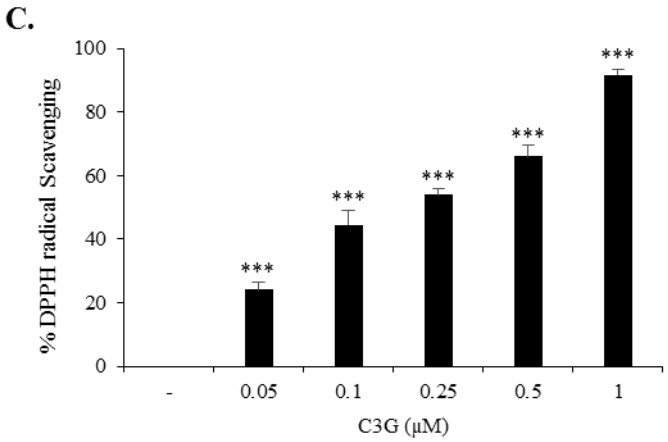


Figure 16. Effect of C3G on glutamate-induced production of intracellular ROS in HT22 cells and antioxidant activity. (A) and (B) The pretreatment of cells with C3G (0-1μM) for 24 h, followed by 5 mM glutamate for 18 h. After that the cells were incubated with 10 μM $DCFH_2$-DA for 45 min at 37ºC. The fluorescence intensity was measured by flow cytometry. Data were expressed as a relative ROS level of non-treated control. Values are the mean ± SD (n=4). $^{\#}p<0.05$ versus non-treated control, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus 5 mM glutamate-treated cells. (C) The free radical scavenging activity of various concentrations of C3G (0-1 μM) was evaluated using DPPH assay. Values are the mean ± SD (n=4). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus non-treated control.

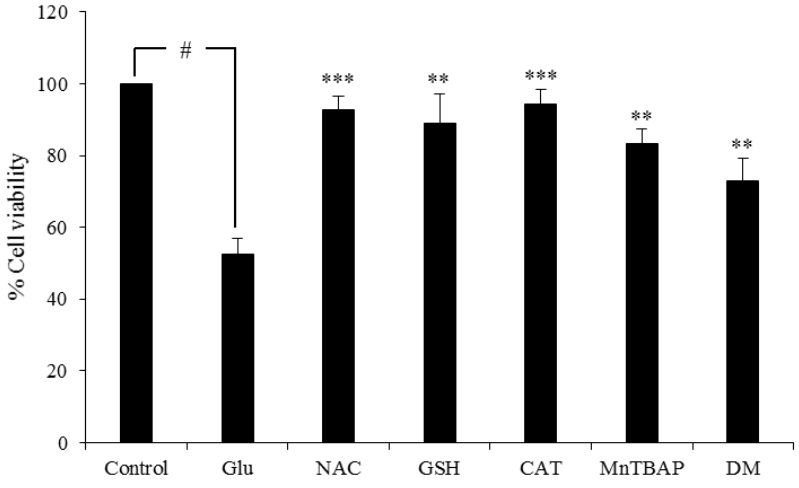


Figure 17. Protection of HT22 cells against glutamate-induced death by specific ROS inhibitors. Cells were pretreated specific ROS inhibitors for 30 min and followed by 5 mM glutamate for 18 h, cell viability was detected by the MTT assay. Glu, glutamate; NAC, N-acetyl-L-cysteine; GSH, glutathione; CAT, catalase; MnTRAP, Mn(III)tetrakis (4-benzoic acid) porphyrin; DM, deferoxamine mesylate. Values are the mean ± SD (n=4). $^{\#}p<0.05$ versus non-treated control, *p<0.05, **p<0.01, ***p<0.001 versus 5 mM glutamate-treated cells.

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3.4 Effect of CNP extract and C3G on glutamate-induced ER stress

The ER is a major organelle that crucially controls for the protein synthesis, folding and trafficking, and calcium homeostasis. It has been revealed that ER stress-induced cell death plays critical roles in the pathogenesis of neurodegenerative diseases (Chaudhari et al., 2014b; Choi et al., 2010; Cullinan & Diehl, 2006). ER stress triggers the calcium ion release, up-regulation of calpain, and the activation of caspase-12 (Momoi, 2004).

In addition, the increase of cellular CHOP, was shown to be another mechanism of ER stress (Oyadomari & Mori, 2004). To determine the effect of CNP extract on ER stress-mediated cell death, the ER stress signatures including calpain, caspase-12 and CHOP were determined by Western blot analysis. The HT22 cells were pre-incubated with various concentrations of CNP extract (0-1 μg/ml) and C3G (0-1 μM) for 24 h and exposed to glutamate for 18 h. Figure 18 and 19 show that glutamate alone significantly increased the levels of calpain, caspases-12 and CHOP. Interestingly, our results indicated that CNP extract at the concentrations of 0-1 μg/ml and C3G at the concentrations of 0-1 μM significantly suppressed glutamate-mediated calpain, cleaved caspase-12 and CHOP inductions (Figure 18A-C and 19A-C). Altogether, CNP extract and C3G were shown to inhibit glutamate-induced apoptosis, at least in part, via suppression of ER stress pathway.

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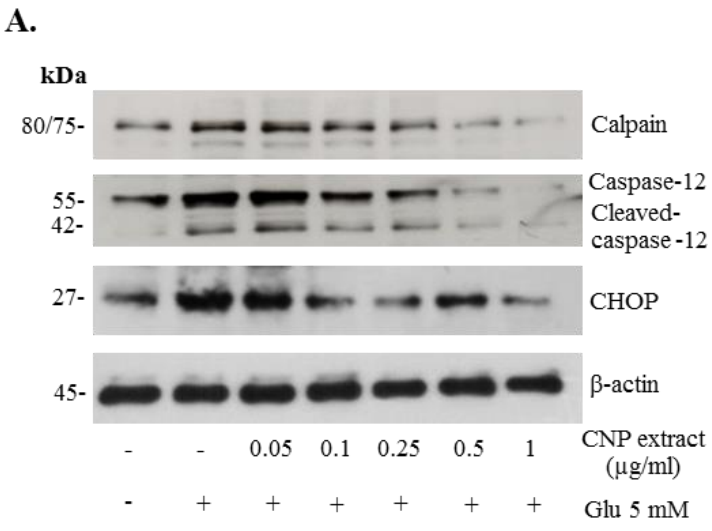

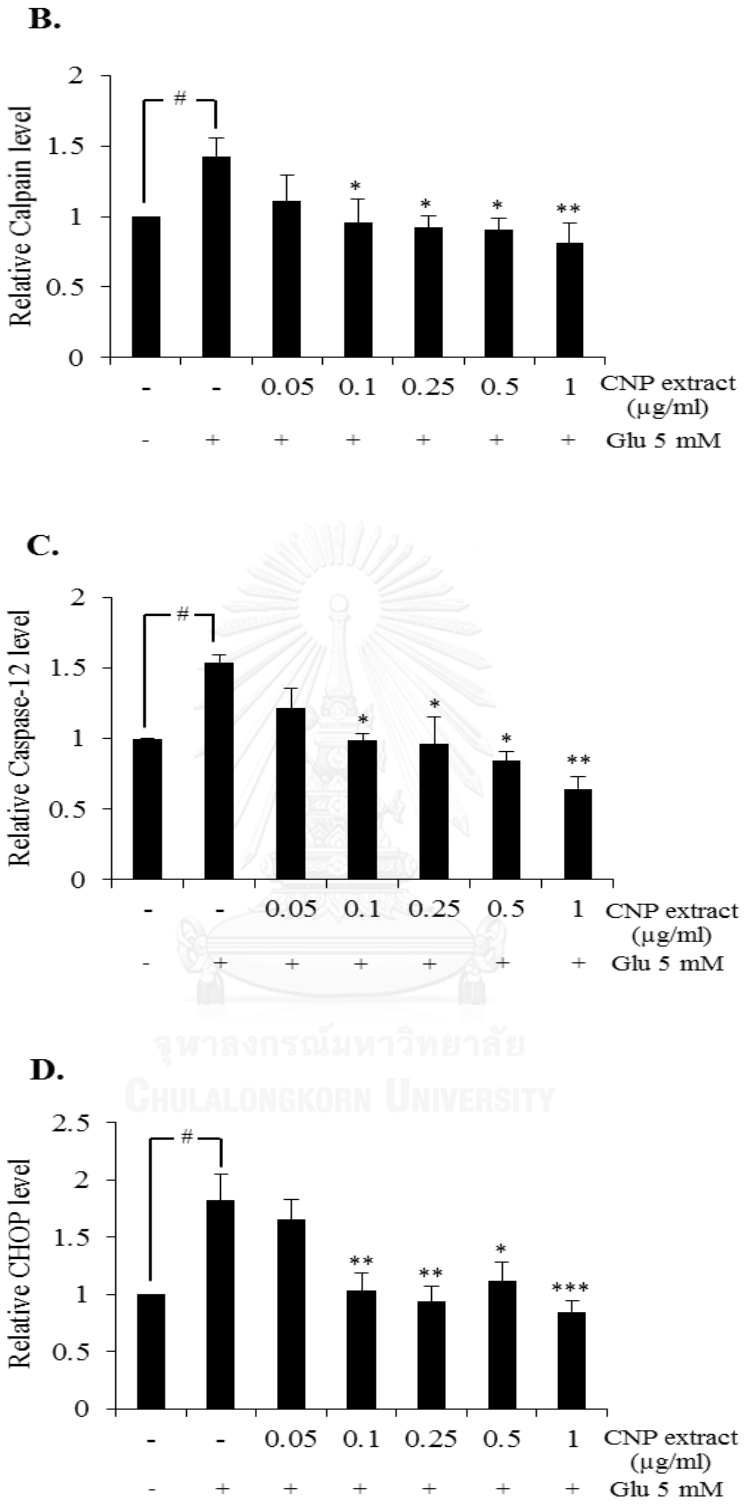

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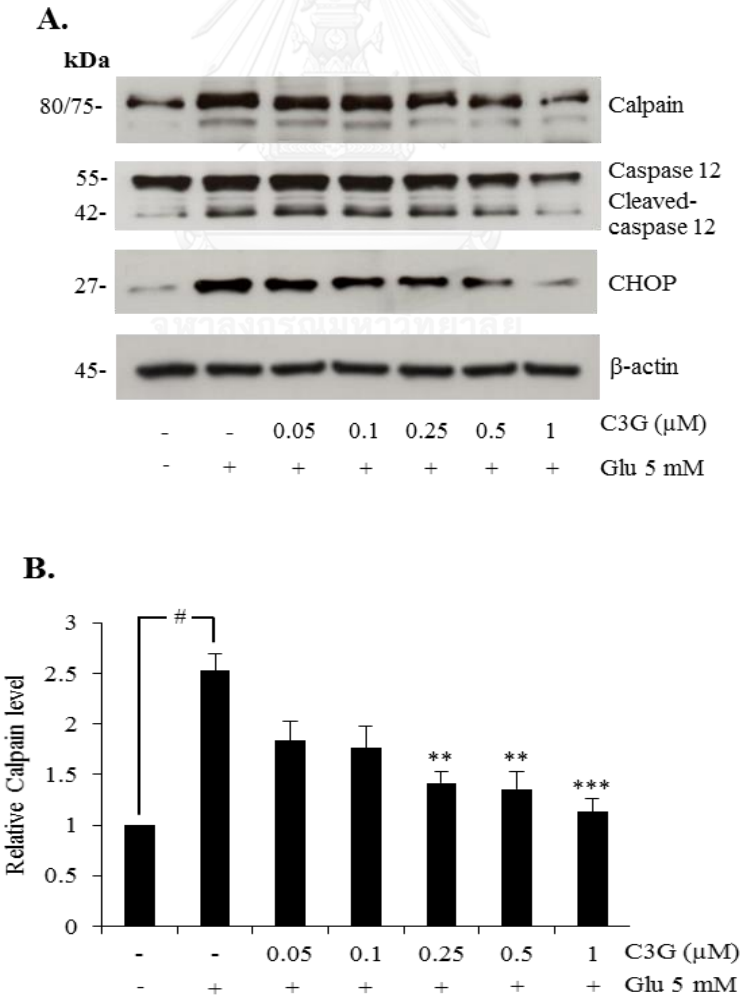
Figure 18. Effect of CNP extract on glutamate-induced ER stress apoptotic proteins expression in HT22 cells. Cells were pretreated with CNP extract (0-1 µg/ml) for 24 h,

followed by 5mM glutamate for 18 h. (A) Representing ER stress apoptotic proteins were determined by Western blot analysis. Quantitative analysis of (B) calpain, (C) caspase-12 and (D) CHOP. β-actin was served as the loading control. The immunoblot signals were quantified by densitometry and the mean data from independent experiments were normalized to the results. The data represent the means of four independent samples ± SD. #p<0.05 versus non-treated control, *p<0.05, **p<0.01, ***p<0.001 versus 5 mM glutamate-treated cells.



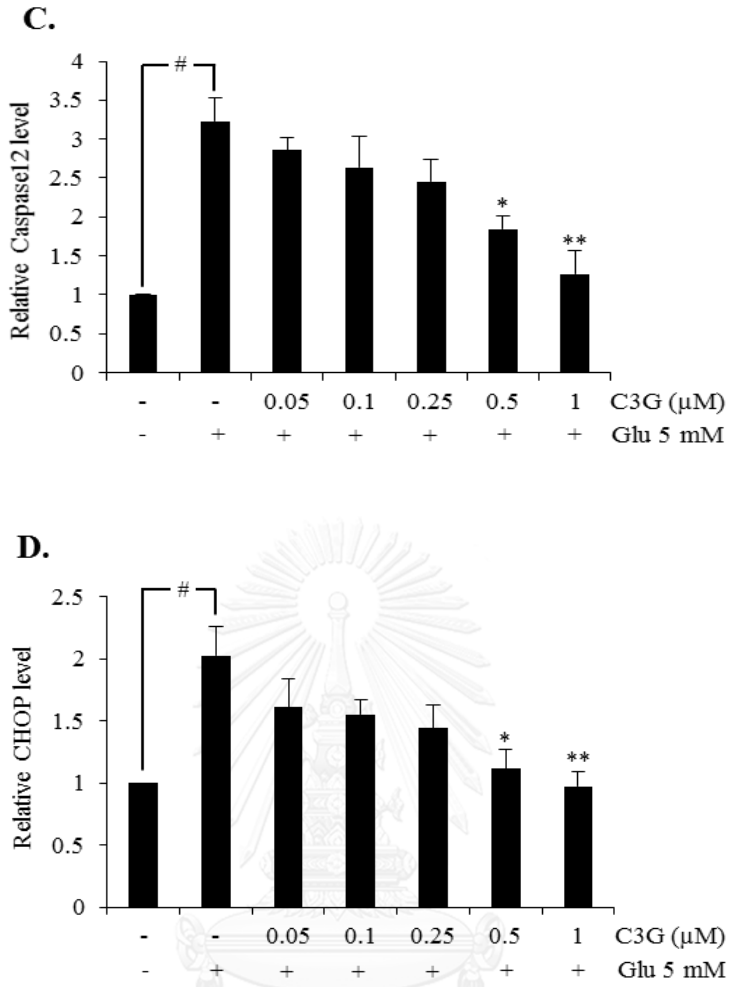


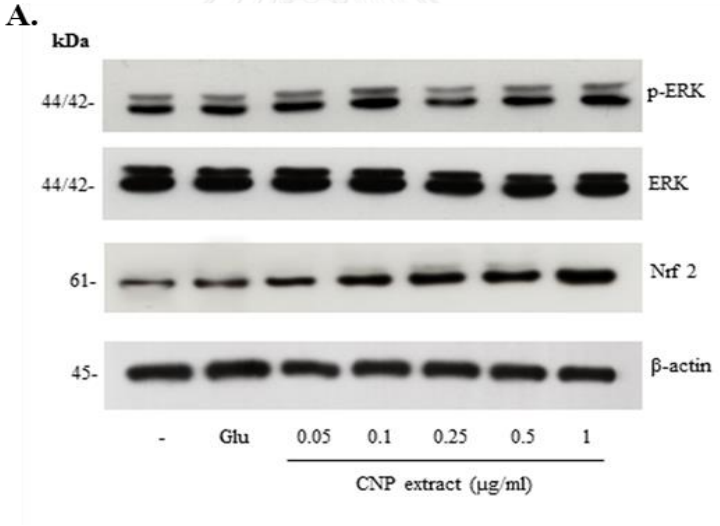
Figure 19. Effect of C3G on glutamate-induced ER stress apoptotic proteins expression in HT22 cells. Cells were pretreated with C3G (0-1 μM) for 24 h, followed by 5mM glutamate for 18 h. After treatment, (A) Representing ER stress apoptotic proteins expression were determined by western blot analysis. Quantitative analysis of (A) calpain, (B) caspase-12 and (C) CHOP. β-actin was served as the loading control. Relative protein levels were quantified by densitometry and the mean data from independent experiments were normalized to the results. The data represent the

means of four independent samples ± SD. $^{\#}p<0.05$ versus non-treated control, *p<0.05, **p<0.01, ***p<0.001 versus 5 mM glutamate-treated cells.

3.5 CNP extract and C3G activate survival antioxidant proteins in HT22 cells

The survival signaling that defense oxidative stress in cellular mechanisms, Nrf2 is known to play an essential role in endogenous antioxidants and phase II detoxification systems for increasing cellular defense against oxidative stress (Behl & Moosmann, 2002; Essa et al., 2012). Activation of Nrf2 pathway represents a promising therapeutic approach to restore the systemic and neuronal redox balance by reducing ROS-mediated neuronal damage (Slemmer & Weber, 2014). In addition, it has been demonstrated that Nrf2 is activated by the MAPKs signaling under conditions of oxidative stress, namely ERK. It has been originally shown that are also involved in the stress response and an important for cell survival in role of anti-apoptotic signals in neuronal cells (Chang & Karin, 2001; Cullinan & Diehl, 2006; Doyle et al., 2011; Leong et al., 2011; J. Li et al., 2013; Stanciu & DeFranco, 2002; Sun, Huang, & Zhang, 2009b). Therefore, we set out to investigate the protective effect of CNP extract and C3G on oxidative/ER stress-mediated death in this HT22 mouse hippocampal neuronal cells, the expression of ERK and Nrf2 in response to the cell treatment was assessed by Western blot analysis. The HT22 cells were treated with various concentrations of CNP extract (0-1 μg/ml) and C3G (0-1 μM) for 24 h. We found that the extract and C3G

treatments led to a significant increase in Nrf2 and the p-ERK/ERK protein levels was found to be slightly increased, as compared with the control cells (Figure 20 for the extract and Figure 21 for C3G). While the positive control as glutamate caused insignificantly elevated expressions in both of proteins versus non-treated control. Taken together, our results suggested that both of CNP extract and C3G can activate the ERK/Nrf2 antioxidant protein signaling, resulting in promote the protective HT22 cells signaling pathway.



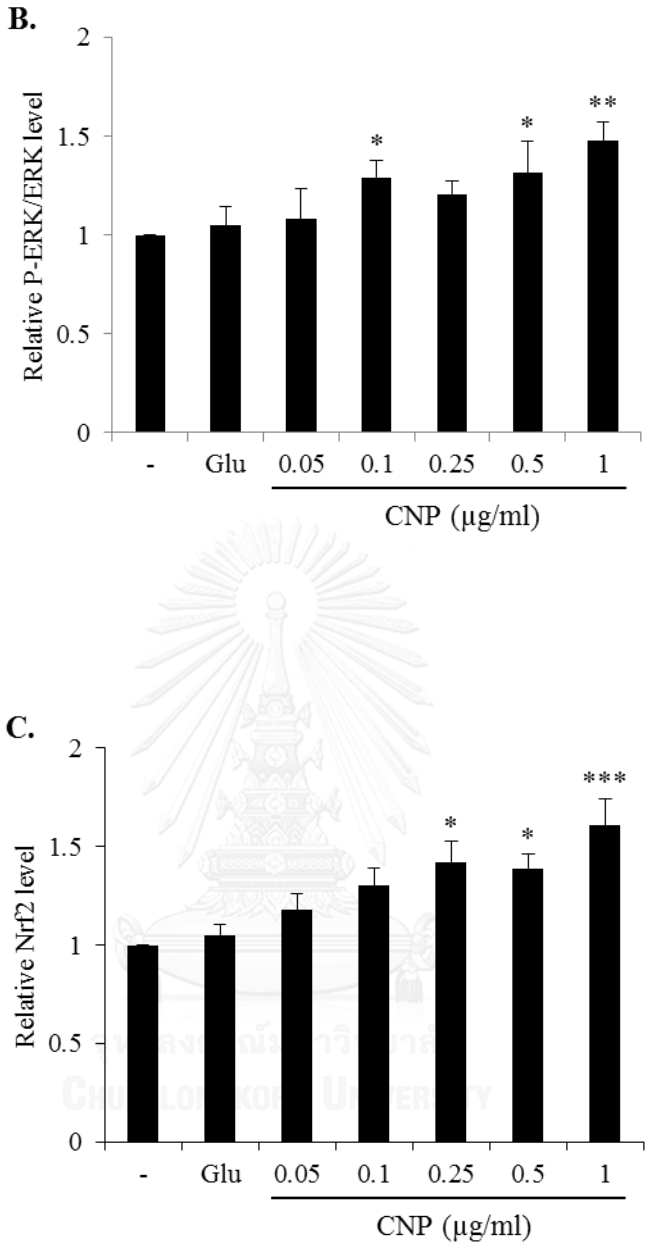
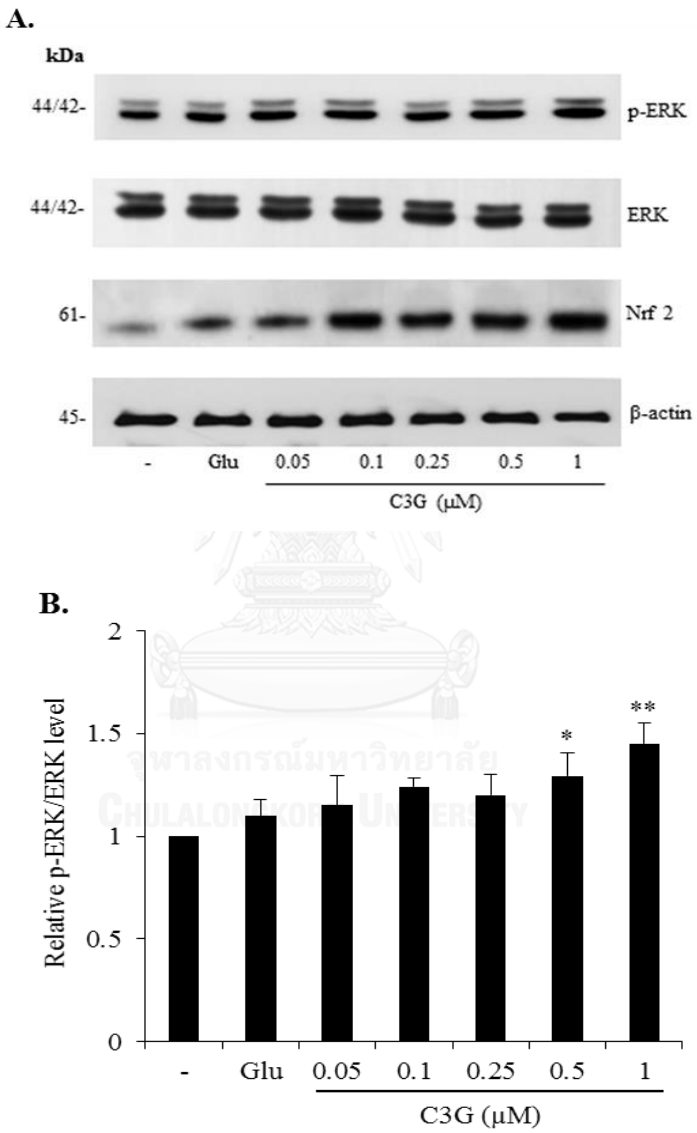


Figure 20. CNP extract activates ERK and Nrf2 signaling proteins in HT22 cells. Cells were pretreated with the CNP extract (0-1 µg/ml) for 24 h, followed by 5mM glutamate for 18 h. After treatment, (A) the expressions of p-ERK/ERK and Nrf2 were determined by Western blot analysis. β-actin was used to ensure the equal loading. (B) Relative protein levels were quantified by densitometry and the mean data from independent

experiments were normalized to the results. The data represent the means of four independent samples ± SD. *p<0.05, **p<0.01, ***p<0.001 versus non-treated control.



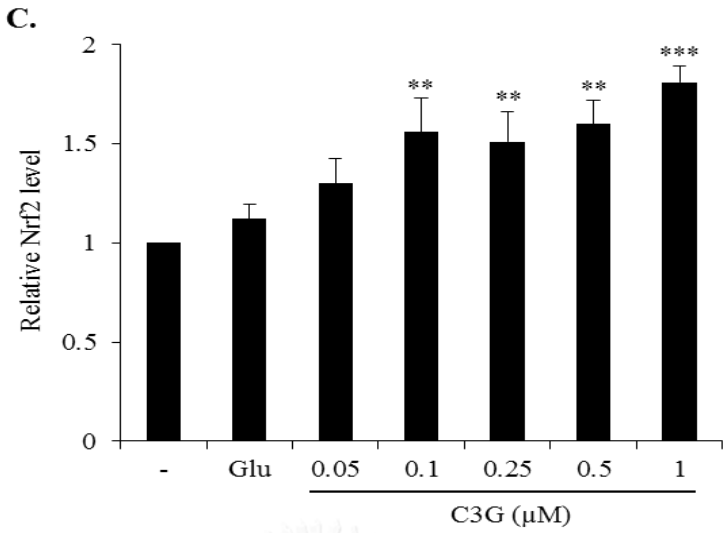


Figure 21. C3G activates ERK and Nrf2 signaling proteins in HT22 cells. Cells were pretreated with the C3G (0-1 μM) for 24 h, followed by 5mM glutamate for 18 h. After treatment, (A) the expression levels of p-ERK/ERK and Nrf2 were determined by Western blot analysis. β-actin was used to ensure the equal loading. (B) Relative protein levels were quantified by densitometry and the mean data from independent experiments were normalized to the results. The data represent the means of four independent samples ± SD. *p<0.05, **p<0.01, ***p<0.001 versus non-treated control.

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3.6 CNP extract and C3G enhance antioxidant and phase II enzymes expression in HT22 cells

The main types of antioxidant and phase II enzymes are SOD, CAT, GPx and GST which have a major role to prevent ROS-mediated cellular damage, as well as in neurons (Slemmer & Weber, 2014). These enzymes act as a downstream signaling of Nrf2 (Kraft, Johnson, & Johnson, 2004; Mota et al., 2015). In order to clarify the antioxidant mechanisms of the CNP extract and C3G, we evaluated the change of endogenous antioxidant enzymes, which are SOD, CAT, and GPx and phase II enzyme (GST) expressions after CNP or C3G treatment. The results showed that both of treatment cells with CNP extract or C3G significantly up-regulated the expressions of endogenous antioxidant enzymes, namely SOD (SOD1 and SOD2), CAT and GPx including phase II enzyme, namely GST (GSTo1 and GSTa2) in a concentration-dependent manner (Figure 22A-F; CNP extract and Figure 23-F; C3G). These results suggested that CNP extract and C3G can enhance the endogenous antioxidant and phase II enzymes of the neuron cells and this mechanism may help protect the cells against oxidative damage from glutamate toxicity.

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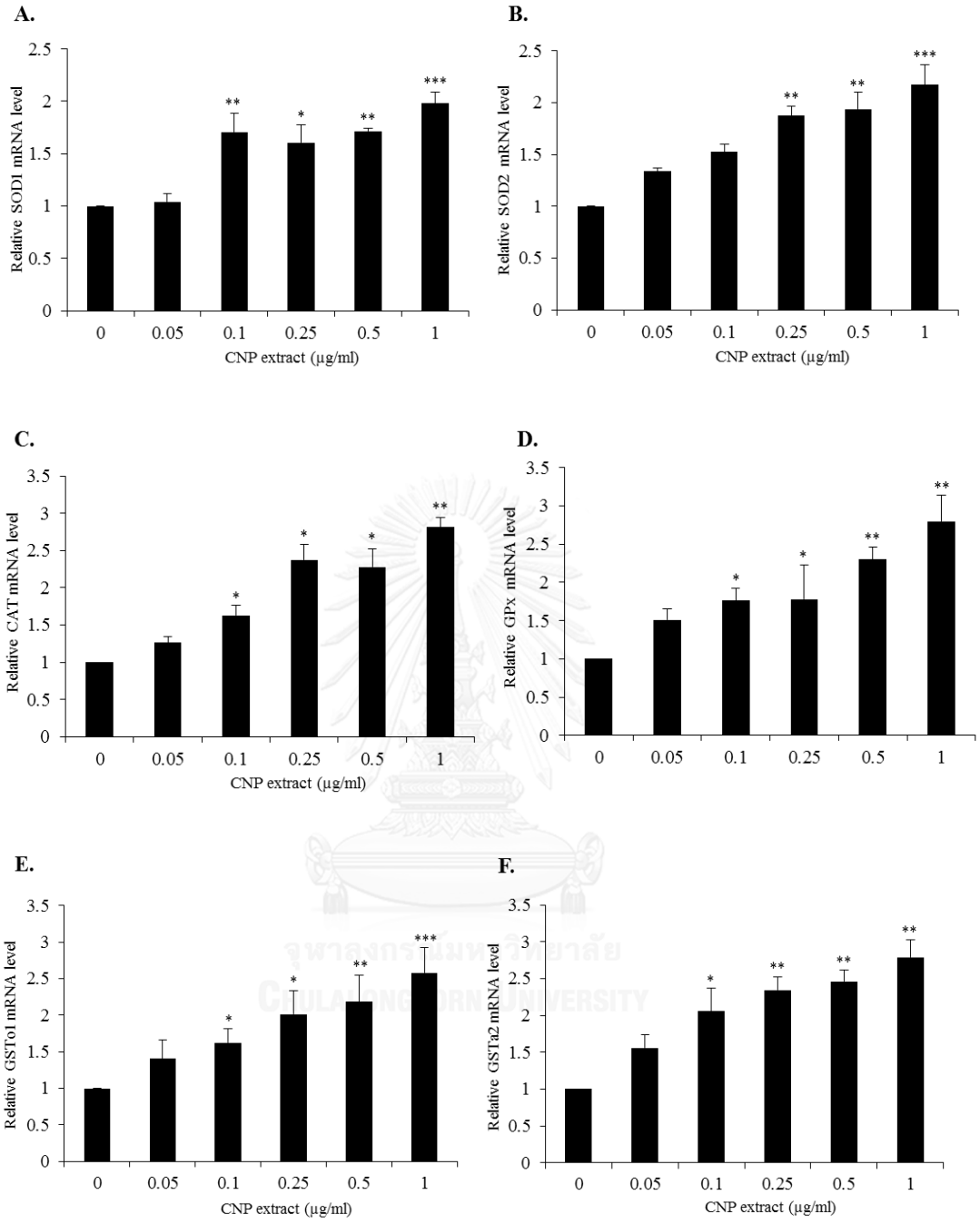

Figure 22. CNP extract enhances gene expression of endogenous antioxidant and phase II enzymes in HT22 cells. After treatment cells with CNP extract (0-1 µg/ml), cells were collected and analyzed for gene expression. The level of (A) SOD1, (B) SOD2, (C) CAT, (D) GPx, (E) GSTo1 and (F) GSTa2 mRNA expression were determined by

quantitative real-time PCR. The data are shown as fold change of mRNA expression normalized with β-actin. Values are mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 versus non-treated control.

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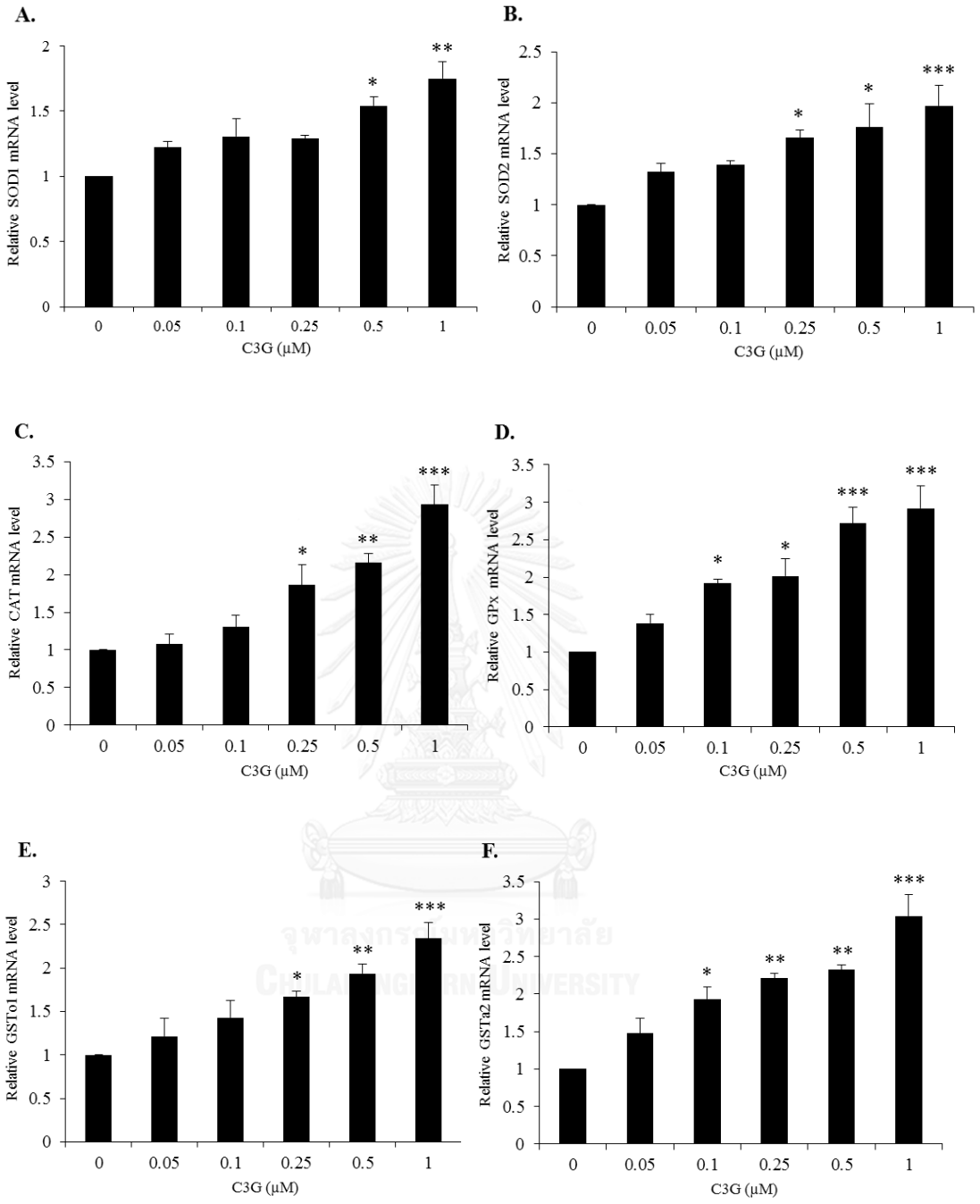

Figure 23. C3G enhances gene expression of endogenous antioxidant and phase II enzymes in HT22 cells. After treatment cells with C3G (0-1 μM), cells were collected and analyzed for gene expression. The levels of (A) SOD1, (B) SOD2, (C) CAT, (D) GPx, (E) GSTo1 and (F) GSTa2 mRNA expression were determined by quantitative real-time

PCR. The data are shown as fold change of mRNA expression normalized with β-actin. Values are mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 versus non-treated control.

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CHAPTER IV

DISCUSSION

Increasing evidence indicates that the death of neurons in neurodegenerative disorders are multifactorial and may include genetic predisposition and environmental factors. The one of important factor is ROS that particularly active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, whose metabolism is factory of ROS, which are unique to the brain and serve as sources of oxidative stress. ROS attack neurons which is post-mitotic cells; therefore, they are especially sensitive to free radicals, leading to neuronal damage (Gilgun-Sherki, Melamed, & Offen, 2001). It has been reported that deleterious effects of ROS on human and animal cells may end in oxidative injury leading to many forms of programmed cell death as well apoptosis (Valko et al., 2007). Glutamate, is a mammalian excitatory neurotransmitter in the central nervous system. Its toxicity has been shown to induce neuronal cell death via both receptor-initiated excitotoxicity and non-receptor-mediated oxidative stress (Choi et al., 2010; Jeong et al., 2010; Rudy et al., 2015). Moreover, an increase extracellular concentrations of glutamate has been shown to cause neuronal cell damage and death by generating cellular oxidative stress (Cheng et al., 2011; Greenwood & Connolly, 2007; Ha & Park, 2006; Jin et al., 2014; Yang et al., 2013). Therefore, glutamate-induced oxidative damage is a major contributor to pathological cell death within the nervous system (Li et al., 2009). Accumulated evidence have

suggested that cellular stress such as the generations of ROS and ER stress death signaling have been long known to play a pivotal role in the neurodegeneration (Chaudhari et al., 2014a; Doyle et al., 2011; Lee et al., 2010; Liu, Zhang, & Yin, 2013). The potential of natural products for the prevention and cure of neurodegeneration is supported by various experimental studies. Fruits, spices, and herbs possess important antioxidants and bioactive compounds necessary for the prevention and cure of various diseases without undesirable side effects (Essa et al., 2012). Also, the antioxidants that have a potent action in inhibition of ROS generation, ROS detoxification, or direct interact with the ROS generated by glutamate are likely to prevent glutamate-mediated neuronal cell death.

In the present study, we used the ripe Thai berry fruit extract-CNP and its major anthocyanin-C3G. The berry and anthocyanin are potent antioxidant capabilities which have been reported to produce health benefits through a wide range of biological effects in brain. However, there is no study on neuroprotective effects and underlying mechanisms of CNP extract and/or C3G against glutamate-induced oxidative and ER stress toxicity in HT22 cells. Natural products have been recognized as principle sources of bioactive compounds for drug discovery (Behl & Moosmann, 2002; Essa et al., 2012; Pratico, 2008; Valko et al., 2007). Although the general antioxidants that direct scavenge radical species have potentials to be used for the treatment or prevention of ROS-mediated disorders and diseases, their instability and short half-life have

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frequently limited their further development. Herein, we have demonstrated the protective effect of CNP extract that not only exerted its antioxidant activities through the direct scavenging property, but the extract also enhanced the expression of cellular antioxidant enzymes. By such means, the extract is likely to possess both short- and long-term antioxidant effects (Slemmer & Weber, 2014; Valko et al., 2007). In our present study, we found that CNP extract was able to directly scavenge reactive oxygen radicals, as indicated by the strong activity in both DPPH and ORAC assays. The potent radical scavenging activities may come from the high content of total phenol and anthocyanin (Table 2). Moreover, the bioactive anthocyanin marker of the CNP extract was identified trace of C3G as evaluated using HPLC. It contained the abundant amount in this fruit extract which this result was consistent with the previous study, Charoensin and colleague (2012) showed that the aqueous extract of CNP fruit was rich in anthocyanins, particularly C3G.

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For neuroprotective approach, we have demonstrated that non-toxic concentrations of CNP extract and C3G protected against glutamate-induced cell apoptosis. In this current study we found that HT22 cells facing glutamate showed higher levels of ROS and CNP extract and/or C3G suppressed the generation of intracellular ROS by glutamate toxicity. Additionally, we observed that glutamate was able to generate all of ROS formation types with specific ROS inhibitors, which confirming that all of ROS formation species can be induced by glutamate treatment in our system HT22 cells.

Consequently, we investigated the expression levels of specific markers of ER stress apoptotic proteins including calpain, caspase-12 and CHOP in response to glutamate treatment, suggesting that ER stress is a primary mechanism mediating apoptosis in these cells. Interestingly, addition of CNP extract and C3G could be able to suppress all ER stress proteins in glutamate-treated cells. This neuroprotective effect was linked with the potent antioxidant properties of the CNP extract and C3G, as oxidative stress is long known as an important inducer of ER stress by triggering the release of calcium ion from ER, resulting in calpain and caspase-12 activations (Lamkanfi, Kalai, & Vandenabeele, 2004; Momoi, 2004). Indeed, caspase-12 is an ER stress-specific caspase activated by the function of calpain (Doyle et al., 2011; Liu, Zhang, & Yin, 2013; Nakagawa et al., 2000; Q. Zhang et al., 2016). Moreover, we investigated the activation of CHOP, another crucial ER-stress regulating protein in glutamate-treated cells. In normal condition, the CHOP is kept at low level; however, CHOP and its accumulation in the nucleus are up-regulated during ER stress (Bernales, Soto, & McCullagh, 2012a; Lee et al., 2010; Oyadomari & Mori, 2004). We have found that CNP extract and C3G could suppress all ER stress mediating proteins induced by glutamate, assuring that CNP exytact and its C3G-anthocyanin type exert its neuroprotection by inhibition of ER stress apoptosis pathway.

Collectively, these findings showed that CNP extract and C3G acted as a neuroprotector in mouse hippocampal HT22 cells experimental model. Our results

suggested that the extract and C3G can significantly decrease glutamate-induced oxidative/ER stress, resulting in apoptosis, by blocking the intracellular ROS generation and enhancing the antioxidant system. Further, we investigated that the protective effect of CNP extract and C3G that not only exerted its antioxidant activity through the direct scavenging property, which inhibited intracellular ROS generation, but both of the extract and C3G also activated the expression level of Nrf2 antioxidant protein by MAPK/ERK signaling. Supporting evidences have demonstrated that activation of the Nrf2 antioxidant pathway protect neurons against oxidative stress from glutamate- and H_2O_2-induced cell death (Kraft, Johnson, & Johnson, 2004). In addition, some previous study found that C3G blocked oxidative stress by inducing Nrf2 and enhancing the cellular antioxidant pathway in human umbilical vein endothelial cells (Fratantonio et al., 2015). Recent work has showed that dimethyl fumarate protected neural stem/progenitor cells and neurons from H_2O_2-induced oxidative damage through MAPK ERK1/2-Nrf2 pathway (Wang et al., 2015). Besides, we found that pretreatment of the cells with CNP extract and/or C3G markedly up-regulated the gene expressions of antioxidant enzymes, namely SOD1, SOD2, CAT and GPx and phase II enzymes, namely GSTa2 and GSTo1 in a dose-dependent manner, which they are the downstream function of Nrf2 (Behl & Moosmann, 2002; Cullinan & Diehl, 2006; Essa et al., 2012) and these antioxidants found in brain tissue for ROS detoxification (Behl & Moosmann, 2002; Essa et al., 2012; Maher & Schubert, 2000; Markesbery & Carney, 1999; Slemmer & Weber, 2014; Valko et al., 2007). This potential of berry fruits to protect the neurons

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from oxidative stress has gained increased attention in recent years, in part due to its high polyphenol content and antioxidant capacity (Andres-Lacueva et al., 2005; Ansari & Khodagholi, 2013; Chen et al., 2009; Chen & Luo, 2010; Di Giacomo et al., 2012; Fang, 2014; Slemmer & Weber, 2014; Valko et al., 2007). Furthermore, previous research suggested that the high levels of anthocyanins in berry extract were able to directly scavenge ROS and provide substantial neuroprotection (Cheng et al., 2011; Miller & Shukitt-Hale, 2012; Talavera et al., 2005). C3G, this anthocyanin has recently gained attention for its multifunctional benefits including antioxidant properties, anti-inflammatory, and prevention of neurodegenerative disorders (Essa et al., 2012; Prior & Wu, 2006; Tan et al., 2014; Valko et al., 2007). Importantly, it is also able to reach the brain, which likely to pass through blood brain barrier as they can be detected in various part of the brain such as cerebellum, cortex, hippocampus or striatum of the rats after blueberry feeding (Andres-Lacueva et al., 2005; Faria et al., 2010; Fornasaro et al., 2016; Kalt et al., 2008; Talavera et al., 2005). Additionally, some study has shown that C3G up-regulated glutamate-cysteine ligase expression, which in turn mediated the reduction in ROS levels (W. Zhu et al., 2012).

Taken together, these data suggest that the protective mechanisms by which Thai berry- CNP extract and its major anthocyanin-C3G inhibit glutamate-induced oxidative/ER stress cell death through ERK/Nrf2 antioxidant pathway in HT22 cells, for

developing new treatment and prevention of neuronal cell death in neurodegenerative diseases such as AD.

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CHAPTER V

CONCLUSION

In conclusion, the present study we propose that the pretreatment with CNP extract and/or its major anthocyanin-C3G have a potential to protect against glutamate-induced oxidative/ER stress mediated HT22 neuronal cell apoptosis by direct scavenging ROS, up-regulating antioxidant and phase II enzymes system via enhancing the ERK/Nrf2 signaling pathway. This research supports that the indigenous CNP-Thai berry fruit might have a neuroprotective property, in addition to also reveal the potential benefit of C3G for the prevention of neurodegenerative diseases. Our findings for the protective pathway of CNP extract and/or C3G in mouse hippocampal neuronal HT22 cells model is summarized in Figure 24. These findings implicated the information and molecular mechanism of CNP extract and/or C3G as a promising neuroprotectant through activation of ERK/Nrf2 antioxidant pathway. However, it remains to be determined if the effects reported herein are CNP berry extract or can be extended to the other anthocyanins or polyphenols family. Further investigations are required the beneficial role of other anthocyanin fractions in this fruit extract on the protective effects in neurons. Accordingly, pharmacodynamics and pharmacokinetic studies of CNP extract and C3G in neurons are needed into clinical use.

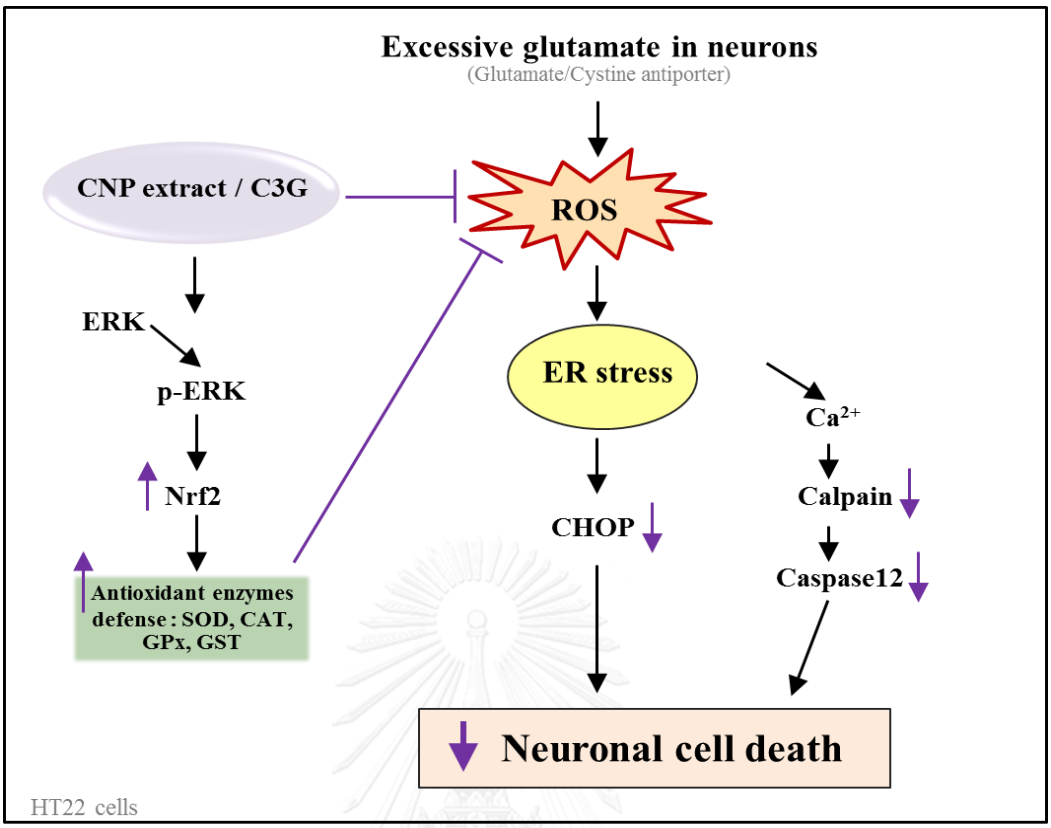

Figure 24. Schematic diagram summarizes the down-regulation of glutamate-induced oxidative and ER stress apoptosis in HT22 neuronal cells by CNP extract and/or C3G. Excessive glutamate-induced intracellular ROS generation evokes oxidative stress lead to ER stress. The resultant oxidative stress trigger the specific ER stress apoptosis mechanisms involving calcium ion-mediated calpain activation lead to caspase-12 cascades apoptosis signaling, together with CHOP apoptosis pathway. The role of CNP extract and/or C3G indicated that it inhibits ROS and activates p-ERK, and Nrf2 expression, which promote the gene expressions of endogenous antioxidant and phase II enzymes resulting in suppress the activity of ER stress apoptosis signaling molecules, to promote HT22 cell survival.

REFERENCES

Abdel-Aal, E.S.M., & Hucl, P. (1999). A Rapid Method for Quantifying Total Anthocyanins in Blue Aleurone and Purple Pericarp Wheats. *Cereal Chem J, 76*(3), 350-354.

Allen, R. G., & Tresini, M. (2000). Oxidative stress and gene regulation. *Free Radic Biol Med, 28*(3), 463-499.

Amarowicz, R., Pegg, R.B., Rahimi-Moghaddam, P., Barl, B., & Weil, J. A. (2004). Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem, 84*(4), 551-562.

Andersen, J. K. (2004). Oxidative stress in neurodegeneration: cause or consequence? *Nat Med, 10 Suppl*, S18-25.

Andres-Lacueva, C., Shukitt-Hale, B., Galli, R. L., Jauregui, O., Lamuela-Raventos, R. M., & Joseph, J. A. (2005). Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutr Neurosci, 8*(2), 111-120.

Ansari, N., & Khodagholi, F. (2013). Natural products as promising drug candidates for the treatment of Alzheimer's disease: molecular mechanism aspect. *Curr Neuropharmacol, 11*(4), 414-429.

Behl, C., & Moosmann, B. (2002). Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. *Free Radic Biol Med, 33*(2), 182-191.

Benzie, Iris F.F., & Strain, J.J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Anal Biochem, 239*(1), 70-76.

Bernales, S., Soto, M. M., & McCullagh, E. (2012a). Unfolded protein stress in the endoplasmic reticulum and mitochondria: a role in neurodegeneration. *Front Aging Neurosci, 4*, 5.

Bernales, S., Soto, M. M., & McCullagh, E. (2012b). Unfolded protein stress in the endoplasmic reticulum and mitochondria: a role in neurodegeneration. *Front Aging Neurosci, 4*, 1-13.

Bishop, N. A., Lu, T., & Yankner, B. A. (2010). Neural mechanisms of ageing and cognitive decline. *Nature, 464*(7288), 529-535.

Breyer, A., Elstner, M., Gillessen, T., Weiser, D., & Elstner, E. (2007). Glutamate-induced cell death in neuronal HT22 cells is attenuated by extracts from St. John's wort (Hypericum perforatum L.). *Phytomedicine, 14*(4), 250-255.

Brimson, J. M., Brimson, S. J., Brimson, C. A., Rakkhitawatthana, V., & Tencomnao, T. (2012). Rhinacanthus nasutus extracts prevent glutamate and amyloid-beta neurotoxicity in HT-22 mouse hippocampal cells: possible active compounds include lupeol, stigmasterol and beta-sitosterol. *Int J Mol Sci, 13*(4), 5074-5097.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Brimson, J. M., & Tencomnao, T. (2011). Rhinacanthus nasutus protects cultured neuronal cells against hypoxia induced cell death. *Molecules, 16*(8), 6322-6338.

Brown, Lockwood, A. H., & Sonawane, B. R. (2005). Neurodegenerative diseases: an overview of environmental risk factors. *Environ Health Perspect, 113*(9), 1250-1256.

Brown, & Naidoo, N. (2012). The endoplasmic reticulum stress response in aging and age-related diseases. *Front Physiol, 3*(263), 1-10.

Butterfield, D. A., Perluigi, M., & Sultana, R. (2006). Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. *Eur J Pharmacol, 545*(1), 39-50.

Calabrese, V., Boyd-Kimball, D., Scapagnini, G., & Butterfield, D. A. (2004). Nitric oxide and cellular stress response in brain aging and neurodegenerative disorders: the role of vitagenes. *In Vivo, 18*(3), 245-267.

Cao, G., Alessio, H. M., & Cutler, R. G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic Biol Med, 14*(3), 303-311.

Chang, L., & Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature, 410*(6824), 37-40.

Charoensin, S., Taya, S., Wongpornchai, S., & Wongpoomchai, R. (2012). Assessment of genotoxicity and antigenotoxicity of an aqueous extract of Cleistocalyx

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

nervosum var. paniala in in vitro and in vivo models. *Interdiscip Toxicol, 5*(4), 201-206.

Chaudhari, N., Talwar, P., Parimisetty, A., Lefebvre d'Hellencourt, C., & Ravanan, P. (2014a). A molecular web: endoplasmic reticulum stress, inflammation, and oxidative stress. *Front Cell Neurosci, 8*, 213.

Chaudhari, N., Talwar, P., Parimisetty, A., Lefebvre d'Hellencourt, C., & Ravanan, P. (2014b). A molecular web: endoplasmic reticulum stress, inflammation, and oxidative stress. *Front Cell Neurosci, 8*, 1-15.

Chen, G., Bower, K. A., Xu, M., Ding, M., Shi, X., Ke, Z. J., & Luo, J. (2009). Cyanidin-3-glucoside reverses ethanol-induced inhibition of neurite outgrowth: role of glycogen synthase kinase 3 Beta. *Neurotox Res, 15*, 321-331.

Chen, G., & Luo, J. (2010). Anthocyanins: are they beneficial in treating ethanol neurotoxicity? *Neurotox Res, 17*, 91-101.

Chen, G., Ma, C., Bower, K. A., Shi, X., Ke, Z. J., & Luo, J. (2008). Ethanol Promotes Endoplasmic Reticulum Stress-Induced Neuronal Death: Involvement of Oxidative Stress. *J Neurosci Res, 86*(4), 937-946.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Cheng, Z., He, W., Zhou, X., Lv, Q., Xu, X., Yang, S., Zhao, C., & Guo, L. (2011). Cordycepin protects against cerebral ischemia/reperfusion injury in vivo and in vitro. *Eur J Pharmacol, 664*(1-3), 20-28.

Chhunchha, B., Fatma, N., Kubo, E., Rai, P., Singh, S. P., & Singh, D. P. (2013). Curcumin abates hypoxia-induced oxidative stress based-ER stress-mediated cell death in mouse hippocampal cells (HT22) by controlling Prdx6 and NF-kappaB regulation. *Am J Physiol Cell Physiol, 304*(7), C636-655.

Choi, J. H., Choi, A. Y., Yoon, H., Choe, W., Yoon, K. S., Ha, J., Yeo, E. J., & Kang, I. (2010). Baicalein protects HT22 murine hippocampal neuronal cells against endoplasmic reticulum stress-induced apoptosis through inhibition of reactive oxygen species production and CHOP induction. *Exp Mol Med, 42*(12), 811-822.

Cullinan, S. B., & Diehl, J. A. (2006). Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. *Int J Biochem Cell Biol, 38*(3), 317-332.

DeGracia, D. J., Kumar, R., Owen, C. R., Krause, G. S., & White, B. C. (2002). Molecular pathways of protein synthesis inhibition during brain reperfusion: implications for neuronal survival or death. *J Cereb Blood Flow Metab, 22*(2), 127-141.

Di Giacomo, Claudia, Acquaviva, Rosaria, Santangelo, Rosa, Sorrenti, Valeria, Vanella, Luca, Li Volti, Giovanni, D'Orazio, Nicolantonio, Vanella, Angelo, & Galvano, Fabio. (2012). Effect of Treatment with Cyanidin-3-O-β-D-Glucoside on Rat

Chulalongkorn University

Ischemic/Reperfusion Brain Damage. *Evid Based Complement Alternat Med 2012*, 1-10.

Doyle, K. M., Kennedy, D., Gorman, A. M., Gupta, S., Healy, S. J., & Samali, A. (2011). Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. *J Cell Mol Med, 15*(10), 2025-2039.

Duran-Aniotz, Claudia, Martínez, Gabriela, & Hetz, Claudio. (2014). Memory loss in Alzheimer's disease: are the alterations in the UPR network involved in the cognitive impairment? *Front Aging Neurosci, 6*, 1-3.

Emerit, J., Edeas, M., & Bricaire, F. (2004). Neurodegenerative diseases and oxidative stress. *Biomed Pharmacother, 58*(1), 39-46.

Essa, M. M., Vijayan, R. K., Castellano-Gonzalez, G., Memon, M. A., Braidy, N., & Guillemin, G. J. (2012). Neuroprotective effect of natural products against Alzheimer's disease. *Neurochem Res, 37*(9), 1829-1842.

Fang, J. (2014). Bioavailability of anthocyanins. *Drug Metab Rev, 46*(4), 508-520.

Faria, A., Pestana, D., Teixeira, D., Azevedo, J., De Freitas, V., Mateus, N., & Calhau, C. (2010). Flavonoid transport across RBE4 cells: A blood-brain barrier model. *Cell Mol Biol Lett, 15*(2), 234-241.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Farooqui, T., & Farooqui, A. A. (2009). Aging: an important factor for the pathogenesis of neurodegenerative diseases. *Mech Ageing Dev, 130*(4), 203-215.

Finley, J. W., Kong, A. N., Hintze, K. J., Jeffery, E. H., Ji, L. L., & Lei, X. G. (2011). Antioxidants in foods: state of the science important to the food industry. *J Agric Food Chem, 59*(13), 6837-6846.

Fornasaro, Stefano, Ziberna, Lovro, Gasperotti, Mattia, Tramer, Federica, Vrhovšek, Urška, Mattivi, Fulvio, & Passamonti, Sabina. (2016). Determination of cyanidin 3-glucoside in rat brain, liver and kidneys by UPLC/MS-MS and its application to a short-term pharmacokinetic study. *Scientific Reports, 6*, 22815.

Fratantonio, D., Speciale, A., Ferrari, D., Cristani, M., Saija, A., & Cimino, F. (2015). Palmitate-induced endothelial dysfunction is attenuated by cyanidin-3-O-glucoside through modulation of Nrf2/Bach1 and NF-kappaB pathways. *Toxicol Lett, 239*(3), 152-160.

Fukui, M., Song, J. H., Choi, J., Choi, H. J., & Zhu, B. T. (2009). Mechanism of glutamate-induced neurotoxicity in HT22 mouse hippocampal cells. *Eur J Pharmacol, 617*(1-3), 1-11.

Fukumoto, L. R., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *J Agric Food Chem, 48*(8), 3597-3604.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Galehdar, Z., Swan, P., Fuerth, B., Callaghan, S. M., Park, D. S., & Cregan, S. P. (2010). Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. *J Neurosci, 30*(50), 16938-16948.

Ghosh, Arindam P., Klocke, Barbara J., Ballestas, Mary E., & Roth, Kevin A. (2012). CHOP Potentially Co-Operates with FOXO3a in Neuronal Cells to Regulate PUMA and BIM Expression in Response to ER Stress. *PLoS ONE, 7*(6), 1-11.

Gilgun-Sherki, Y., Melamed, E., & Offen, D. (2001). Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology, 40*(8), 959-975.

Granholm, A. C., Boger, H., & Emborg, M. E. (2008). Mood, memory and movement: an age-related neurodegenerative complex? *Curr Aging Sci, 1*(2), 133-139.

Greenwood, S. M., & Connolly, C. N. (2007). Dendritic and mitochondrial changes during glutamate excitotoxicity. *Neuropharmacology, 53*(8), 891-898.

Gross, A., McDonnell, J. M., & Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes Dev, 13*(15), 1899-1911.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Ha, J. S., & Park, S. S. (2006). Glutamate-induced oxidative stress, but not cell death, is largely dependent upon extracellular calcium in mouse neuronal HT22 cells. *Neurosci Lett, 393*(2-3), 165-169.

Heo, H.J., & Lee, C.Y. (2005). Strawberry and Its Anthocyanins Reduce Oxidative Stress-Induced Apoptosis in PC12 Cells. *J Agric Food Chem, 53*(6), 1984-1989.

http://laboratoryinfo.com/hplc/#prettyPhoto.).

Hui, L. (2015). Aging and chronic disease as independent causative factors for death and a programmed onset for chronic disease. *Arch Gerontol Geriatr, 60*(1), 178-182.

Hung, C. W., Chen, Y. C., Hsieh, W. L., Chiou, S. H., & Kao, C. L. (2010). Ageing and neurodegenerative diseases. *Ageing Res Rev, 9 Suppl 1*, S36-46.

Inboot, W., Taya, S., Chailungka, A., Meepowpan, P., & Wongpoomchai, R. (2012). Genotoxicity and antigenotoxicity of the methanol extract of Cleistocalyx nervosum var. paniala seed using a Salmonella mutation assay and rat liver micronucleus tests. *Mol Cell Toxicol, 8*(1), 19-24.

Jansom, C., Bhamarapravati, S. , & Itharat, A. (2008). Major anthocyanin from ripe berries of cleistocalyx nervosum var. paniala. *Thammasat Med J, 8*(3), 364-370.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Jeong, G. S., Byun, E., Li, B., Lee, D. S., Kim, Y. C., & An, R. B. (2010). Neuroprotective effects of constituents of the root bark of Dictamnus dasycarpus in mouse hippocampal cells. *Arch Pharm Res, 33*(8), 1269-1275.

Jia, Xiao, Y., Wang, W., Qing, L., Xu, Y., Song, H., Zhen, X., Ao, G., Alkayed, N. J., & Cheng, J. (2013). Differential mechanisms underlying neuroprotection of hydrogen sulfide donors against oxidative stress. *Neurochem Int, 62*(8), 1072-1078.

Jia, Zhu, H., Li, J., Wang, X., Misra, H., & Li, Y. (2012). Oxidative stress in spinal cord injury and antioxidant-based intervention. *Spinal Cord, 50*(4), 264-274.

Jin, M. L., Park, S. Y., Kim, Y. H., Oh, J. I., Lee, S. J., & Park, G. (2014). The neuroprotective effects of cordycepin inhibit glutamate-induced oxidative and ER stress-associated apoptosis in hippocampal HT22 cells. *Neurotoxicology, 41*, 102-111.

Kalt, W., Blumberg, J. B., McDonald, J. E., Vinqvist-Tymchuk, M. R., Fillmore, S. A., Graf, B. A., O'Leary, J. M., & Milbury, P. E. (2008). Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. *J Agric Food Chem, 56*(3), 705-712.

Kim, Geon Ha, Kim, Jieun E., Rhie, Sandy Jeong, & Yoon, Sujung. (2015). The Role of Oxidative Stress in Neurodegenerative Diseases. *Experimental Neurobiology, 24*(4), 325-340.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Kohri, S., Fujii, H., Oowada, S., Endoh, N., Sueishi, Y., Kusakabe, M., Shimmei, M., & Kotake, Y. (2009). An oxygen radical absorbance capacity-like assay that directly quantifies the antioxidant's scavenging capacity against AAPH-derived free radicals. *Anal Biochem, 386*(2), 167-171.

Kraft, A. D., Johnson, D. A., & Johnson, J. A. (2004). Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. *J Neurosci, 24*(5), 1101-1112.

Kritis, A. A., Stamoula, E. G., Paniskaki, K. A., & Vavilis, T. D. (2015). Researching glutamate - induced cytotoxicity in different cell lines: a comparative/collective analysis/study. *Front Cell Neurosci, 9*, 1-18.

Kropat, C., Mueller, D., Boettler, U., Zimmermann, K., Heiss, E. H., Dirsch, V. M., Rogoll, D., Melcher, R., Richling, E., & Marko, D. (2013). Modulation of Nrf2-dependent gene transcription by bilberry anthocyanins in vivo. *Mol Nutr Food Res, 57*(3), 545-550.

Lamkanfi, M., Kalai, M., & Vandenabeele, P. (2004). Caspase-12: an overview. *Cell Death Differ, 11*(4), 365-368.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Lee, Won, S. M., Suh, J., Son, S. J., Moon, G. J., Park, U. J., & Gwag, B. J. (2010). Induction of the unfolded protein response and cell death pathway in Alzheimer's disease, but not in aged Tg2576 mice. *Exp Mol Med, 42*(5), 386-394.

Lee, Jaewon, Jo, Dong-Gyu, Park, Daeui, Chung, Hae Young, & Mattson, Mark P. (2014). Adaptive Cellular Stress Pathways as Therapeutic Targets of Dietary Phytochemicals: Focus on the Nervous System. *Pharmacological Reviews, 66*(3), 815.

Leonarduzzi, G., Sottero, B., & Poli, G. (2010). Targeting tissue oxidative damage by means of cell signaling modulators: the antioxidant concept revisited. *Pharmacol Ther, 128*(2), 336-374.

Leong, P. K., Chiu, P. Y., Chen, N., Leung, H., & Ko, K. M. (2011). Schisandrin B elicits a glutathione antioxidant response and protects against apoptosis via the redox-sensitive ERK/Nrf2 pathway in AML12 hepatocytes. *Free Radic Res, 45*(4), 483-495.

Li, Jeong, G. S., Kang, D. G., Lee, H. S., & Kim, Y. C. (2009). Cytoprotective effects of lindenenyl acetate isolated from Lindera strychnifolia on mouse hippocampal HT22 cells. *Eur J Pharmacol, 614*(1-3), 58-65.

Li, O, W., Li, W., Jiang, Z. G., & Ghanbari, H. A. (2013). Oxidative stress and neurodegenerative disorders. *Int J Mol Sci, 14*(12), 24438-24475.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Li, J., O, W., Li, W., Jiang, Z. G., & Ghanbari, H. A. (2013). Oxidative stress and neurodegenerative disorders. *Int J Mol Sci, 14*(12), 24438-24475.

Liu, D., Zhang, M., & Yin, H. (2013). Signaling pathways involved in endoplasmic reticulum stress-induced neuronal apoptosis. *Int J Neurosci, 123*(3), 155-162.

Maher, P., & Davis, J. B. (1996). The role of monoamine metabolism in oxidative glutamate toxicity. *J Neurosci, 16*(20), 6394-6401.

Maher, P., & Schubert, D. (2000). Signaling by reactive oxygen species in the nervous system. *Cell Mol Life Sci, 57*(8-9), 1287-1305.

Mahmood, Tahrin, & Yang, Ping-Chang. (2012). Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences, 4*(9), 429-434.

Marczylo, T. H., Cooke, D., Brown, K., Steward, W. P., & Gescher, A. J. (2009). Pharmacokinetics and metabolism of the putative cancer chemopreventive agent cyanidin-3-glucoside in mice. *Cancer Chemother Pharmacol, 64*, 1261-1268.

Markesbery, W. R., & Carney, J. M. (1999). Oxidative alterations in Alzheimer's disease. *Brain Pathol, 9*(1), 133-146.

Mattson, Mark P., Chan, Sic L., & Duan, Wenzhen. (2002). Modification of Brain Aging and Neurodegenerative Disorders by Genes, Diet, and Behavior. *Physiological Reviews, 82*(3), 637.

Mazza, G., Kay, C. D., Cottrell, T., & Holub, B. J. (2002). Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J Agric Food Chem, 50*(26), 7731-7737.

McCubrey, J. A., Lahair, M. M., & Franklin, R. A. (2006). Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxid Redox Signal, 8*(9-10), 1775-1789.

Milbury, P. E., & Kalt, W. (2010). Xenobiotic metabolism and berry flavonoid transport across the blood-brain barrier. *J Agric Food Chem, 58*(7), 3950-3956.

Miller, M. G., & Shukitt-Hale, B. (2012). Berry fruit enhances beneficial signaling in the brain. *J Agric Food Chem, 60*(23), 5709-5715.

Momoi, T. (2004). Caspases involved in ER stress-mediated cell death. *J Chem Neuroanat, 28*(1-2), 101-105.

Mota, S. I., Costa, R. O., Ferreira, I. L., Santana, I., Caldeira, G. L., Padovano, C., Fonseca, A. C., Baldeiras, I., Cunha, C., Letra, L., Oliveira, C. R., Pereira, C. M., & Rego, A. C.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

(2015). Oxidative stress involving changes in Nrf2 and ER stress in early stages of Alzheimer's disease. *Biochim Biophys Acta, 1852*(7), 1428-1441.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., & Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature, 403*(6765), 98-103.

Nikitina, T. V., Nazarova, NIu, Tishchenko, L. I., Tuohimaa, P., & Sedova, V. M. (2003). [Use of the real-time RT-PCR method for investigation of small stable RNA expression level in human epidermoid carcinoma cells A431]. *Tsitologiia, 45*(4), 392-402.

Niture, S. K., Khatri, R., & Jaiswal, A. K. (2014). Regulation of Nrf2-an update. *Free Radic Biol Med, 66*, 36-44.

Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J.A., & Deemer, E.K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *J Agric Food Chem, 50*(11), 3122-3128.

Oyadomari, S., & Mori, M. (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ, 11*(4), 381-389.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Panee, J., Liu, W., Nakamura, K., & Berry, M. J. (2007). The responses of HT22 cells to the blockade of mitochondrial complexes and potential protective effect of selenium supplementation. *Int J Biol Sci, 3*(5), 335-341.

Patthamakanokporn, O., Puwastien, P., Nitithamyong, A., & Sirichakwal, P.P. (2008). Changes of antioxidant activity and total phenolic compounds during storage of selected fruits. *J Food Compost Anal, 21*(3), 241-248.

Poontawee, W., Natakankitkul, S., & Wongmekiat, O. (2016). Protective Effect of Cleistocalyx nervosum var. paniala Fruit Extract against Oxidative Renal Damage Caused by Cadmium. *Molecules, 21*(2), 1-13.

Pratico, D. (2008). Oxidative stress hypothesis in Alzheimer's disease: a reappraisal. *Trends Pharmacol Sci, 29*(12), 609-615.

Prior, R. L., & Wu, X. (2006). Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic Res, 40*(10), 1014-1028.

Rudy, C. C., Hunsberger, H. C., Weitzner, D. S., & Reed, M. N. (2015). The role of the tripartite glutamatergic synapse in the pathophysiology of Alzheimer's disease. *Aging Dis, 6*(2), 131-148.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Yoga Latha, L. (2011). Extraction, Isolation and Characterization of Bioactive Compounds from Plants'

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Extracts. *African Journal of Traditional, Complementary, and Alternative Medicines, 8*(1), 1-10.

Schonthal, A. H. (2012). Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. *Scientifica (Cairo), 2012*, 1-27.

Shah, S. A., Lee, H. Y., Bressan, R. A., Yun, D. J., & Kim, M. O. (2014). Novel osmotin attenuates glutamate-induced synaptic dysfunction and neurodegeneration via the JNK/PI3K/Akt pathway in postnatal rat brain. *Cell Death Dis, 5*, e1026.

Shetty, A. K., Hattiangady, B., Rao, M. S., & Shuai, B. (2011). Deafferentation enhances neurogenesis in the young and middle aged hippocampus but not in the aged hippocampus. *Hippocampus, 21*(6), 631-646.

Shih, Johnson, D. A., Wong, G., Kraft, A. D., Jiang, L., Erb, H., Johnson, J. A., & Murphy, T. H. (2003). Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J Neurosci, 23*(8), 3394-3406.

Shih, P. H., Yeh, C. T., & Yen, G. C. (2005). Effects of anthocyanidin on the inhibition of proliferation and induction of apoptosis in human gastric adenocarcinoma cells. *Food Chem Toxicol, 43*, 1557-1566.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Shiraishi, H., Okamoto, H., Yoshimura, A., & Yoshida, H. (2006). ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. *J Cell Sci, 119*(Pt 19), 3958-3966.

Slemmer, J. E., & Weber, J. T. (2014). Assessing Antioxidant Capacity in Brain Tissue: Methodologies and Limitations in Neuroprotective Strategies. *Antioxidants (Basel), 3*(4), 636-648.

Sriwanthana, B., Treesangsri, W. , Boriboontrakul, B. , Niumsakul, S., & Chavalittumrong, P. (2007). In vitro effects of Thai medicinal plants on human lymphocyte activity. *Songklanakarin J Sci Technol, 29*, 17-28.

Stanciu, M., & DeFranco, D. B. (2002). Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. *J Biol Chem, 277*(6), 4010-4017.

Sun, Z., Huang, Z., & Zhang, D. D. (2009a). Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response. *PLoS One, 4*(8), 1-9.

Sun, Z., Huang, Z., & Zhang, D. D. (2009b). Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response. *PLoS One, 4*(8), e6588.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Talavera, S., Felgines, C., Texier, O., Besson, C., Gil-Izquierdo, A., Lamaison, J. L., & Remesy, C. (2005). Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J Agric Food Chem, 53*(10), 3902-3908.

Tan, L., Yang, H. P., Pang, W., Lu, H., Hu, Y. D., Li, J., Lu, S. J., Zhang, W. Q., & Jiang, Y. G. (2014). Cyanidin-3-O-galactoside and blueberry extracts supplementation improves spatial memory and regulates hippocampal ERK expression in senescence-accelerated mice. *Biomed Environ Sci, 27*(3), 186-196.

Taya, S., Punvittayagul, C., Chewonarin, T., & Wongpoomchai, R. . (2009). Effect of aqueous extract from *Cleistocalyx nervosum* on oxidative status in rat liver. *Thai J Toxicol, 24*, 101-105.

Trachootham, Dunyaporn, Lu, Weiqin, Ogasawara, Marcia A., Valle, Nilsa Rivera-Del, & Huang, Peng. (2008). Redox Regulation of Cell Survival. *Antioxidants & Redox Signaling, 10*(8), 1343-1374.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol, 39*(1), 44-84.

Wada, T., & Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. *Oncogene, 23*(16), 2838-2849.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Wang, Chuikov, S., Taitano, S., Wu, Q., Rastogi, A., Tuck, S. J., Corey, J. M., Lundy, S. K., & Mao-Draayer, Y. (2015). Dimethyl Fumarate Protects Neural Stem/Progenitor Cells and Neurons from Oxidative Damage through Nrf2-ERK1/2 MAPK Pathway. *Int J Mol Sci, 16*(6), 13885-13907.

Wang, Wen, L. L., Huang, Y. N., Chen, Y. T., & Ku, M. C. (2006). Dual effects of antioxidants in neurodegeneration: direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation. *Curr Pharm Des, 12*(27), 3521-3533.

Weiner, M. W., Veitch, D. P., Aisen, P. S., Beckett, L. A., Cairns, N. J., Green, R. C., Harvey, D., Jack, C. R., Jagust, W., Liu, E., Morris, J. C., Petersen, R. C., Saykin, A. J., Schmidt, M. E., Shaw, L., Siuciak, J. A., Soares, H., Toga, A. W., & Trojanowski, J. Q. (2012). The Alzheimer's Disease Neuroimaging Initiative: a review of papers published since its inception. *Alzheimers Dement, 8*(1 Suppl), S1-68.

Xu, Mei, Bower, Kimberly A., Wang, Siying, Frank, Jacqueline A., Chen, Gang, Ding, Min, Wang, Shiow, Shi, Xianglin, Ke, Zunji, & Luo, Jia. (2010). Cyanidin-3-Glucoside inhibits ethanol-induced invasion of breast cancer cells overexpressing ErbB2. *Molecular Cancer, 9*(1), 285.

Yang, E. J., Lee, J. Y., Park, S. H., Lee, T., & Song, K. S. (2013). Neuroprotective effects of neolignans isolated from Magnoliae Cortex against glutamate-induced apoptotic stimuli in HT22 cells. *Food Chem Toxicol, 56*, 304-312.

Zhang, An, C., Gao, Y., Leak, R. K., Chen, J., & Zhang, F. (2013). Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Prog Neurobiol, 100*, 30-47.

Zhang, Q., Liu, J., Chen, S., Liu, J., Liu, L., Liu, G., Wang, F., Jiang, W., Zhang, C., Wang, S., & Yuan, X. (2016). Caspase-12 is involved in stretch-induced apoptosis mediated endoplasmic reticulum stress. *Apoptosis, 21*(4), 432-442.

Zhu, W., Jia, Q., Wang, Y., Zhang, Y., & Xia, M. (2012). The anthocyanin cyanidin-3-O-beta-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway. *Free Radic Biol Med, 52*(2), 314-327.

Zhu, X., Raina, A. K., Lee, H. G., Casadesus, G., Smith, M. A., & Perry, G. (2004). Oxidative stress signalling in Alzheimer's disease. *Brain Res, 1000*(1-2), 32-39.

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APPENDIX

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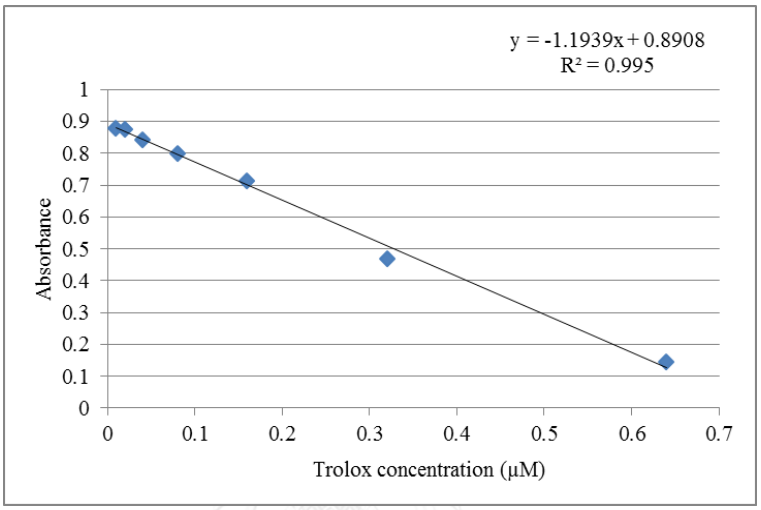
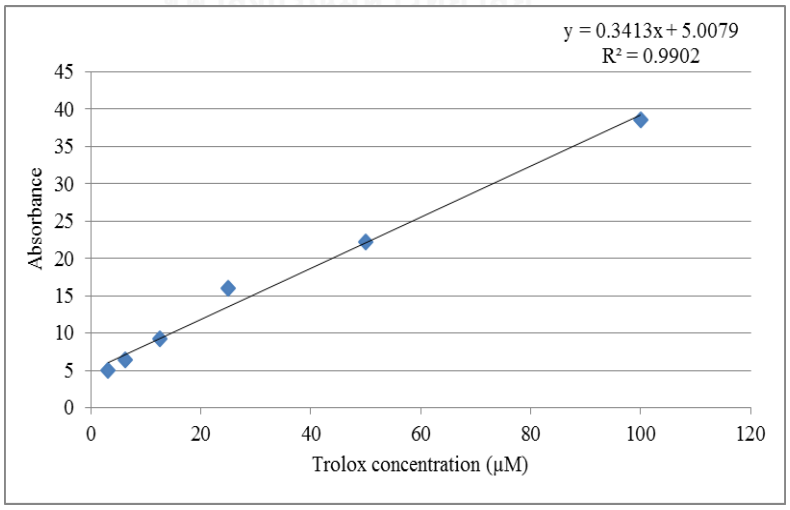
1. Standard calibration curve of antioxidant capacity assay

To use for calculation of the content of antioxidant values from the CNP crude extract.

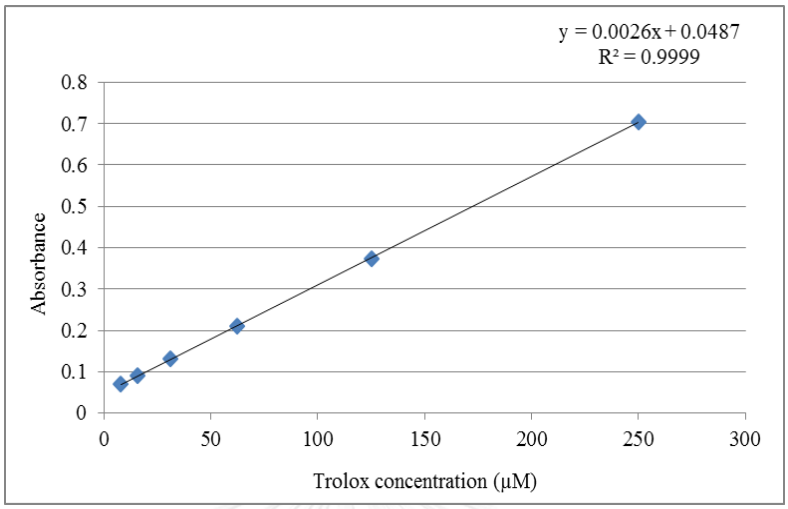
1.1 DPPH assay



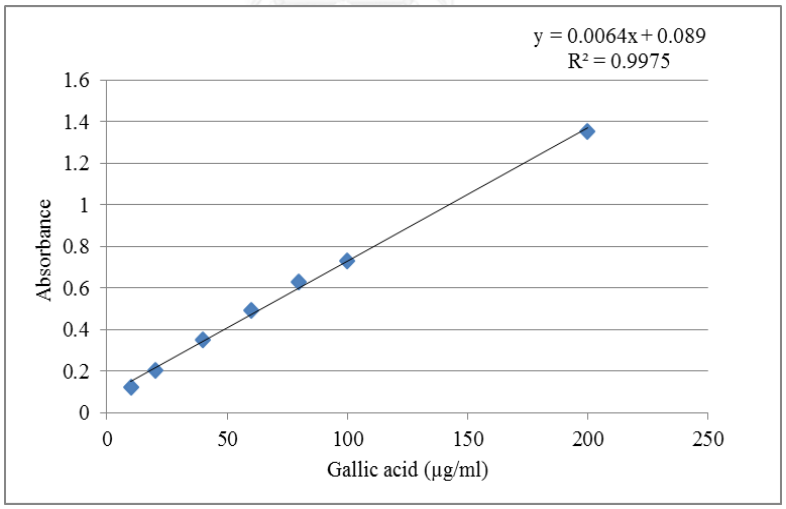
1.2 ORAC assay



1.3 FRAP assay

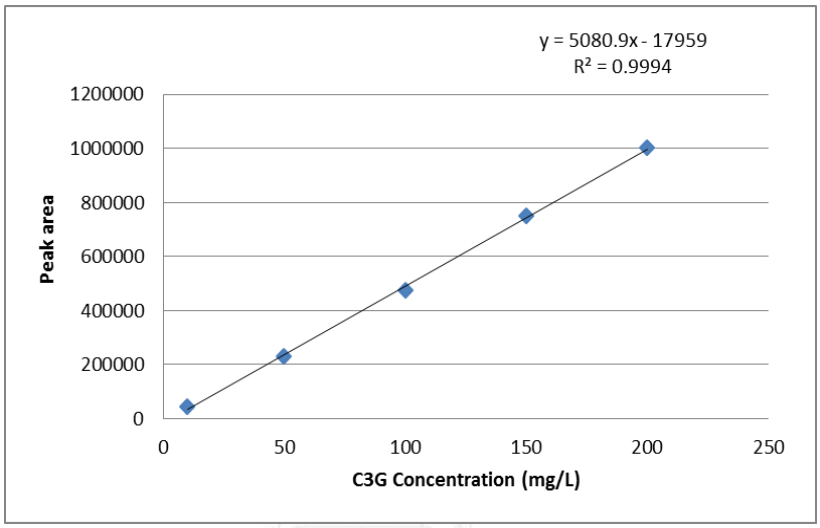


1.4 Total phenolic content

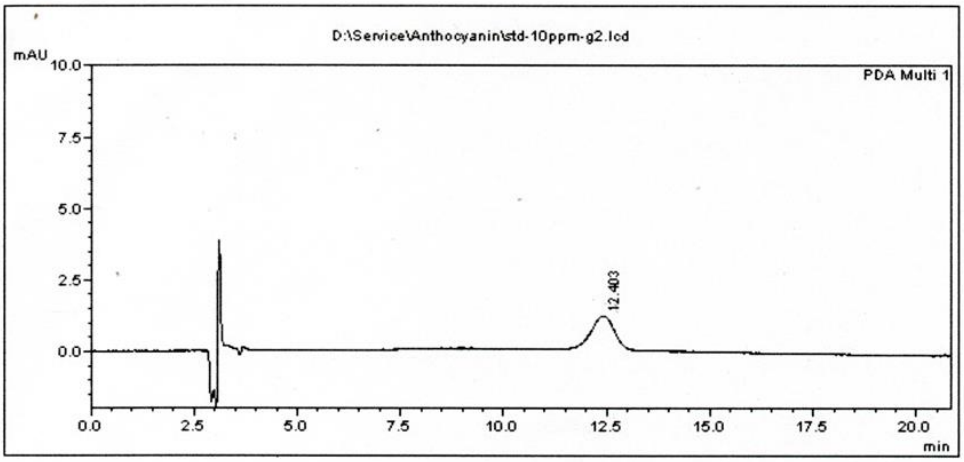


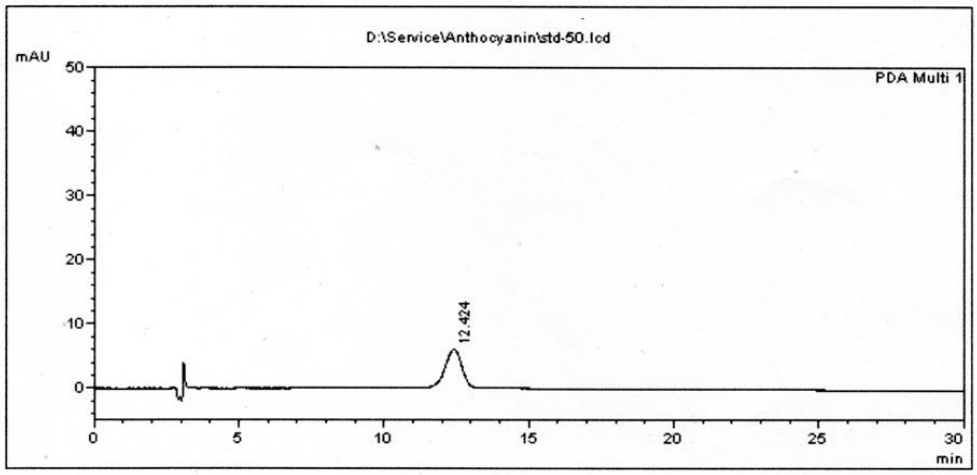
2. Standard calibration curve of HPLC analysis

To use for calculation of the standard marker, anthocyanin (C3G) content from the CNP crude extract.



2.1 Standard-C3G concentrations (0-200 mg/L) data graph from HPLC analysis

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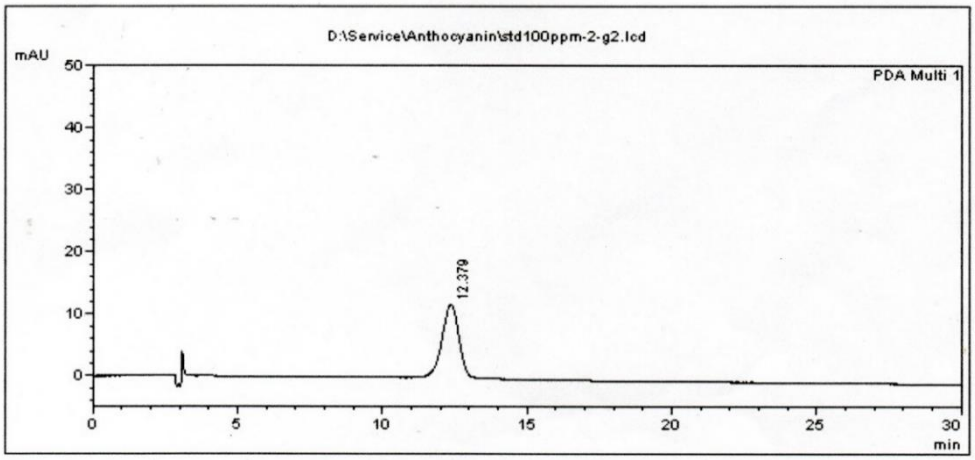
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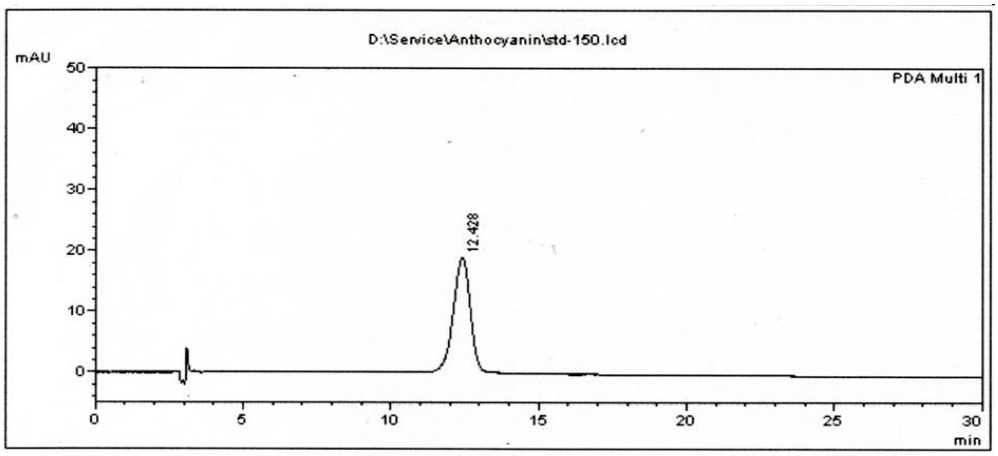
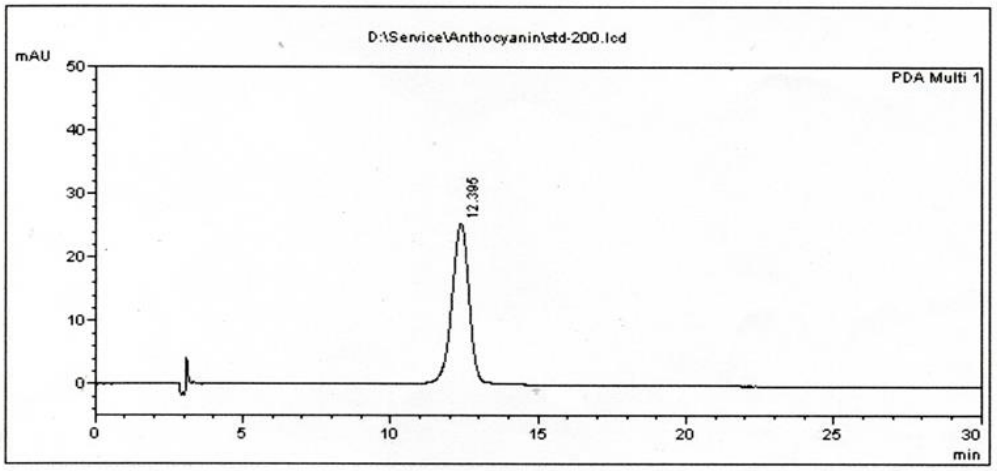
12.379
min


D:\Service\Anthocyanin\std-150.lcd
mAU
PDA Multi 1
12.428
min



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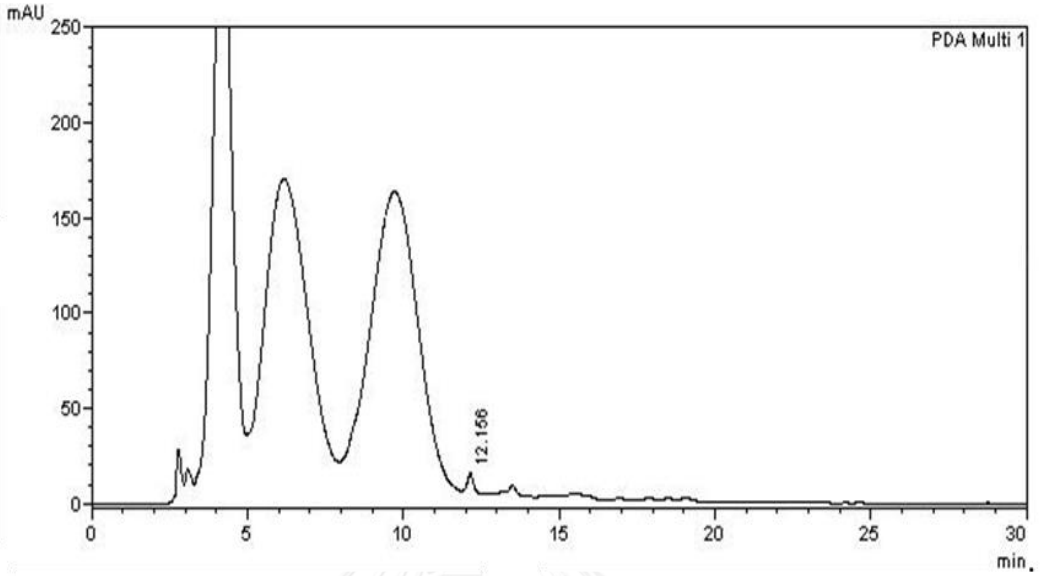
2.2 Spiked sample of CNP extract with reference standard (C3G) data graph from HPLC analysis



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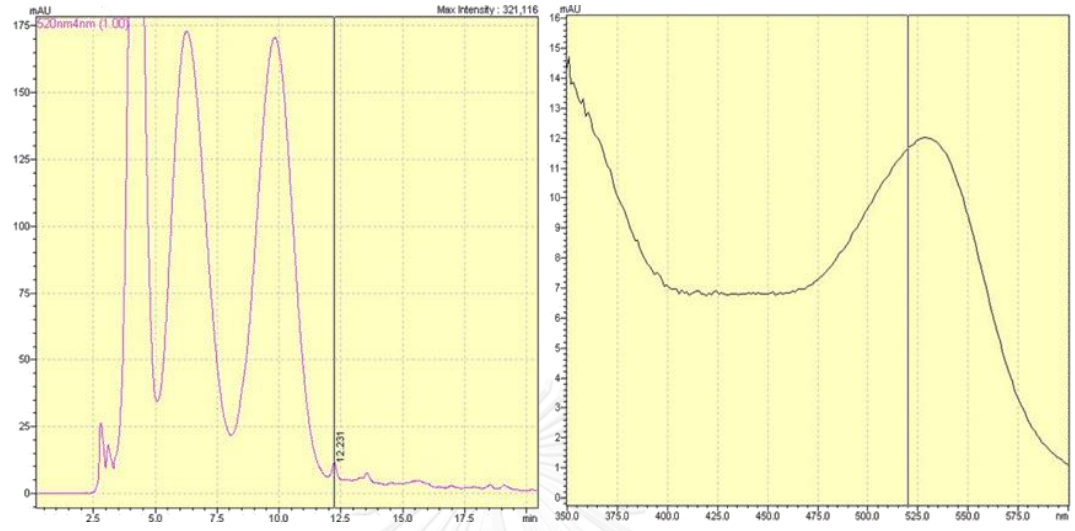
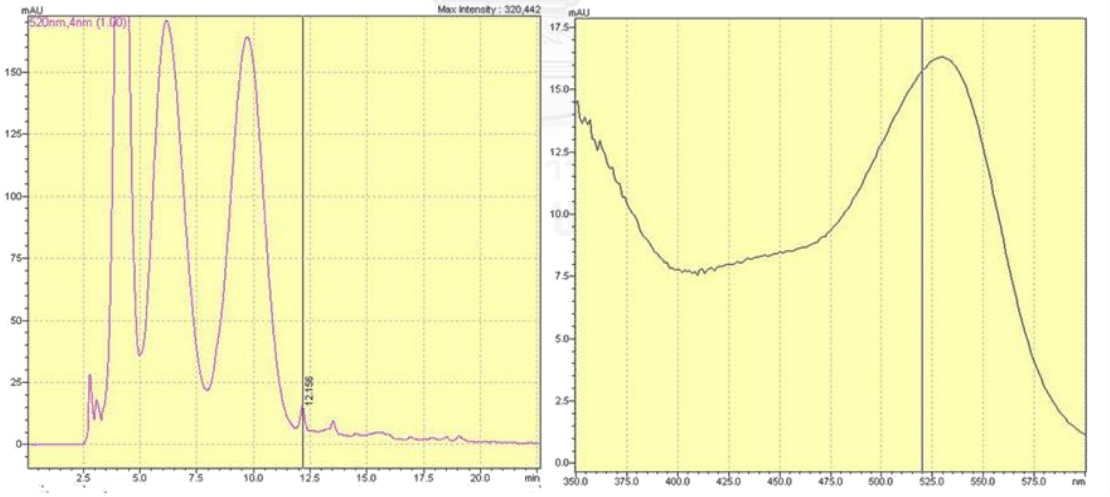
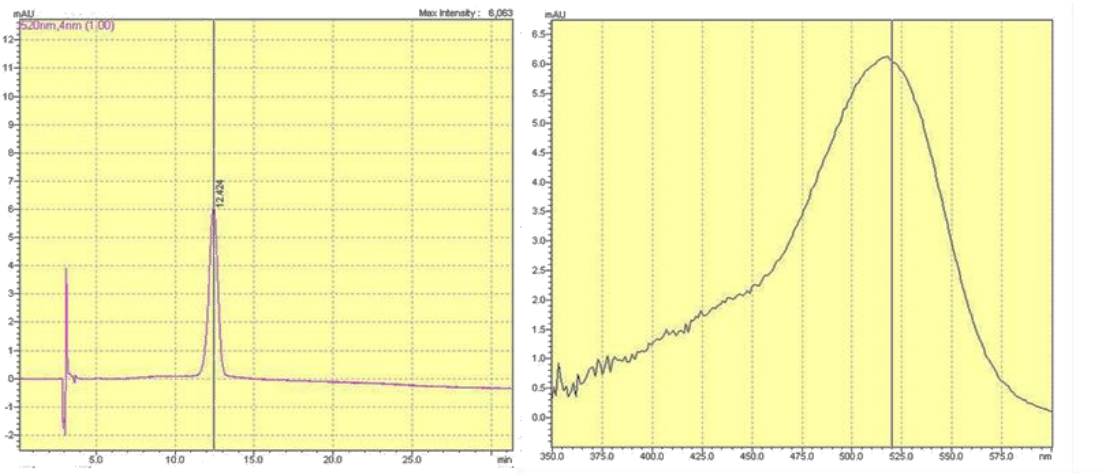
2.3 Confirmation identified sample by relation time and spectrum 520 nm with C3G-standard marker and CNP extract data graph from HPLC analysis.



CNP extract



Spiked CNP extract with reference standard (C3G)



Reference standard (C3G)

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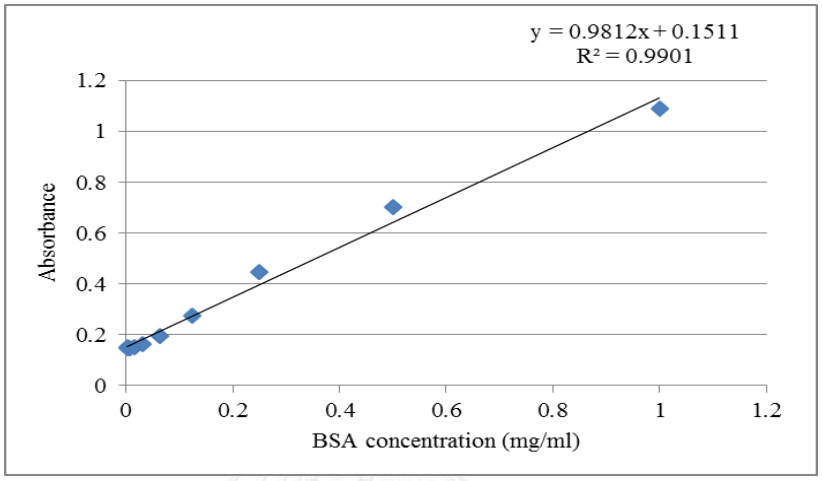
3. Standard calibration curve of total protein content by Bradford assay

To use for calculation of total protein content of the cell treatments for further Western blotting analysis.



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VITA

My name is Miss Monruedee Sukprasansap. I was born on September 3rd, 1980 in Maesod, Tak, Thailand. I graduated with bachelor degree (Food Science and Technology), King Mongkut's University of Technology North Bangkok, Thailand and obtained Master degree (Food and Nutritional Toxicology), Mahidol University, Bangkok, Thailand. After that I furthered study in doctoral degree program in Clinical Biochemistry and Molecular Medicine, Chulalongkorn University, Bangkok, Thailand. My field of Ph.D study is biochemistry and molecular mechanism of edible plant, especially in term of prevention of apoptosis cell death. I work as a researcher at Institute of Nutrition, Mahidol University (2008-present).

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