THE EFFECT OF NAC AND CARNOSINE ON ALZHEIMER'S DISEASE-RELATED *IN VITRO* MODEL THROUGH RAGE-AGE SIGNALING PATHWAY

Miss Suphasarang Sirirattanakul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Clinical Biochemistry and Molecular Medicine
Department of Clinical Chemistry
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2018

ผลของ NAC และ CARNOSINE ในแบบจำลองภายนอกร่างกายของโรคสมองเสื่อมอัลไซเมอร์ ผ่านวิถีสัญญาณของ RAGE-AGE

น.ส.สุภสรางค์ สิริรัตนากุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมีคลินิกและอณูทางการแพทย์ ภาควิชาเคมีคลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	THE EFFECT OF NAC AND CARNOSINE ON ALZHEIMER'S DISEASE-RELATED *IN VITRO* MODEL THROUGH RAGE-AGE SIGNALING PATHWAY
By	Miss Suphasarang Sirirattanakul
Field of Study	Clinical Biochemistry and Molecular Medicine
Thesis Advisor	Associate Professor RACHANA SANTIYANONT, Ph.D.

Accepted by the Faculty of Allied Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the Faculty of Allied Health Sciences

(Assistant Professor PALANEE AMMARANOND, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman

(Assistant Professor TEWIN TENCOMNAO, Ph.D.)

..... Thesis Advisor

(Associate Professor RACHANA SANTIYANONT, Ph.D.)

..... Examiner

(SUPANTITRA CHANPRASERT, Ph.D.)

..... Examiner

(Assistant Professor VIROJ BOONYARATANAKORNKIT, Ph.D.)

..... External Examiner

(Warunya Arunotayanun, Ph.D.)

สุภสรางค์ สิริรัตนากุล : ผลของ NAC และ CARNOSINE ในแบบจำลองภายนอกร่างกายของโรคสมองเสื่อมอัลไซเมอร์ ผ่านวิถีสัญญาณของ RAGE-AGE. (THE EFFECT OF NAC AND CARNOSINE ON ALZHEIMER'S DISEASE-RELATED *IN VITRO* MODEL THROUGH RAGE-AGE SIGNALING PATHWAY) อ.ที่ปรึกษาหลัก : รศ. ดร.รัชนา ศานติยานนท์

โรคอัลไซเมอร์ (Alzheimer's disease) เป็นโรคสมองเสื่อมชนิดหนึ่งที่พบได้บ่อยที่สุด ซึ่งพบมากขึ้นตามอายุ โดยจะมีการเสื่อมของเซลล์สมองทุกส่วน โดยเฉพาะอย่างยิ่งจะมีความผิดปกติเกี่ยวกับความจำ เมื่อเป็นแล้วจะไม่มีวันหาย นอกจากนั้นยังมีความผิดปกติในด้านอื่นๆ ซึ่งแตกต่างจากโรคสมองเสื่อมชนิดอื่น โดยนอกจากจะมีภาวะความจำเสื่อมแล้ว จะมีการสูญเสียสติปัญญาอย่างอื่นร่วมด้วย เช่น ไม่สามารถเรียนรู้สิ่งใหม่ๆ ขาดความคิดริเริ่ม เบื่อหน่ายในสิ่งที่เคยชอบ รวมทั้งจะสูญเสียความสามารถในการตัดสินใจ ความคิดที่เป็นเหตุผล การคำนวณ การประกอบกิจวัตรประจำวัน สมาธิ การรับรู้บุคคล สถานที่ เวลา อารมณ์ การใช้ภาษา และการเข้าสังคม เนื่องจากโรคอัลไซเมอร์ก่อให้เกิดผลกระทบรุนแรงหลายด้าน ทั้งต่อผู้ป่วย ครอบครัว และผู้ใกล้ชิด ดังนั้นผู้วิจัยจึงสนใจศึกษากลไกของการเกิดการเสื่อมทำลายของเซลล์ประสาท เมื่อเซลล์อยู่ในภาวะเครียดออกซิเดชันที่มีผลต่อการชักนำให้เกิดการอักเสบในเซลล์ประสาทผ่านทางตัวรับของ Advanced glycation end products (Receptor for Advanced glycation end products; RAGE) ที่ส่งผลต่อเนื่องไปทาง NF-kB และ MAPK signaling pathway กับความเชื่อมโยงของภาวะเครียดออกซิเดชัน จากการศึกษาพบว่า AGEs สามารถกระตุ้นการแสดงออกของ RAGE ให้เพิ่มขึ้น และจากกลไกนี้ส่งผลให้มีการเพิ่มขึ้นของภาวะเครียดออกซิเดชัน ซึ่งพบว่าทำให้ nuclear factor-kappa B (NF-**K**B) มีการส่งสัญญาณเพิ่มขึ้น เป็นผลให้มีการเพิ่มการแสดงออกของ pro-inflammatory cytokines ประกอบด้วย IL1β, IL6 และ TNFα นอกจากนี้ยังพบว่าการกระตุ้นนี้ทำให้เกิดการตายแบบ apoptosis ที่มากขึ้นด้วย จากการทดสอบสมุนไพร *F. ovata*, NAC และ Carnosine พบว่า *F. ovata* (ส่วนที่สกัดได้ด้วย petroleum ether และ methanol) 100 μg/mL สามารถลดการเกิด superoxide radical ได้ และยังพบว่า *F. ovata* (ส่วนที่สกัดได้ด้วย dichloromethane และ methanol) 100 μg/mL, NAC, และ Carnosine ลดการกระตุ้นการส่งสัญญาณผ่าน MAPK และลดการตายแบบ apoptosis ($p<0.05$). นอกจากนี้ *F. ovata* (ส่วนที่สกัดได้ด้วย petroleum ether, dichloromethane, และ ethanol) และ NAC ช่วยเพิ่มการเคลื่อนของของ NF**K**B จากไซโตพลาสมเข้าสู่นิวเคลียส ($p<0.05$).

สาขาวิชา	ชีวเคมีคลินิกและอณูทางการแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5576956437 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE
KEYWORD: Alzheimer's disease, oxidative stress, NAC, Carnosine, F. ovata, AGEs
Suphasarang Sirirattanakul : THE EFFECT OF NAC AND CARNOSINE ON ALZHEIMER'S DISEASE-RELATED *IN VITRO* MODEL THROUGH RAGE-AGE SIGNALING PATHWAY. Advisor: Assoc. Prof. RACHANA SANTIYANONT, Ph.D.

Alzheimer's disease (AD) is the most common dementia which found increasing with age. It involves neurodegeneration which leads to various abnormalities especially memory loss, inability to learn new things, lack of creativity, lack of decision making ability, lack of critical thinking, calculation inability, inability to live normal daily life, inability to recognize person, time and place, emotional disturbance, and difficulty to use language. Since AD has severe impact in various aspects on both the patients and their families, so we are interested to study the neurodegenerative mechanism when neuronal cells are under oxidative stress which will induce neuroinflammation through the binding of advanced glycation endproducts (AGEs) and its receptor (receptor for advanced glycation end products; RAGE) via NF-kB and MAPK signaling pathway and integration of oxidative stress. We found that upon exposure of the optimized AGEs, RAGE expression was increased. In response to this activation, accumulation of ROS was also enhanced. The activation of nuclear factor-kappa B (NF-**κ**B) which is a primary transcription factor that plays an important role in regulating cellular responses was triggered. Hence, pro-inflammatory cytokines including IL1β, IL6 and TNFα were increased. In addition, increase in program cell death is likely to follow this activation. In this study, *Fimbristylis ovata* (*F. ovata)* (petroleum ether and methanol extract) 100 μg/mL was found to be able to reduce superoxide radical. Moreover, *F. ovata* (dichloromethane and methanol extract) 100 μg/mL, NAC, and Carnosine were found to decrease MAPK activation and cell apoptosis ($p<0.05$). In addition, *F. ovata* (petroleum ether, dichloromethane, and ethanol extract) and NAC were revealed to enhance NF**κ**B translocation ($p<0.05$).

Field of Study:	Clinical Biochemistry and Molecular Medicine	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

ACKNOWLEDGEMENTS

The successful of my thesis is dedicated to my beloved family. All best gratefulness to my advisor, Associate Professor Dr. Rachana Santiyanont, and my co-advisor, Assistant Professor Dr. Tewin Tencomnao for giving me an opportunity to be a part of this research, their support, advice, and encouragement during the entire of my work. Moreover, I would like to thank Dr. Supantitra Chanprasert, a chairman of the thesis committee, Dr. Warunya Arunotayanun and Assistant Professor Dr. Viroj Boonyaratanakornkit, the committee members, for their kindness, time, guidance and recommendations thorough my thesis.

In addition, I would like to thanks Faculty of Allied Health Sciences, Chulalongkorn University for supporting facilities. Second, I would like to thanks The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) for research funding. Lastly, I would like to thanks Chulalongkorn University Graduate Scholarship to Commemorate The 72nd Anniversary of His Majesty King Bhumibol Adulyadej.

Officially done with my thesis, I'm truly grateful to all my teachers, friends, sisters and brothers for recommending and helping me complete my work. Special thanks to my beloved family for supporting and encouraging me to keep going through the ups and downs during the study. My thesis would not be possible without support from all of you.

Suphasarang Sirirattanakul

TABLE OF CONTENTS

Page

CHAPTER I
INTRODUCTION

Background and Rationale

Alzheimer's disease (AD) is the most common dementia which found increasing with age. It involves neurodegeneration which leads to various abnormalities especially memory loss, inability to learn new things, lack of creativity, lack of decision making ability, lack of critical thinking, calculation inability, inability to live normal daily life, inability to recognize person, time and place, emotional disturbance, and difficulty to use language. In the United States, more than 3-4 million people living with AD were estimated and the incidence will continuously increase owing to the longer life span of people. AD was found 2-4% in population with age more than 60 years old and doubly increase in people after 60 years old (1). In Thailand, Health System Research Institute reported more than 7 million aging people of over 60 years old and forecasted that the incidence of dementia was 2.1 million, among these, more than 1 million have AD (2).

At present, the causes of AD are still unclear, there were many hypotheses that explained causes of AD, and the most cited hypothesis is Amyloid hypothesis which suggested that accumulation of Amyloid-β protein (Aβ protein) is the principal cause of AD development. This theory is interesting since Amyloid protein precursor (APP) resides at chromosome No.21 and people who have one more of chromosome 21 (trisomy 21) which have Down Syndrome will express symptoms of AD earlier than normal, at age about 40 years old. In addition, Apolipoprotein E4 (ApoE4) gene which is genetic risk factor of AD can largely increase the production of Aβ protein in brain

before the onset of AD. Thus, it is believed that accumulation of Aβ protein leads to clinical symptoms of AD (3). Another supported evidence came from animal study in mice with mutated gene of Aβ precursor protein (APP) which produced large amount of Aβ protein and developed amyloid plaques in brain giving AD-resembled pathology in brain (4). Production of Aβ protein started from APP which are cleaved by the enzymes β-secretase and γ-secretase. Recent studies explained the role of oxidative stress which increases with age as an important cause of AD development. AD is an example of pathology caused from antioxidant imbalance with supporting evidence that AD patients have less antioxidants in brain and high oxidative stress (5, 6). At present, there is no drug or treatment that can prevent or cure AD, thus it is important to study pathogenesis mechanism of AD and exploring substance that can inhibit this mechanism.

Since AD has severe impact in various aspects on both the patients and their families, so we are interested to study the neurodegenerative mechanism when neuronal cells are under oxidative stress which will induce neuroinflammation through the binding of advanced glycation end products (AGEs) and its receptor (receptor for advanced glycation end products; RAGE) via NF-kB signaling pathway and integration of oxidative stress and production of Aβ protein through amyloidogenic pathway. The injuries caused by oxidative stress occur as a result of the imbalance between pro-oxidants and anti-oxidants leading to the accumulation of cytotoxic compounds and their counterpart such as reactive oxygen species (ROS). Antioxidant compounds are raising agent which believed to play a part in rescuing Alzheimer's disease progression. N-acetyl cysteine (NAC) is a precursor of glutathione (GSH), an antioxidant molecule which scavenges reactive oxygen species (ROS). Carnosine, a natural occurring

compound which acts as an antioxidant, a metal-chelator, an anti-glycation agent, as well as inhibitor of Aβ toxicity. These two compounds may significantly augment the clinical efficacy on Alzheimer's disease treatment through their detoxifying properties. Therefore, the aim of this study was to define whether NAC and carnosine exerted part of their efficacy by suppressing oxidative stress level, then down-regulate the expression of pro-inflammatory genes in the Alzheimer's disease-related in vitro model through the regulation of RAGE-AGE pathway.

Previous studies in cultured monocytes and epithelial cells showed that when both cell types were treated with AGEs to induce oxidative stress, the binding of AGEs and RAGE will increase which then activate signaling through NF-kB pathway then increase gene expression of pro-inflammatory cytokines. *Fimbristylis ovata (F. ovata),* a grass-like leaves can significantly decrease gene expression as well as protein production from those genes in both cell types thus can decrease or prevent inflammation in those cells (7). Since there has never been report on *Fimbristylis ovata* study in cultured neuronal cells and it is likely that this extract can protect neuronal cells from oxidative stress and its downstream signaling regarding neuroinflammation, thus we interested to study the effect of *Fimbristylis ovata* extract on this regard. As a consequence, the results from this study give a new insight to understand the mechanism of NAC, carnosine and *Fimbristylis ovata* extract on anti-oxidation and inhibition of inflammation under oxidative stress condition through AGEs enhancement in SH-SY5Y neuroblastoma cell line.

Objectives

1. To investigate the possible mechanism of AGEs in the Alzheimer's disease-related *in vitro* model through the regulation of RAGE-AGE pathway
2. To investigate abilities of the selected antioxidant compounds against AGEs-induced Alzheimer's disease-related mechanism

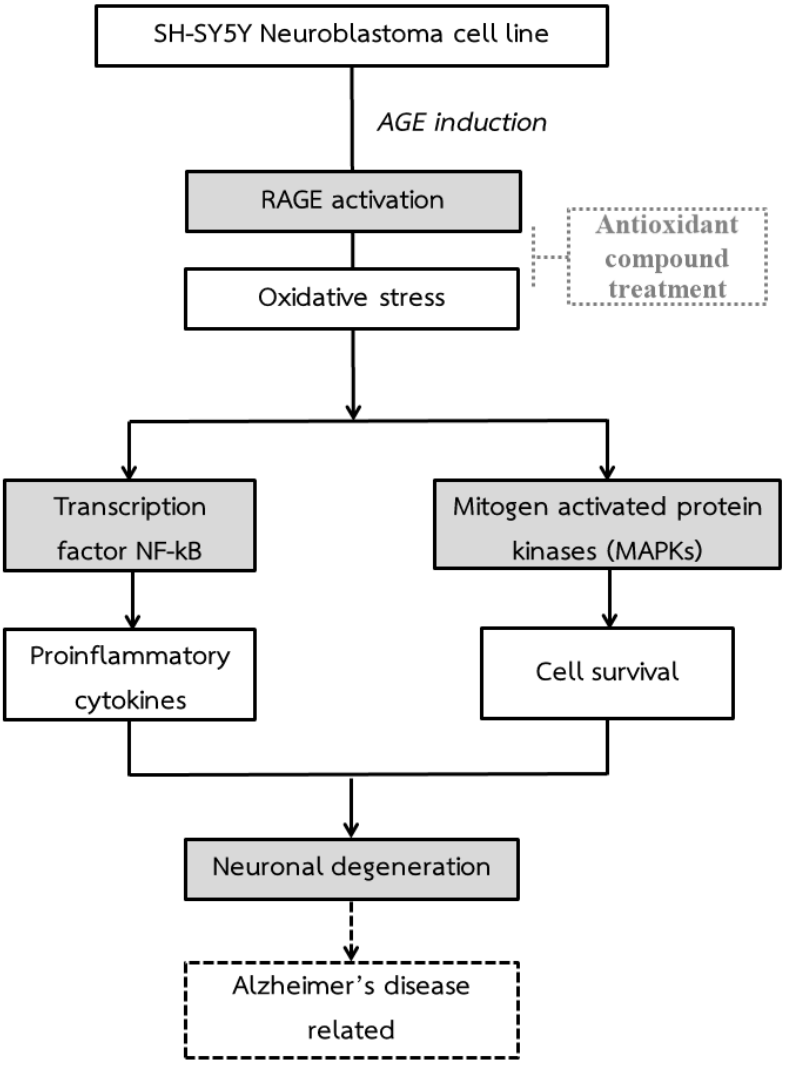
Hypotheses

1. AGEs induce Alzheimer-like disease through the regulation of RAGE-AGE pathway in the *in vitro* model
2. Selected antioxidant compounds alleviate the Alzheimer-like disease induced by AGEs through the regulation of RAGE-AGE pathway in the *in vitro* model

Expected Benefits and Application

This study will delineate understanding about pathophysiology of Alzheimer's disease based on inflammation, oxidative stress and the RAGE-AGE signaling pathway. The results from this study may have the potential to open possibilities for new treatments of Alzheimer's disease by targeting the above mentioned pathways, as well; the results from Alzheimer's disease-associated RAGE-AGE pathway may lead to the development of new biomarkers for clinical diagnosis which will be beneficial for the early prevention of the disease.

Conceptual framework



CHAPTER II
REVIEW OF THE RELATED LITERATURES

Alzheimer's Disease

Alzheimer's disease, an age-related neurodegenerative disorder, is the most common form of dementia. In 2010, it was estimated that 35.6 million people worldwide living with dementia, particularly in older people. Prevalence and incidence projections indicate that the number of people with dementia will be increasing over time, with prediction of 115.4 million people being affected in 2050 (8). Neurodegenerative disorders are characterized by progressive and irreversible loss of neurons from specific brain region. Neuronal dysfunction and cell loss in Alzheimer's disease are most vulnerable in the medial temporal lobe including entorhinal cortex and hippocampus. Since these brain regions involved in forming memories, the most common initial symptom of Alzheimer's disease is episodic memory loss (e.g. misplaced items, difficulty remembering recent conversations, names or events, forgotten details of daily life). As neurons malfunction in other parts of the brain are occurred, individuals experience other difficulties (e.g. difficulty completing familiar tasks, problems with words in speaking or writing, changes in mood and personality, including apathy and depression). Various hypotheses have been proposed for Alzheimer's pathogenesis including amyloid cascade, excitotoxicity, mitochondrial dysfunction, oxidative stress, and inflammation hypothesis (9-14). However, none of these hypotheses clearly describes for all aspects of the actual pathological processes underlying AD causality. Currently, there are no available clinical treatments that halt or reverse the progression of the disease, which worsens as it progresses, and

eventually leads to death. Continuing efforts on clinical efficacy are still required. This includes developing new drug target or compound that would prevent, stop, or slow progression of the Alzheimer's disease from occurring.

Pathophysiology of Alzheimer's disease is characterized by the presence of extracellular neuritic plaques containing amyloid-β (Aβ) fibril deposit and intracellular neurofibrillary tangles composed of the hyperphosphorylated microtubule-associated protein tau which lead to impaired synaptic function and neurons injury (10). Aβ deposition, a biomarker related to this early pathophysiological process, is a secreted protein which was resulting from the proteolytic processing of amyloid precursor protein (APP) involving proteolytic enzyme β and γ secretase. APP is a cell surface protein with a single transmembrane domain. Aβ portion of the protein is located at the cell surface with part of transmembrane domain. Almost all peripheral cells express APP and the production of Aβ is a normal metabolic event influenced by the developmental and physiological state of the cells. There are two potential pathways of the APP proteolytic process; nonamyloidogenic and amyloidogenic pathway (figure 1). Both pathways begin with an extracellular cleavage, followed by an intramembranous cleavage. The proteolytic event at the extracellular domain will determined the APP pathway. If the cleavage occurs at the α-secretase site within the Aβ domain, then Aβ is not generated (the nonamyloidogenic pathway). On the other hand, APP can undergo cleavage by β-secretase (beta-site amyloid precursor protein–cleaving enzyme 1; BACE1), which cuts at the N-terminal region of the Aβ sequence to produce Aβ (amyloidogenic pathway). Following the APP cleavage at either of these sites, an intramembranous cleavage at the C-terminus of Aβ by the γ-secretase

complex (containing presenilin, nicastrin, Pen-2, and Aph-1) will be carried out. In the nonamyloidogenic pathway, a secreted sAPPα and an 83-residue carboxy-terminal APP fragment (C83) will be created upon the first cut. C83 is then cleaved by γ-secretase to produce extracellular peptide P3 and the APP intracellular domain (AICD) which further translocate to the nucleus to trigger transcription activation. In contrast, during a first cut of the amyloidogenic pathway, a secreted sAPPβ and a 99-residue carboxy-terminal APP fragment (C99) will be formed. After that, Aβ and AICD will be generated (11, 15, 16).

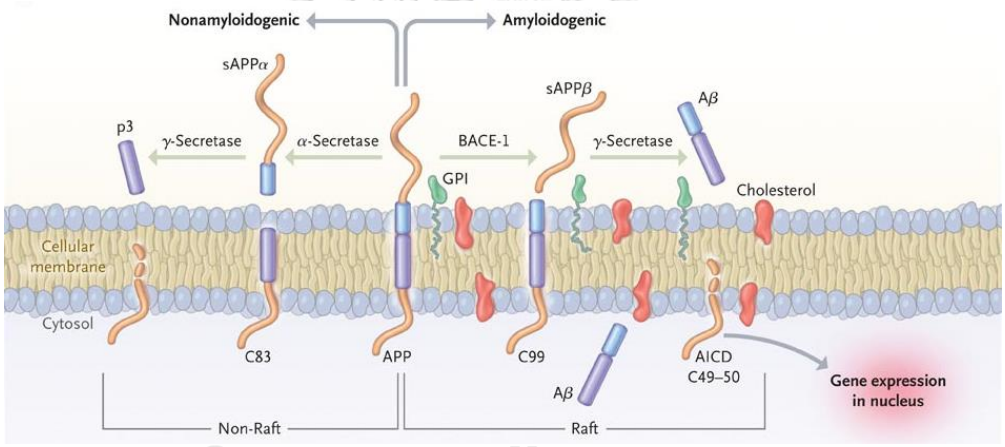


Figure 1 The proteolytic processing of amyloid precursor protein (APP) (16)

The Advanced Glycation End Products (AGEs) and the Receptor for Advanced Glycation End Products (RAGE)

Advanced glycation end products (AGEs) are reactive derivatives of the nonenzymatic glycation of proteins, lipids, and nucleic acids known as the Maillard reaction. In the classical pathway of AGEs formation (figure 2), reaction between aldehyde groups of reducing sugars (R-CHO) and amino groups of proteins (R'-NH_2) initiates the reversible formation of a Schiff base. This imine product (R-CH=N-R') is then rearrange to form a more stable Amadori product (R-CH_2-NH-R'). Multiple rearrangements cause alteration in the sugar portion resulting in the irreversible formation of AGEs which has ability to crosslink with proteins leading to structural perturbation in the proteins. Alternatively, AGEs can be created via glycolytic intermediate compounds such as highly reactive dicarbonyl compounds (3-deoxyglucosome, methylglyoxal and glyoxal). AGEs formation through this pathway requires several mechanisms including decomposition of phosphorylated intermediates and oxidative degradation of Amadori products (17, 18). AGEs formation has been known to progress in normal aging process. The generation of AGEs triggers several pathological processes involving inflammatory and oxidative stress pathway which are believed to play a pivotal role in various degenerative diseases, for example, diabetes mellitus, skin aging, diabetic neuropathy, and Alzheimer's disease (17-20).

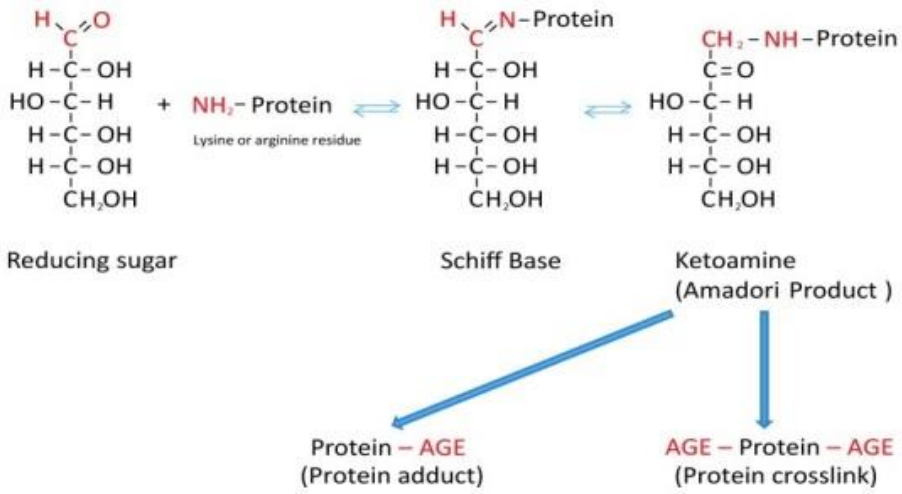


Figure 2 Schematic presentation of the Maillard reaction (18)

In addition to be protein adduct and protein crosslink, AGEs can act as a ligand to trigger their specific receptor. The receptor for advanced glycation end products (RAGE or AGER) is a major receptor for AGEs. There are other cell surface receptors for AGEs such as dolichyl-diphosphooligosaccharide-protein glycosyltransferase (AGE-R1), protein kinase C substrate, 80KH phosphoprotein (AGE-R2), galectin-3 (AGE-R3), and class A macrophage scavenger receptors type I and II (21). RAGE is a multi-ligand receptor which can bind various molecules such as AGEs, S-100 family of calcium binding proteins, high motility group protein B1 (HMGB1; amphoterine), and Aβ peptides (22, 23). The interaction between AGEs and RAGE activates a number of signaling pathway in different cell types including p38 mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-**K**B), CDC42/Rac, SAPK/JNK, and JAK/STAT pathways (21). Moreover, AGEs regulation involving intracellular ROS generation was found to enhance RAGE expression, create positive feedback loops of AGEs signaling, and subsequently induce inflammatory reactions (24). Hence, the therapeutic strategy aiming to alleviate AGEs formation, AGEs/RAGE activation, and/or RAGE downstream pathway might be helpful for several diseases.

AGEs, RAGE, and Alzheimer's Disease

The brain is considered as a particularly place vulnerable to oxidative injury due to its high oxygen utilization. Mitochondria are a major source of free radical in the brain as a byproduct of energy production. Alteration in mitochondria function leads to increase oxidative stress levels. Moreover, neurotransmitter activity also generates free radicals by auto-oxidation of dopamine and excitotoxicity related to glutamatergic signaling (25, 26). Thus, brain is a place where confounded by the

generation of free radicals, and insufficient antioxidant defense mechanism. The excessive of free radicals and disturbance of antioxidant defense mechanism lead to neurotoxicity. As a consequence of cellular injury and degeneration, neuronal cells undergo apoptotic event. AGEs may also employ their part in neurotoxicity through the capable of oxidative stress induction. Additionally, previous studies demonstrated that AGEs levels were higher in the brain of Alzheimer's patients compared to the normal one, and also contribute to Aβ aggregation and plaque deposition (27, 28). The previous studies demonstrated that the presence of AGEs in plaque enriched fractions isolated from frontal cortex samples of AD brains contained 8.9 ± 1.4 AGE units/mg of protein which significantly about 3-fold higher than corresponding preparations from healthy controls.

RAGE is a member of the immunoglobulin superfamily of cell surface protein expressed by neurons, microglia, astrocytes, cerebral endothelial cells, pericytes, and smooth muscle cells. Besides being a receptor for AGEs, RAGE acts as a multi-ligand receptor that binds a number of other ligands which include Aβ (22, 23, 29, 30). Growing evidence supports that RAGE involved in various pathogenesis of neurological diseases such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, and neurological complications of diabetes (31-37). The central player of RAGE activation in contributing to Alzheimer's disease is Aβ leading to different cellular signaling pathway. The major feature of this event is NF-**K**B and SP1 elements which are located on RAGE gene promoter (38). These transcription elements are responding to proinflammatory cellular signaling by increased RAGE expression. Hence, a positive feedback mechanism driven by cell-surface RAGE/ligand interaction is set up. The effects of the RAGE/ligand mechanism on a specific disease are perhaps

depended on the binding ligands. Moreover, a number of reports have been demonstrated that RAGE can mediate transport of Aβ across the blood-brain barrier (BBB) (32, 39-42). As a result, transportation of the circulating Aβ across the BBB also enhances Aβ accumulation in the brain and in the cerebral vasculature. Although Aβ is the major ligand for RAGE/ligand interaction in Alzheimer's disease, various work also point that RAGE/AGE interaction might exert its role on Alzheimer's disease pathology (43-46). It is important to note that additional study is needed before this pathological stage of the disease can be identified that may lead to the development of new biomarkers for clinical diagnosis which will be beneficial for the early prevention of the disease.

N-Acetyl Cysteine (NAC)

Amino acids produced by digestion of dietary proteins are absorbed through intestinal epithelial cells and enter blood stream. Various cell types take up these amino acids, and enter their cellular pools. They are used for the synthesis of proteins and other nitrogen-containing compounds including neurotransmitters. Cysteine is one of the ten amino acid which can be produced from glucose, however required its sulfur atom from essential amino acid. The carbons and nitrogen for cysteine synthesis are provided by glucose through serine formation, and the sulfur is provided by the essential amino acid methionine. Serine reacts with homocysteine produced from methionine to form cystathionine. Then, cystathionine is cleaved by cystathionase to form cysteine (figure 3). Because cysteine derives its sulfur from the essential amino acid methionine, cysteine production is limited by the availability of methionine. Moreover, the deficiency of enzyme in this pathway, and the inadequate dietary intake

of folate or B12 lead to the defect in methionine degradation to cysteine (47, 48). Hence, the availability of cysteine is very limited.

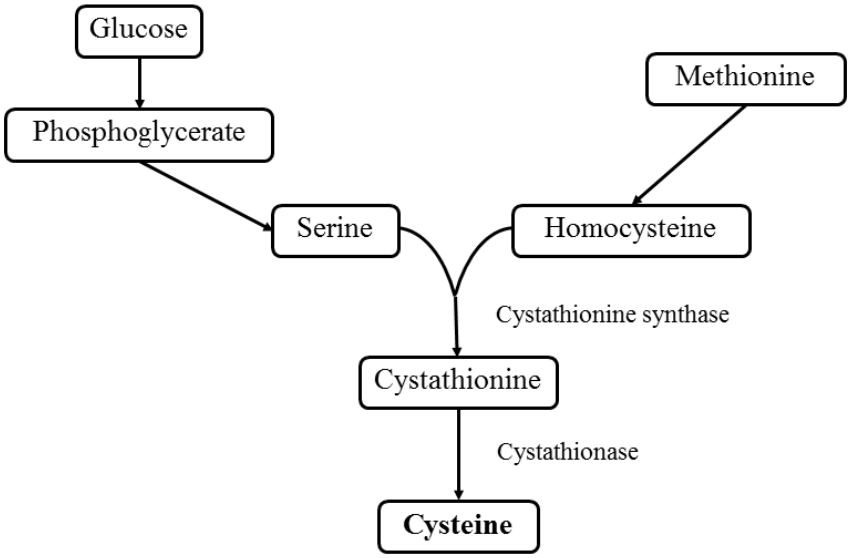


Figure 3 The synthesis of cysteine

N-Acetyl Cysteine (NAC), a dietary supplement, is a precursor to the amino acid cysteine. Through the pharmacokinetic studies on NAC obtained from various formulations of administration and dose regimens, they found that the plasma levels of NAC are rapidly decline, and only low levels of oxidized NAC are detectable for several hours after administration, suggesting that NAC itself does not accumulate in the body, but rather its oxidized forms and other metabolites (49-51). NAC plays two important metabolic roles. First, exposure to NAC leads to a large increase in intracellular glutathione (GSH) production which participates in the general antioxidant activities of the body (52). Second, NAC plays a crucial role as a modulator of the glutamatergic system by supplying cystine (53). NAC is a membrane-permeable cysteine precursor that does not require active transport to deliver cysteine into the cell (54). Once it enters the cell, NAC is rapidly hydrolyzed to release cysteine, a

precursor of GSH. The intracellular synthesis of GSH (γ- glutamyl-cysteine-glycine) is mainly regulated by 2 consecutive processes; (i) γ- glutamylcysteine synthetase (glutamate-cysteine ligase), and (ii) GSH synthetase. GSH is a reducing agent (electron donor) necessary for the removal of H_2O_2 and lipid peroxides created by reactive oxygen species (ROS), as a consequence, oxidized form of GSH (glutathione disulfide; GSSG) is generated. GSSG can be reduced back by glutathione reductase activity requiring NADPH as cofactor (figure 4) (54). Cysteine is usually a limiting precursor, and the synthesis of GSH is limited by the availability of its substrates. Thus, NAC could serve as an antioxidant and a free radical scavenger by increasing intracellular GSH. In addition to its role as an antioxidant agent, NAC supplies cysteine as a substrate for the cystine/glutamate antiporter or x(c)-system (55, 56). Through the cystine/cysteine cycle, a part of cysteine is oxidized to cystine. Cystine acts through the cystine/glutamate antiporter, by which shuttles glutamate out of the cell in exchange for cystine. The regulation of glutamate synthesis influences the reward-reinforcement pathway which is implicated in various psychiatric and neurodegenerative disorders including Alzheimer's disease (53, 57-60). As a consequence, NAC could serve its role as a modulator of the glutamatergic system.

NAC and Alzheimer's Disease

The major endogenous antioxidant molecule in the brain is GSH (61-64). GSH, a cysteine derivative, is a very efficient redox scavenger. GSH plays a role as an oxidative damage protection by removing H_2O_2 and lipid peroxides generated via ROS which are produced during normal metabolism. GSH can be formed inside cells from the amino acids cysteine, glutamate (derived from α-ketoglutarate mediated

tricarboxylic acid (TCA) cycle), and glycine (primarily produced by serine but can also be produced from threonine) (figure 4). However, by ingesting GSH or cysteine orally is not effectively transported into the cells (54, 65). Moreover, administer cysteine orally is potentially toxic. In contrast to NAC, a precursor to the amino acid cysteine can be ingested orally and acts as a precursor for the GSH synthesis (54, 65). Additionally, NAC itself shows a detoxifying property as a scavenger for ROS, particularly the reduction of the hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). This is partly due to its thiol group (66, 67). As a result, NAC could serve as an antioxidant and a free radical scavenger by increasing intracellular GSH to protect the cells from oxidative stress. Moreover, NAC has been previously demonstrated its property as a protective agent against apoptotic death of N18 neuroblastoma cells induced by Sindbis virus and of cultured neonatal sympathetic neurons and pheochromocytoma PC12 cells in the absence of nerve growth factor (68). These effects appear to be GSH-independent. Study in fibroblasts from patients with Alzheimer's disease revealed that NAC has an ability to decrease oxidative stress and apoptotic markers (caspase 9 and Bax) (69). The induction of Aβ toxicity in cultured astrocytes was also testified to be prevented by NAC (70). Recently reports have suggested that NAC may significantly augment the clinical efficacy on neurological disorders including Alzheimer's disease treatment (71-77). These effects are perhaps due to the abovementioned functions.

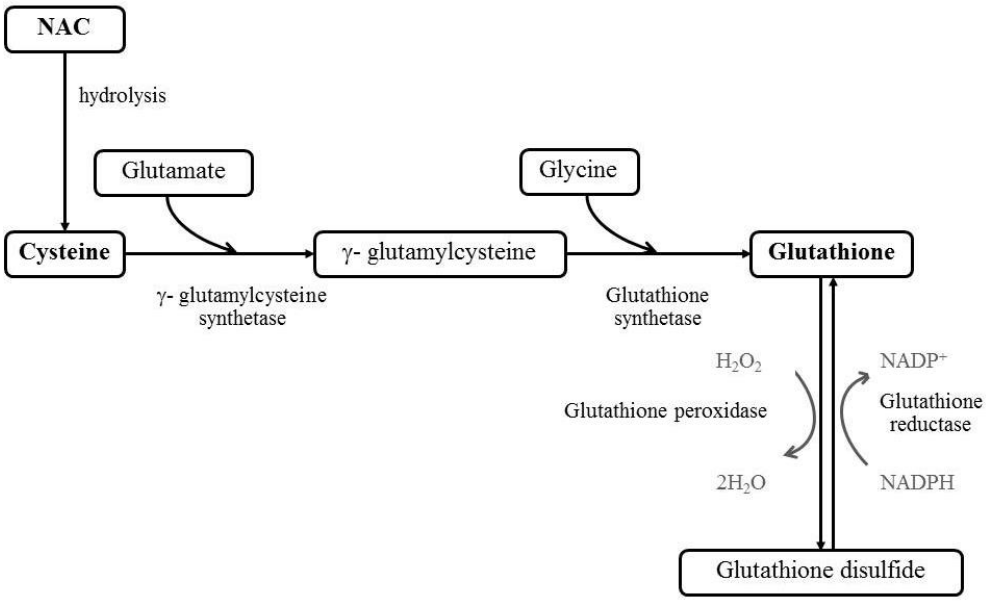


Figure 4 The intracellular synthesis of glutathione

Carnosine

Carnosine is a water-soluble natural occurring compound which contain of β-alanine and L-histidine. Other subsequently isolated carnosine-related dipeptides are anserine, ophidine/balenine (mostly found in marine mammals), and homocarnosine (an analog in which β-alanine is replaced by GABA) (figure 5). Carnosine along with its analog have been found to be widely distributed in excitable tissues of several animal tissues such as muscle and nervous tissue (78). The carnosine synthesis is starting from its component amino acids; β-alanine and L-histidine mediated by the enzyme called carnosine synthase (CS) (figure 6). This process requires Mg^{2+} and ATP involvement. CS is cytosolic enzyme mainly present in muscle and brain. Both carnosine and homocarnosine are synthesized by CS and degraded by carnosinase (CN). In the brain, homocarnosine is more abundantly than carnosine, and CS is believed to be responsible for the homocarnosine synthesis (78). Previous studies demonstrated that

carnosine exerts antioxidant activity mediated by its metal ion chelation and ROS scavenging property (79-81). Several studies indicate that supplemented carnosine exerts protective actions against organ injury and dysfunction induced by oxidative damage by acting as antioxidant and free-radical scavenger, increase GSH levels, and even decrease pro-inflammatory cytokines (IL-6 and TNF-α) (82-84). Moreover, carnosine has been reported its ability to inhibit advanced lipoxidation end-products (ALEs) and advanced glycoxidation end-products (AGEs) formation in both in vitro and in vivo models (85-88).

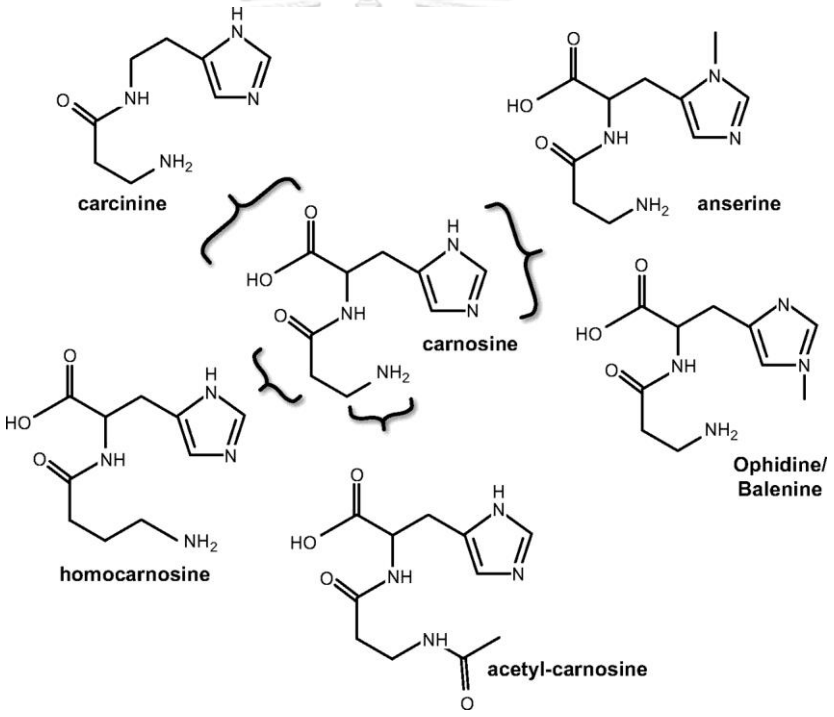


Figure 5 The chemical structures of carnocine and its analog (78)

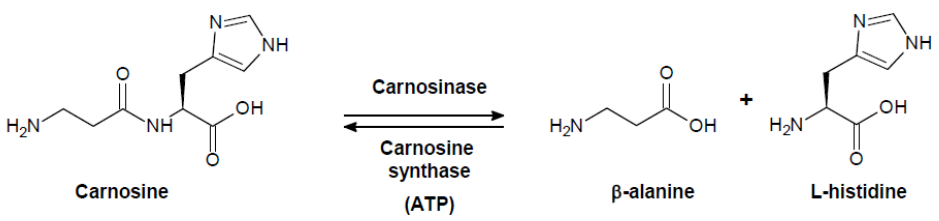


Figure 6 Synthesis and hydrolysis scheme of carnosine (89)

Carnosine and Alzheimer's Disease

Carnosine (β-alanyl-L-histidine) is a natural dipeptide which probably only present in animals especially, in skeletal muscle and the brain. Even though, its physiological role is not completely understood, several studies indicate that the carnosine may exert its protective effect on oxidative damage-related disorders including Alzheimer's disease (90-92). Carnosine level was found to be lower in individuals with Alzheimer's disease compared to normal control (91). On the other hand, carnosinase activity, a carnosine proteolysis was significantly higher in aging brain (93). Study in Alzheimer animal model has indicated that carnosine decrease intraneuronal Aβ deposition, show a positive trend in rescuing long-term memory deficits (94). Furthermore, supplemented carnosine was found to prevent cognitive decline by decreasing RAGE expression and microglia activation in Alzheimer mice model fed with high fat diet (95). Study in another type of dementia involving excessive zinc also found to be protected by carnosine (96, 97). Considering carnosine as an antioxidant, a metal-chelator, an anti-glycation agent, as well as inhibitor of Aβ toxicity, this compound is perhaps a relevant drug that could enhance clinical efficacy in Alzheimer's disease treatment.

Fimbristylis ovata (Burm.f.) Kern

Fimbristylis ovata (Burm.f.) Kern belongs to the genus *Fimbristylis* (family *Cyperaceae* or sedges) (Figure 7). Plants usually herbs with triangular stem; leaves 3-

ranked, ligule absent; flowers in spikelets of cyme; bracts glumaceous; lodicules absent; fruit an achene or nut, seed endospermic (98). This family consists of more than 90 genera and over 4000 species, which are commonly inhabitants of damp places. The plants in this family have long been reported wide range of medicinal and pharmacological applications according to the Ayurvedic system of medicine (99). The usage activities have been reported for this plant including anti-Candida, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, antibacterial, antioxidant, cytotoxic and apoptotic, anti-pyretic and analgesic activities. Total oligomeric flavonoids (TOFs) isolated from the plant belongs to *Cyperaceae* family has been shown its neuroprotective potential in rat model of cerebral ischemia-reperfusion which involved various pathophysiological mechanisms including excitotoxicity, inflammation and apoptosis. The post-ischemic status has significant forecasts toward complications that can lead to impaired cognition and memory. Treatment with TOFs increased the glutathione content in a dose dependent approach, increased SOD activity in all the brain structures, reduced the neurological deficits and reversed the anxiogenic behavior in rats (100). Previous studies of *F. ovata* extract which has been firstly reported in cultured monocytes and epithelial cells showed that this extract significantly decreases those inflammatory cytokines under oxidative stress induction (7). Hence this extract might as well exert its protective effects in neuronal cells undergoing oxidative stress condition by AGEs.

Figure 7 *Fimbristylis ovata (Burm.f.) Kern* (Photo by R. Santiyanont)

CHAPTER III

RESEARCH METHOD

Instruments, Devices, and/or Apparatus

0.2 mL, 0.5 mL, and 1.5 mL nuclease-free microcentrifuge tubes	Bioscience Inc., US and Axygen Inc., US
12 well cell culture plate	Corning, USA
24 well cell culture plate	Corning, USA
6 well cell culture plate	Corning, USA
96 well cell culture plate	Corning, USA
-20°C Non-frost-free freezers	Sanyo Electric, Japan
-80°C Non-frost-free freezers	Ilshin Lab, South Korea
Aerosol resistant tips for P10, P20, P100, P200, and P1000	Molecular BioProducts, Thermo Fisher Scientific, US
Analytical balance	Mettler Toledo, Switzerland
Auto pipettes P10, P20, P100, P200, and P1000	Gilson, France
Block heater	Wealtec Corp., US
Cell culture flask (25 cm^3)	Corning, USA
Cell culture flask (75 cm^3)	Corning, USA
Centrifuge	Hitachi, Japan
Centrifuge tube (15, 50 ml)	Corning, USA
CO_2 incubator	Thermo Scientific, USA
Cryovial tube 2.0 ml	Corning, USA
Disposable serological pipette (5, 10, 25 ml)	Corning, USA
Electrophoresis power supply	Bio-Rad Laboratories, US

ELISA plate reader	BioTek, USA
Evaporator	Genevac, USA
Freeze dryer	Thermo Electron, USA
Gel Doc system	Syngene, United Kingdom
Glassware	Pyrex, US
Hemocytometer	Hausser Scientific, USA
Hot air oven	Memmert, Germany
ImageStreamX Mark II Imaging Flow Cytometer	Merck, Germany
Inverted microscope	Olympus, Japan
Light microscope	Olympus, Japan
Laminar flow cabinet	E.S. I Flufrance, France
Microcentrifuge	Denver Instrument, US
Microcentrifuge	Eppendorf, Germany
Muse Cell Analyzer	Merck, Germany
NanoDrop™ 1000 spectrophotometer	Thermo Fisher Scientific, US
pH meter	Mettler Toledo, Switzerland
Plate shaker	Desaga, Germany
Refrigerator	Sharp, Japan
Rotary evaporator	Heidolph Instruments, Germany
Serological pipette (1, 5, 10, 25 ml)	Corning, USA
Sterile syringes filter	Corning, USA
Thermocycler with heated lid	Bio-Rad Laboratories, US

Tip (10, 20µl)	Sorenson, USA
Tip (100, 200 µl)	Gilson, France
Tip (1000 µl)	Hycon, USA
Ultrapure Lab Water Systems	Merck, Germany
Vortex mixer	FINEPCR, South Korea
Waterbath	Memmert, Germany

Chemicals and Reagents

AccuPower® CycleScript RT PreMix	Bioneer, Republic of Korea
AccuPower® 2X GreenStar Master Mix Solution	Bioneer, Republic of Korea
Amersham™ ECL Select™ Western Blotting Detection Reagent	GE healthcare, UK
Amersham™Hybond™ P 0.45 PVDF	GE healthcare, UK
AmershamHyperfilm™ ECL	GE healthcare, UK
Amnis® NFkB Translocation Kit	Merck, Germany
Antibody against β-actin	Cell Signaling Technology, USA
Antibody against MnSOD	Merck, Germany
Antibody against RAGE	Merck, Germany
Blotting-grade blocker	Bio-Rad, USA
Bovine serum albumin (BSA)	Capricorn Scientific, Germany
CellTiter 96(R) AQueous One SolutionAssay	Promega, USA
Chloroform	Sigma Aldrich, USA

DC Protein assay	Bio-Rad, USA
DEPC	Bio Basic, Canada
Dichloromethane	Merck, Germany
Dimethyl sulphoxide (DMSO) molecular grade	Sigma Aldrich, US
Enhanced chemiluminescence (ECL) substrate	Thermo Fisher Scientific, USA
Ethyl alcohol	Merck, Germany
Ethanol (molecular grade)	PROLABO, France
Fetal bovine serum (FBS)	Gibco Invitrogen, USA
Ham's F12 Nutrient Mixture	Hyclone, USA
Isopropanol (ultra pure)	Merck, German
L-Carnosine	Sigma Aldrich, USA
Methyl alcohol	Merck, Germany
Minimum Essential Media (MEM)	Hyclone, USA
Muse® Annexin V and Dead Cell Assay Kit	Merck, Germany
Muse® MAPK Activation Dual Detection Kit	Merck, Germany
Muse® Oxidative Stress Kit	Merck, Germany
N-Acetyl-L-cysteine (NAC) cell culture grade	Sigma Aldrich, USA
Oligo dT(18) primer	Bio Basic, Canada
Penicillin-Streptomycin	Hyclone, USA
Petroleum benzine	Merck, Germany
Phosphate buffered saline(10X)	Hyclone, USA
Protein Assay Dye Reagent Concentrate	Bio-Rad, USA

RNase away	Molecular BioProducts, Thermo Fisher Scientific, US
Sodium chloride (NaCl)	Merck, Germany
Sodium hydroxide (NaOH)	Merck, Germany
Sodium lauryl sulfate	Sigma Aldrich, USA
Specific primer	Bio Basic, Canada
Tris	Vivantis, USA
TRIzol® Reagent	Thermo Fisher Scientific, USA
Trypan blue	Gibco, USA
Trypsin	Hyclone, USA
Tween20	Merck, Germany

F. ovata extracts

F. ovata was collected from a single source in Bangkok, Thailand. The plant was identified by Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand (voucher No. 013431, BCU). The procedures to obtain the plant extracts were done as the following; 1) the fresh plants were cleaned by water, then dried in laboratory oven at 45 °C for 5 days, and finally ground into fine powder. 2) The plant powder was extracted with petroleum ether, dichloromethane, and methanol 1:10 (w/v) by soxhlet extraction. 3) The extracts were filtered, all filtered extracts were collected and evaporated by rotary evaporator. 4) Crude extracts were dissolved in dimethyl sulfoxide (DMSO) and kept as stock solution (100 mg/mL), at -20°C and protected from light until use.

Figure 8 Dried *Fimbristylis ovata (Burm.f.) Kern* (Photo by S. Sukjamnong)

Cell Culture and Treatment

The human neuroblastoma cell line SH-SY5Y were used for all experiments. The cells were grown in the MEM/F12 (1:1) culture medium supplemented with 15% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. The cultured was incubated under a humidified 5% (*v/v*) CO_2-air environment at 37 °C. Prior to treatment, dormant stage was conducted by a 24-h incubation with medium supplemented with 5% FBS. The cell culture model was exposed to either AGEs-BSA with/without antioxidant compounds pre-treatment, and then subjected to the following experiments.

Cell Viability/Cytotoxicity Determination

After the cells were exposed to each treatment including AGEs-BSA, NAC, Carnosine, and F. ovata extracts cell viability/cytotoxicity will be examined for each condition using the following methods;

MTS Tetrazolium Assay

MTS assay was used to determine cell viability. The method is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. This set of tetrazolium reagents is used in combination with intermediate electron acceptor reagents such as phenazine methyl sulfate (PMS) or phenazine ethyl sulfate (PES) which transfers electron from NADH in the cytoplasm to reduce MTS in the culture medium into an aqueous soluble formazan. The formazan dye produced by viable cells can be quantified by measuring the absorbance at 490-500 nm. The level of activity reflected the proportion of viable cells. The percentage of cellular activity was calculated according to the following formula:

cellular activity (%) = [(absorbance of treatment group – blank/absorbance of control group – blank)] × 100.

Trypan Blue Exclusion Assay

Trypan blue exclusion assay is based on the trypan blue molecule, which is cell membrane impermeable and only enters cells with compromised membranes, thereby rendering the cells a bluish color. Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemocytometer. If cells take up trypan blue, they are considered non-viable. The

percentage of cellular viability was calculated according to the following formula: % viable cells = [1.00 – (Number of blue cells/ Number of total cells)] × 100

Measurement of intracellular superoxide radicals

The Muse® Oxidative Stress Kit was used to measure superoxide radicals in cells undergoing oxidative stress. The kit is based on dihydroethidium (DHE) dye, a fluorescence dye which can be permeated the cell membrane. Once this dye reacts to superoxide radicals, oxyethidium will be formed. Briefly, SH-SY5Y cells were seeded in a density of $4x10^5$ cell/mL in 12-well plates. After 24 hours, the medium was changed to fresh 5%FBS medium. The cells were pretreated with either NAC, Carnosine or *F. ovata* extracts for 3 hours then incubated with AGEs-BSA (200 µg/mL) for an hour before proceeding to manufacture's protocol with minor modification.

Measurement of NF-**K**B translocation

The activation of NF-**K**B and its translocation to the nucleus plays a crucial role in regulating many key processes in mammalian cells which led to studying cellular function, signal transduction pathways, disease mechanisms and drug discovery. NF-**K**B p50/p65 heterodimer, the most abundant form, is kept in inactive state by its inhibitor IkB. Once the IkB is phosphorylated, NF-**K**B complex becomes activated and translocated into nucleus which can be detected by The Anti-HuNF**K**B Alexa Fluor® 488 antibody. Briefly, SH-SY5Y cells were seeded in a density of $1x10^6$ cell/mL in 6-well plates. After 24 hours, the medium was changed to fresh 5%FBS medium. The cells were pretreated with either NAC, Carnosine, or *F. ovata* extracts for 3 hours then

incubated with AGEs-BSA (200 µg/mL) at for 3 hours before proceeding to manufacture's protocol with minor modification.

Measurement of MAPK activation

The extracellular signal-regulated kinases 1 and 2 (ERK) cascade are member of the mitogen-activated protein kinases superfamily of signaling pathways. In particular, ERKs phosphorylation are associated with the regulation of proliferation and differentiation as well as survival of various cell types. In this part, Muse® MAPK Activation Dual Detection Kit was used. The kit includes two directly conjugated antibodies, a phospho-specific anti-phospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187)-Phycoerythrin and an anti-ERK1/2-PECy5 conjugated antibody to measure total levels of ERK. This two color kit is designed to measure the extent of MAPK phosphorylation relative to the total MAPK expression in any given cell population. The results of percentage of inactivated cells, percentage of activated cells (via phosphorylation), and percentage of non-expressing cells were generated. Briefly, SH-SY5Y cells were seeded in a density of $1x10^6$ cell/mL in 6-well plates. After 24 hours, the medium was changed to fresh 5%FBS medium. The cells were pretreated with either NAC or carnosine at different concentrations for 3 hours then incubated with AGEs-BSA (200 µg/mL) for an hour before proceeding to MAPK/ERK activation measurement according to manufacturer's protocol with minor modification.

Identification of apoptotic cells

Apoptosis is a highly regulated mechanism of cell death that is essential to ensure normal development and homeostasis of all multicellular organisms. It counterbalances cell proliferation and differentiation pathways and removes unwanted cells. An excess level of apoptosis is a factor in neurodegenerative diseases and autoimmune disorders, while failure of apoptosis to destroy aberrant cells is a key element of cancer.

Muse® Annexin V and Dead Cell Assay Kit was used for this test. The kit is based on the observation of universal phenomenon during cellular apoptosis in which the loss of phospholipid asymmetry in the cell membrane is occurred. Detection can be analyzed by flow cytometry. Annexin V binds to Phosphatidylserine exposed on the cell membrane, enabling quantification of apoptotic cells by staining them with fluorochrome-conjugated Annexin V. To further differentiate apoptosis from necrosis, cells can be stained with nuclear dyes7-AAD (7-amino-actinomycin D). Briefly, SH-SY5Y cells were seeded in a density of $2x10^5$ cell/mL in 24-well plates. After 24 hours, the medium was changed to fresh 5%FBS medium. The cells were pretreated with either NAC, Carnosine, or *F. ovata* extracts for 3 hours then incubated with AGEs-BSA (400 µg/mL) for 24 hours before proceeding to manufacture's protocol with minor modification.

Western Blot Analysis

Expression levels of RAGE, Mn-SOD and β-actin were measured by Western blot analysis. Briefly, whole protein was extracted from the cell culture model. Protein assay using Bradford assay was performed to ensure equal loading of protein samples

as a pre-determination step. Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a nitrocellulose membrane. The membranes were blocked in BSA solution and the blots were probed with specific primary antibodies overnight at 4 ℃. Next, the blots were incubated with secondary antibodies including horseradish peroxidase conjugated. Densitometry analysis is then used to quantify the intensities of specific bands. The processes are as the following;

1. SH-SY5Y cells were seeded in density of 1 x 10^6 cells/mL in 6-well plates
2. After 24 hours, the medium was changed to fresh 5%FBS medium.
3. Cell were pretreated with either NAC, Carnosine, or *F. ovata* extracts for 3 hours and then treated with AGEs-BSA for 24 hours.
4. Protein were isolated from the treated cells as the following steps

4.1 Cells were washed with cold Phosphate Buffered Saline (PBS).

4.2 Whole cell extracts were prepared using 100 µl lysis buffer with phosphatase inhibitor, plate was incubated on ice for 10 min.

4.3 The lysate was centrifuged for 10 minutes at 13,000 x g, 4 ℃ and the supernatant was transferred to new tubes. The cell debris were discarded.

4.4 The proteins were stored at -80℃ for later analysis.

5. The protein concentration was determined by Bio-Rad Protein Assay.
6. Equal amounts of proteins (15 µg) were separated on 10% SDS-PAGE.
7. Protein were transferred to PVDF blotting membranes.
8. The membranes were blocked with TBS-T containing 5% nonfat dry milk for 1 hour.

9. The membranes were incubated with primary antibodies overnight at 4°C using the following antibodies, as listed in Table 1

Table 1. Antibodies used in Western blot analysis

Primary antibody	Antibody dilution
Mn-SOD	1:2,000
RAGE	1:2,000
β-actin	1:10,000

10. The membranes were incubated with secondary antibodies (peroxidase-conjugated goat anti-mouse or anti-rabbit IgG).
11. The blots were incubated in enhanced chemiluminescence (ECL) substrate, and the membranes were then visualized using high performance chemiluminescence film.
12. ImageJ software (National Institute of Health) was used for protein band density determination, and β-actin was used as the housekeeping protein.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

To detect the gene encoded the target proteins, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used. Briefly, total RNA was purified from cell culture model using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To measure mRNA levels of RAGE gene, the genes encoded pro-inflammatory cytokines including TNF-α, IL-1, and IL-6, the known early-onset familial Alzheimer's disease genes including APP, PSEN1, the gene encoding BACE (β-secretase), ADAM10, TACE and β-actin as an internal control, total RNA will be reverse

transcribed into cDNA using AccuPower® CycleScript RT PreMixReverse Transcription System. The cDNA products were then being amplified with each gene-specific primer pairs and quantitated using using the SYBR Green based Exicycler™ 96 Real-Time Quantitative Thermal Block. The mRNA levels were then calculated. β-actin as a housekeeping gene was used to normalize for the expression level of each gene. The expression level was quantified as fold-change using the ΔΔCt method (2-ΔΔCt). The processes were done as the following steps;

1. SH-SY5Y cells were seeded in density of 1 x 10^6 cells/mL in 6-well plates
2. After 24 hours, the medium was changed to fresh 5%FBS medium.
3. The cells were pretreated with either NAC, Carnosine, or *F. ovata* extracts for 3 hours and then treated with AGEs-BSA for 24 hours.
4. Total RNA was isolated from the treated cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

4.1 Cells were washed twice times with Phosphate Buffered Saline (PBS). Cold TRIzol reagent 1,000 µl was added. The lysate was homogenized by scrapping and pipetting the lysate up and down several time. Then incubated the lysate at RT for 5 minutes.

4.2 200 µl of ice-cold chloroform was then added, mixed, and incubated at RT for 5 minutes.

4.3 The lysate was centrifuged for 15 minutes at 12,000 x g, 2-8 °C and then transferred the aqueous phase containing the RNA to a new tube (approximately 400 µl was transferred).

4.4 500 µl of ice-cold isopropanol was added, inverse-mixed, and incubated at -20 °C for 30 minutes.

4.5 Centrifuged for 10 minutes at 12,000 x g, 2-8 °C.

4.6 The supernatant was removed and then the RNA pellet was washed by adding 1,000 µl of 75% ethanol in DEPC-treated water. Vortex the sample briefly, then centrifuge for 5 minutes at 7500 × g at 2-8 °C. Repeated RNA washing step for 3 times.

4.7 Removed the supernatant, air dry the RNA pellet for an hour.

4.8 Resuspended the pellet in 32 µL of DEPC-treated water, Incubated at 65°C for 5 minutes.

4.9 RNA yield was determined using Nanodrop spectrophotometer.

5. The RNA template was used to synthesize cDNA using AccuPower® CycleScript RT PreMixReverse Transcription System.

Table 2. cDNA synthesis procedure using AccuPower® CycleScript RT PreMix

Step	Condition
Mix the template RNA 1,000 ng and Oligo dT_{18}	70°C, 5 min
Transfer the incubated mixture to an AccuPower® RT PreMix tube, and then fill up the reaction volume with DEPC-treated water	
cDNA synthesis	42°C, 60 min
RTase inactivation	94°C, 5 min

6. For the amplification reaction, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in standard 96-well plates using the Exicycler™ 96 Real-Time Quantitative Thermal Block. To analyze mRNA expression levels, the SYBR green system was used incorporated with gene-

specific primer pairs. A melting curve analysis was also performed to ensure the specificity of the amplified product.

Table 3. Specific nucleotide primers used in qRT-PCR

	Accession number and gene name		Primer pair sequence (5'->3')
1	NM_001101.4 Homo sapiens actin beta (ACTB), mRNA	Forward primer	CTTCCTGGGCATGGAGTCCTGT
		Reverse primer	CTTTGCGGATGTCCACGTCAC
		Product length	80 bp
2	NM_001136.4 Homo sapiens advanced glycosylation end-product specific receptor (AGER), transcript variant 1, mRNA	Forward primer	CAGCATCAGCATCATCGAACCA
		Reverse primer	CGCCTTTGCCACAAGATGACC
		Product length	150 bp
3	NM_000576.2 Homo sapiens interleukin 1 beta (IL1B), mRNA	Forward primer	AGTACCTGAGCTCGCCAGTG
		Reverse primer	GGTCCTGGAAGGAGCACTTCAT
		Product length	107 bp
4	NM_000600.4 Homo sapiens interleukin 6 (IL6), transcript variant 1, mRNA	Forward primer	CTTCTCCACAAGCGCCTTCG
		Reverse primer	TGTGGGGCGGCTACATCTTT
		Product length	120 bp
5	NM_000594.3	Forward primer	GCTGCACTTTGGAGTGATCGG

	Homo sapiens tumor necrosis factor (TNF), mRNA	Reverse primer	CTCAGCTTGAGGGTTTGCTACA
		Product length	148 bp
6	NM_012104.4 Homo sapiens beta-secretase 1 (BACE1), transcript variant a, mRNA	Forward primer	CCACGGGCACTGTTATGGGA
		Reverse primer	ATCGTGCACATGGCAAGCG
		Product length	107 bp

Table 4. qRT-PCR procedure for all gene

Step	Condition	Cycle(s)
Pre-denaturation	95 ℃, 10 min	1
Denaturation	95 ℃, 15 s	35
Annealing/extension	64 ℃, 30 s	
Detection	Scan	
Melting		1

7. The expression for each gene was normalized to that of the control gene, β-actin. Expression was quantified as fold-change using the ΔΔCt method ($2^{-\Delta\Delta Ct}$).

Statistical Analysis

Data were analyzed using SPSS statistical software. When comparing two groups the unpaired two-tailed, Student's t-test was used. When comparing three or more groups, ANOVA followed by post hoc Bonferroni test was used. Differences between the values were considered significant at $p<0.05$.

CHAPTER IV
RESULT

Effect of AGEs on cell viability of human neuroblastoma, SH-SY5Y cells

As a toxic substance, AGEs-BSA was tested on its toxicity upon the exposure of SH-SY5Y neuroblastoma cell line to the compound. SH-SY5Y cells were exposed to AGEs-BSA at various concentrations for 24 hours and 48 hours. After that the cells viability were examined using MTS Tetrazolium assay. Cell viability was greater than 80% in all treatments (Fig. 9).

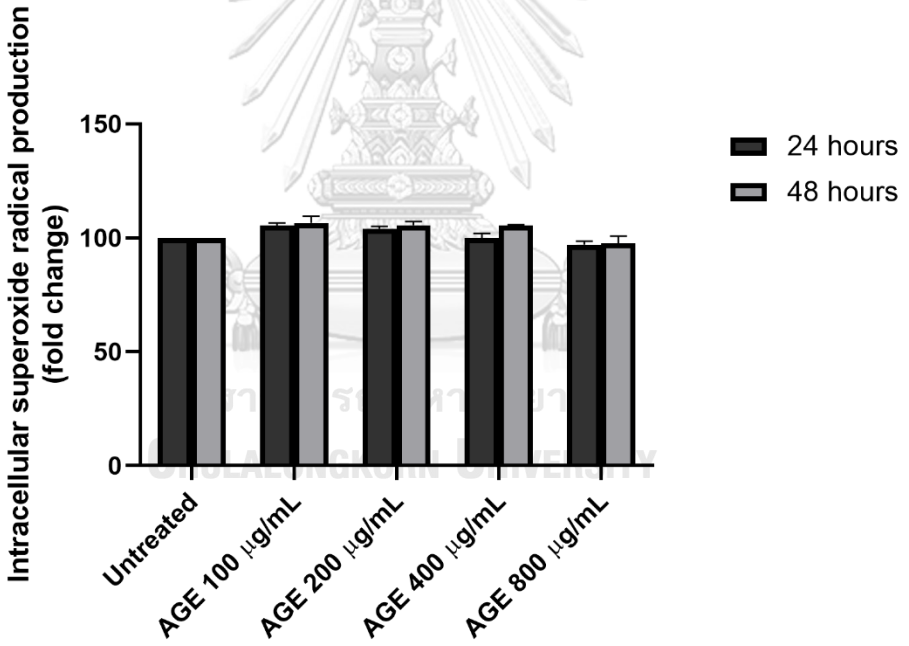


Figure 9: The effect of AGEs-BSA treatment on SH-SY5Y cells viability. SH-SY5Y cells were incubated with AGEs-BSA at various concentrations ranging from 100 μg/mL – 800 μg/mL for 24 hours and 48 hours to test on AGEs-BSA toxicity by MTS assay. The differences in the mean values among the treatment groups are reported as clustered

columns with their standard error of mean (SEM) (mean ± SEM). All experiments were done in triplicate (N=3).

The ralative expression levels of RAGE mRNA and protein upon AGEs-BSA treatment

The relative expression level of RAGE mRNA in SH-SY5Y cells were assessed by qRT-PCR. Our data revealed that, treatment of AGE-BSA ranging from 100, 200, and 400 µg/mL for 24 hours induced RAGE mRNA expression in a dose-dependent manner as shown in Figure 10. The relative expression level of RAGE protein in SH-SY5Y cells were assessed by Western blotting. As shown in Figure 11, RAGE protein levels were significantly increased in SH-SY5Y cells after treatment of 200 µg/ml AGEs-BSA for 24 hours ($P < 0.05$). The induction of RAGE protein showed a dose-dependent manner upon AGEs-BSA treatment.

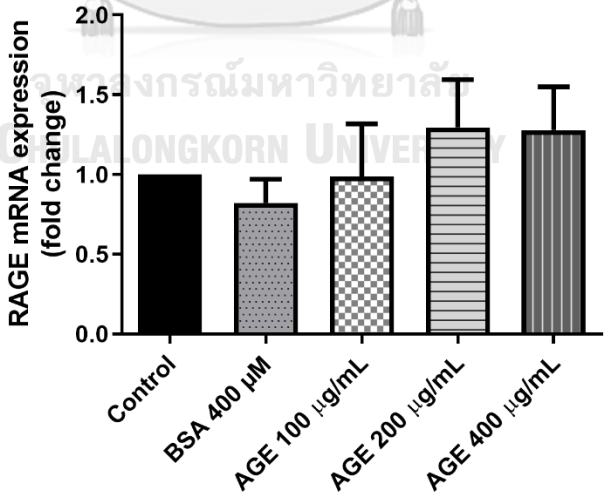


Figure 10: The relative of AGER (RAGE) mRNA expression levels to ACTB (β-actin) mRNA expression levels

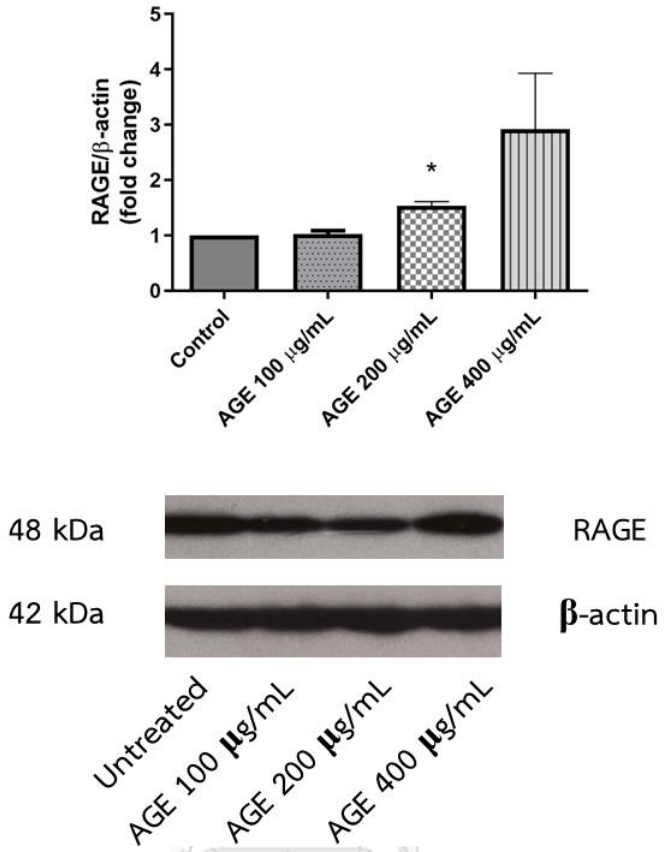


Figure 11: The ralative expression levels of RAGE protein to β-actin protein

Intracellular superoxide radical production upon AGEs treatment on SH-SY5Y cells

The brain is considered as a particularly place vulnerable to oxidative injury due to its high oxygen utilization. Mitochondria are a major source of free radical in the brain as a byproduct of energy production. Alteration in mitochondria function leads to increase oxidative stress levels. Moreover, neurotransmitter activity also generates free radicals by auto-oxidation of dopamine and excitotoxicity related to glutamatergic signaling (25, 26). Thus, brain is a place where confounded by the generation of free radicals, and insufficient antioxidant defense mechanism. The

excessive of free radicals and disturbance of antioxidant defense mechanism lead to neurotoxicity. In this part, the cellular populations undergoing oxidative stress with either H_2O_2 or AGEs (200, 400, and 600 μg/mL) were measured quantitatively by flow cytometer based on a relative detection of intracellular superoxide radical production percent cells positive and negative (Figure 12).

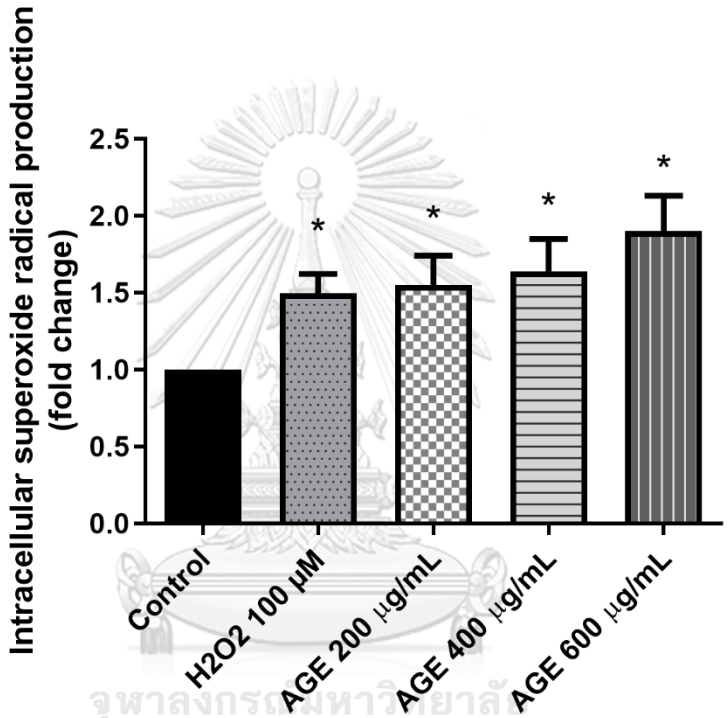


Figure 12: The relative percentage of cells exhibiting intracellular superoxide radical. The relative percentage of cells exhibiting intracellular superoxide radical was determined after SH-SY5Y cells undergoing AGEs treatment for an hour. Flow cytometry analyzer was used based on dihydroethidium (DHE) reagent detection. The differences in the mean values among the treatment groups are reported as clustered columns with their standard error of mean (SEM) (mean ± SEM). All experiments were done in triplicate (N=3).

NF-kB translocation determination in response to AGEs treatment on SH-SY5Y cells

RAGE is a multi-ligand receptor which can bind various molecules (22, 23). The interaction between AGEs and RAGE activates a number of signaling pathway in different cell types including p38 mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-**K**B), CDC42/Rac, SAPK/JNK, and JAK/STAT pathways. Moreover, AGEs regulation involving intracellular ROS generation was found to enhance RAGE expression, create positive feedback loops of AGEs signaling, and subsequently induce inflammatory reactions. The quantitative measurement of NF-kB translocation from the cytoplasm to the nucleus was performed using the combination of flow cytometry and microscopic performance in one system to generate a quantitative result together with image data. These NF-kB translocation analysis is detected based on the correlation of the nuclear 7AAD image to the anti-Hu NF-kB Alexa Fluor® 488 image (Fig. 13). The longer length of incubation time, the higher translocation scores will be (Figure 14a, b).

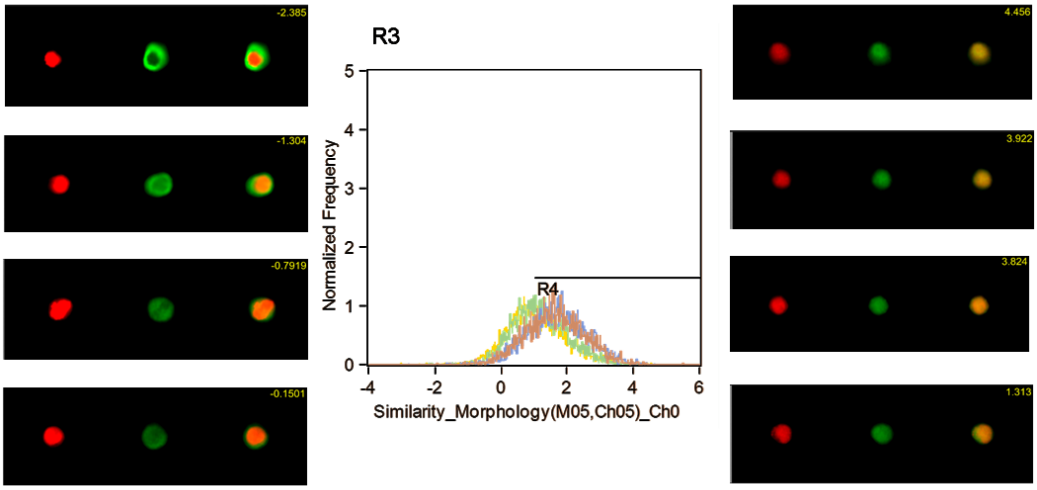


Figure 13: Histogram and 40x composite images of NFkB and 7AAD demonstrating varying similarity scores upon treatment

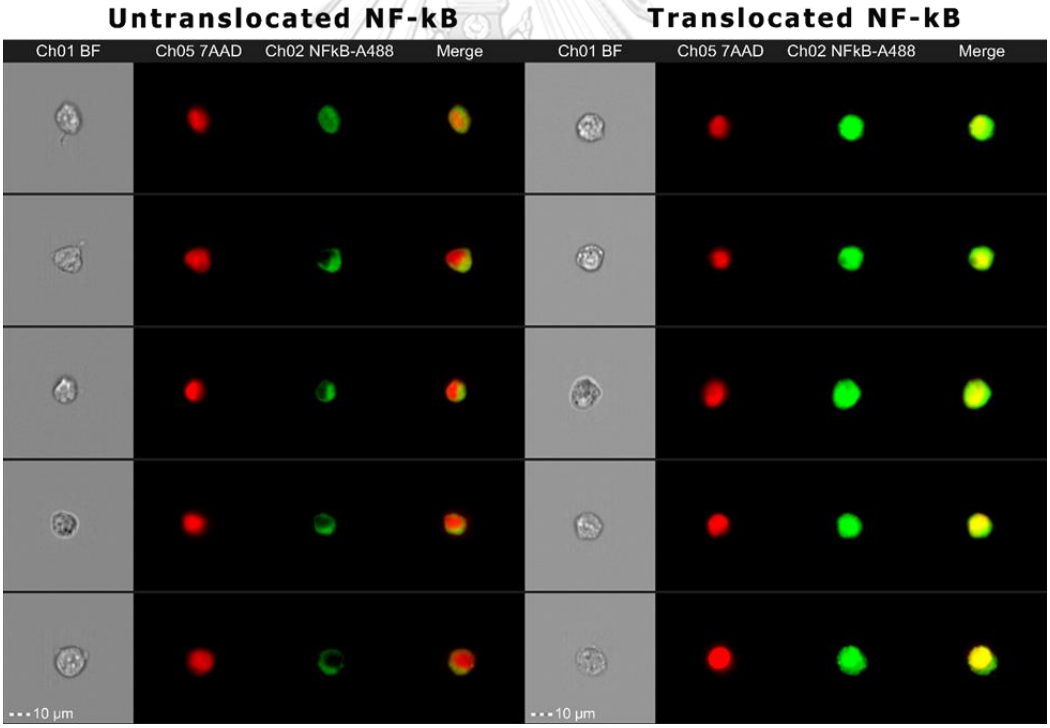


Figure 14a

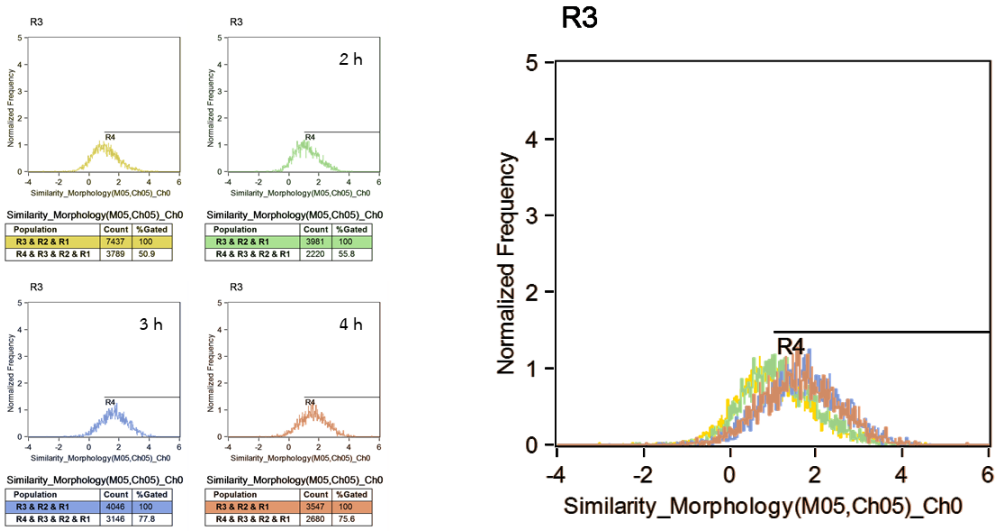


Figure 14b

Figure 14: **a)** Un-translocated and translocated NF-kB. The 40x composite images of the anti-Hu NF-kB Alexa Fluor® 488 (green) and the nuclear 7AAD (red) is delivered from Amnis® Flowsight® & ImageStream® combined with the Nuclear Localization Wizard in the Amnis® IDEAS® software. **b)** The histogram of similarity scores upon AGEs treatment. SH-SY5Y cells were induced with AGEs for 2 (green), 3 (blue) and 4 hours (red) compared to untreated control (yellow). The histogram demonstrates that the longer length of incubation time, the higher similarity score will be. The similarity scores were created using the Nuclear Localization Wizard in the Amnis® IDEAS® software showing the similarity feature which calculates a pixel by pixel of the correlation between the anti-Hu NF-kB Alexa Fluor® 488 and the nuclear 7AAD.

Effect of AGEs on MAPK activation

Erk (Extracellular signal-Related Kinase) is a family of two, highly homologous proteins denoted as Erk1 (p44, MAPK3) and Erk2 (p42, MAPK1) that both function in

the same pathway. The two proteins are often referred to collectively as Erk1/2 or p44/p42 MAP kinase. The Erk pathway is considered the classical, canonical MAPK (Mitogen-Activated Protein Kinase) signaling pathway. It is an evolutionarily conserved pathway that controls and is a critical regulator of the growth and survival through the promotion of cell proliferation and the prevention of apoptosis. Erk is involved in the control of many fundamental cellular processes including cell proliferation, survival, differentiation, apoptosis, motility and metabolism. The result show that AGEs are significantly increased MAPK/Erk activivation starting at 15 minute of treatment.

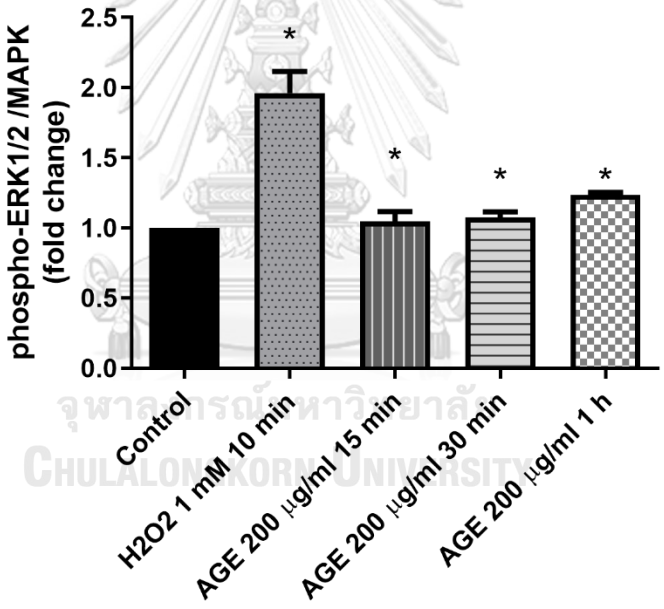


Figure 15 The relative percentage of phospho-ERK1/2 to total ERK was determined after incubated SH-SY5Y cells with AGEs treatment for an hour. Flow cytometry analyzer was used. The differences in the mean values among the treatment groups are reported as clustered columns with their standard error of mean (SEM) (mean ± SEM). All experiments were done in triplicate (N=3).

The effect of AGEs treatment on cell apoptosis

Apoptosis is a highly regulated mechanism of cell death that is essential to ensure normal development and homeostasis of all multicellular organisms. It counterbalances cell proliferation and differentiation pathways and removes unwanted cells. An excess level of apoptosis is a factor in neurodegenerative diseases and autoimmune disorders, while failure of apoptosis to destroy aberrant cells is a key element of cancer. A flow cytometry-based method stained with Annexin V and 7-AAD dye was used for this approach. The result shows that upon AGEs treatment cells were significantly increased their total apoptotic event against normal control (Figure 16).

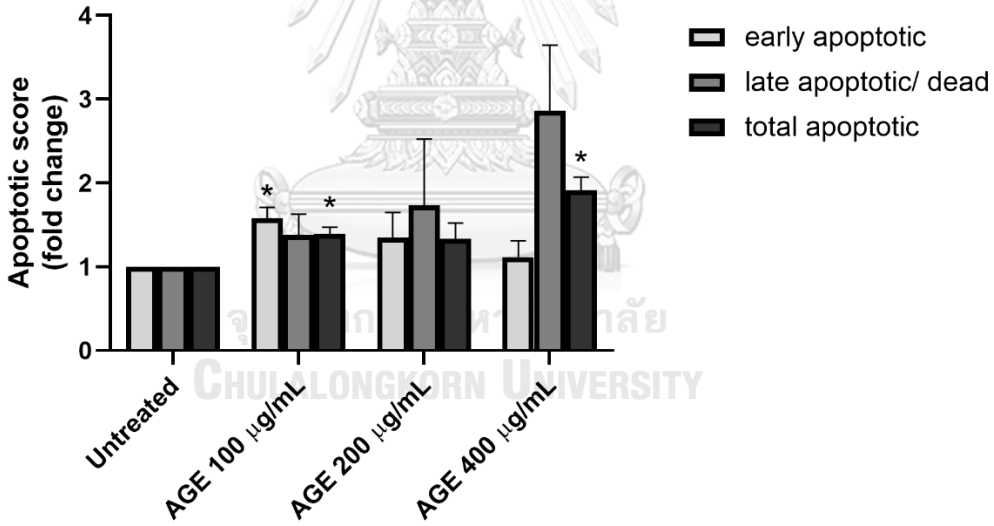


Figure 16 Change in cell apoptosis during AGEs treatment. The relative percentage of cells undergoing apoptotic event was determined after SH-SY5Y cells incubated with AGEs for 24 hours. A flow cytometry-based method was used on this purpose. The differences in the mean values among the treatment groups are reported as clustered columns with their standard error of mean (SEM) (mean ± SEM). All experiments were done in triplicate (N=3).

mRNA expression levels of specific genes to β-actin upon AGE treatments

mRNA expression levels in SH-SY5Y cells upon AGEs treatment were assessed by qRT-PCR. RAGE is a gene involving receptor for AGEs. BACE is a gene responsible for enzyme involved in Aβ production. While, IL1β, IL6, and TNFα are proinflammatory cytokines. As shown in Figure 17, treatment of 100, 200, and 400 μg/ml AGEs-BSA for 24 hours increased RAGE, BACE, IL1B, IL6, and TNF mRNA expression in a dose-dependent manner. Statistical difference was found in BACE gene in response to 200 and 400 μg/mL AGEs-BSA, and TNF treated with 100, 200 and 400 μg/mL AGEs-BSA.

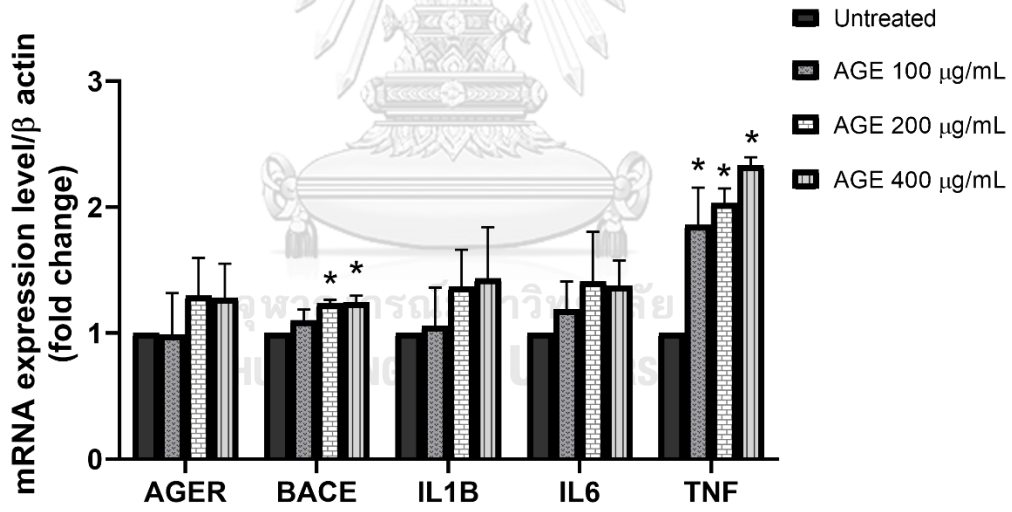


Figure 17: mRNA expression levels of specific genes to β-actin upon AGE treatments

Cell survival upon *F. ovata* extracts treatment

As mentioned previously that *F. ovata* might exert its role as a protective agent against oxidative stress condition induced by AGEs-BSA. Hence, we were tested

the cytotoxicity of these compounds in SH-SY5H using MTS assay. The results demonstrated that cell viability was greater than 80% in all treatments (Figiure 18-20).

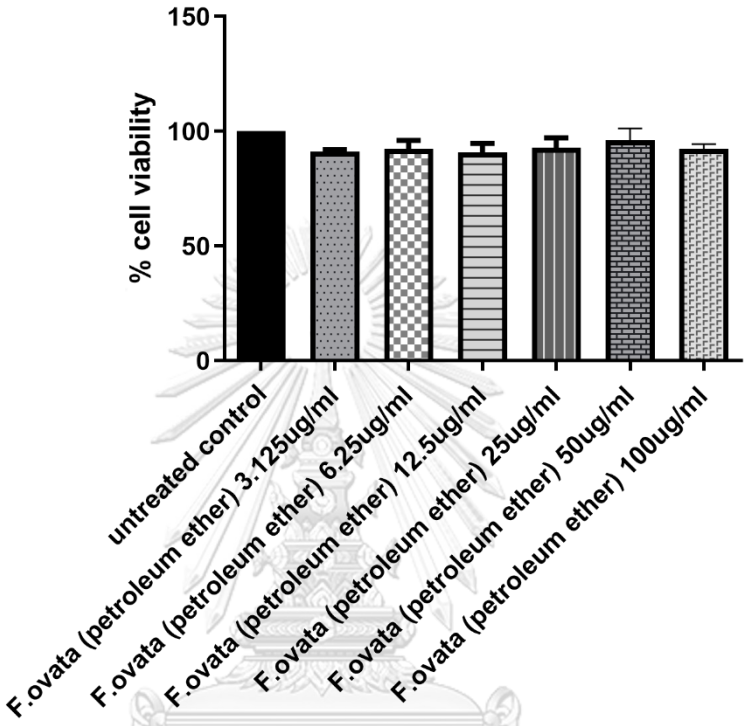


Figure 18: % cell viability upon *F. ovata* (petroleum ether) extract treatment at different concentration for 24 hours

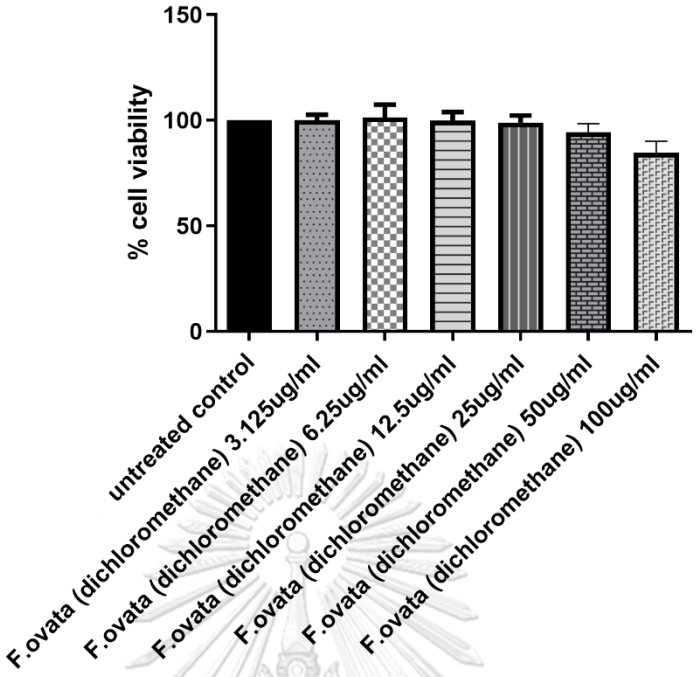


Figure 19: % cell viability upon *F. ovata* (dichloromethane) extract treatment at different concentration for 24 hours

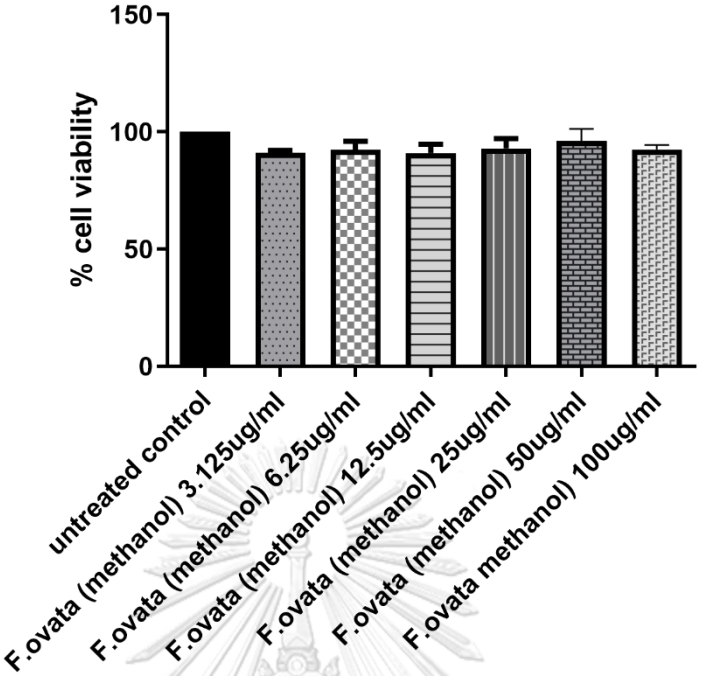


Figure 20: % cell viability upon *F. ovata* (methanol) extract treatment at different concentration for 24 hours

The effect of *F. ovata* extracts on intracellular superoxide generation

In this part, SH-SY5Y cells were pretreated with either *F. ovata* (petroleum ether), *F. ovata* (dichloromethane), or *F. ovata* (methanol) extract 100 μg/mL for 3 hours. The cells were then incubated with 200 μg/mL AGEs-BSA for an hour. The cellular populations undergoing oxidative stress were measured quantitatively by flow cytometer based on a relative detection of intracellular superoxide radical production percent cells positive and negative. The results showed that *F. ovata* obtained from petroleum ether and methanol fractions significantly decreased oxidative stress induced by AGEs-BSA (Figure 21).

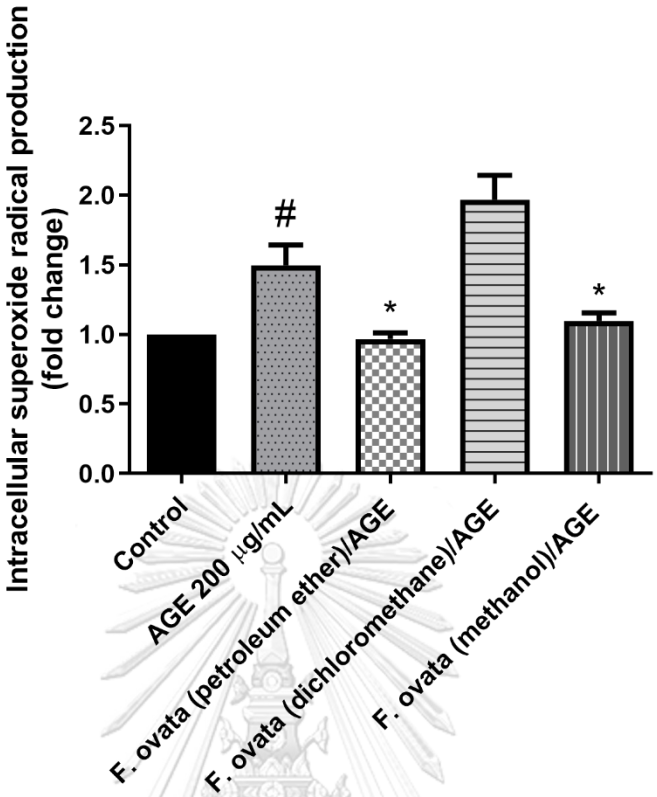


Figure 21: The effect of *F. ovata* extracts on intracellular superoxide generation

Cytotoxicity of SH-SY5Y cells upon NAC and Carnosine treatments

The survival rate of the cells undergoing either NAC or Carnosine treatment for 24 hours was assessed by MTS assay. The results demonstrated that cell viability was greater than 80% in all treatments (Figiure 22-23).

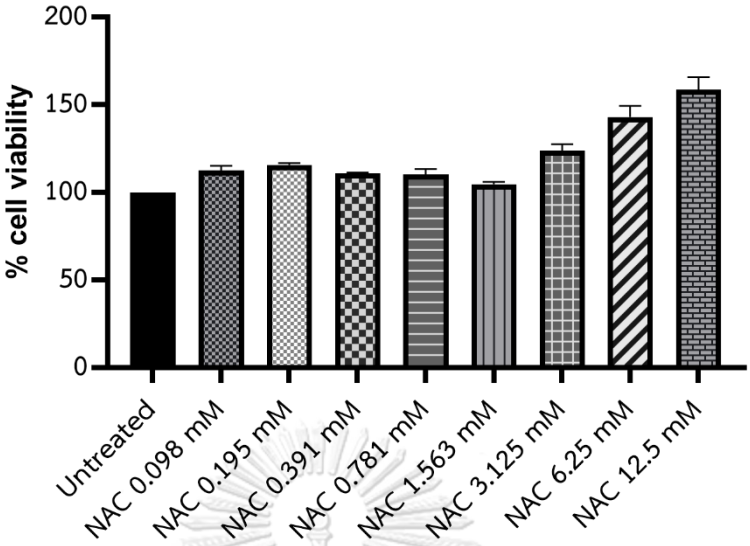


Figure 22: % cell viability upon NAC treatment at different concentrations for 24 hours

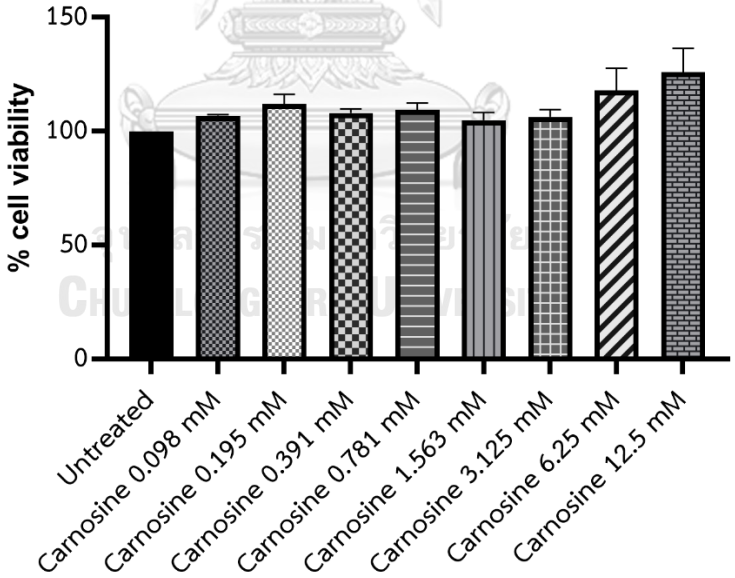


Figure 23: % cell viability upon Carnosine treatment at different concentrations for 24 hours

The effect of *F. ovata* extracts, NAC, and Carnosine on RAGE mRNA (AGER) expression level

In this part, SH-SY5Y cells were pretreated with either *F. ovata* extracts, NAC, or Carnosine for 3 hours, then treated with 200 μg/mL AGEs-BSA for 24 hours. We found that the 100 μg/mL petroleum ether and methanol extracts have possibility to decrease RAGE expression levels. NAC and Carnosine at the lower concentration demonstrated a trend to lessen RAGE expression, while, the higher dose of NAC and Carnosine increased RAGE expression levels in a dose-dependent manner (Figure 24).

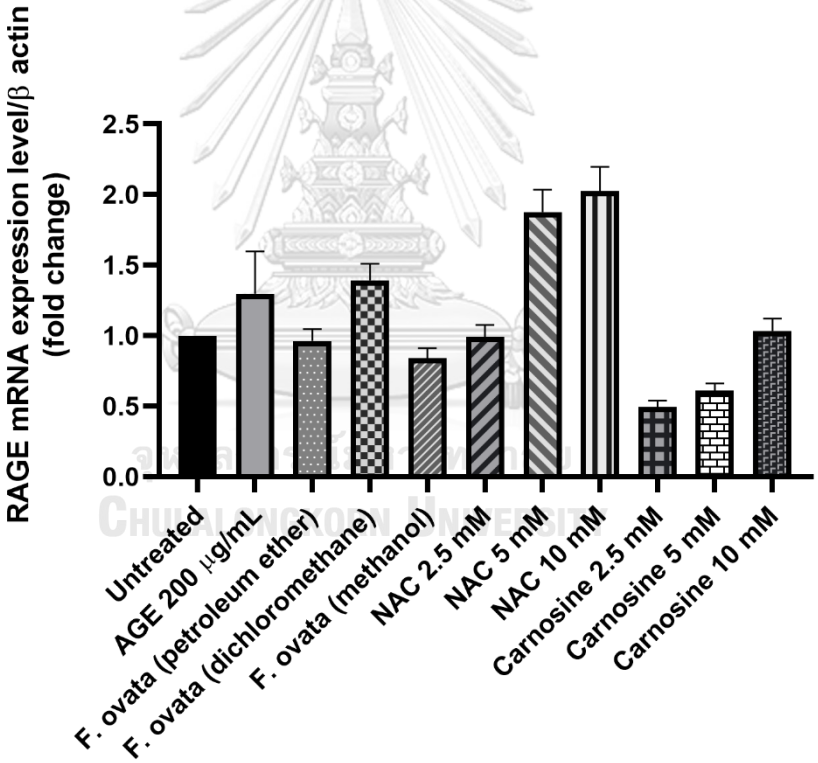


Figure 24: The effect of *F. ovata* extracts, NAC, and Carnosine of RAGE mRNA (AGER) expression level

The effect of *F. ovata* extracts, NAC, and Carnosine on NF**K**B translocation under AGE treatment condition

In this part, SH-SY5Y cells were pretreated with either *F. ovata* extracts, NAC, or Carnosine for 3 hours, then treated with 200 μg/mL AGEs-BSA for 3 hours. We found that the 100 μg/mL of F. ovata extracts from all fractions including petroleum ether, dichloromethane, and methanol significantly increased NF**K**B translocation. 5 and 10 mM of NAC also significantly increased NF**K**B translocation. In contrast to Carnosine which demonstrated a possibility to decrease NF**K**B translocation (Figure 25).

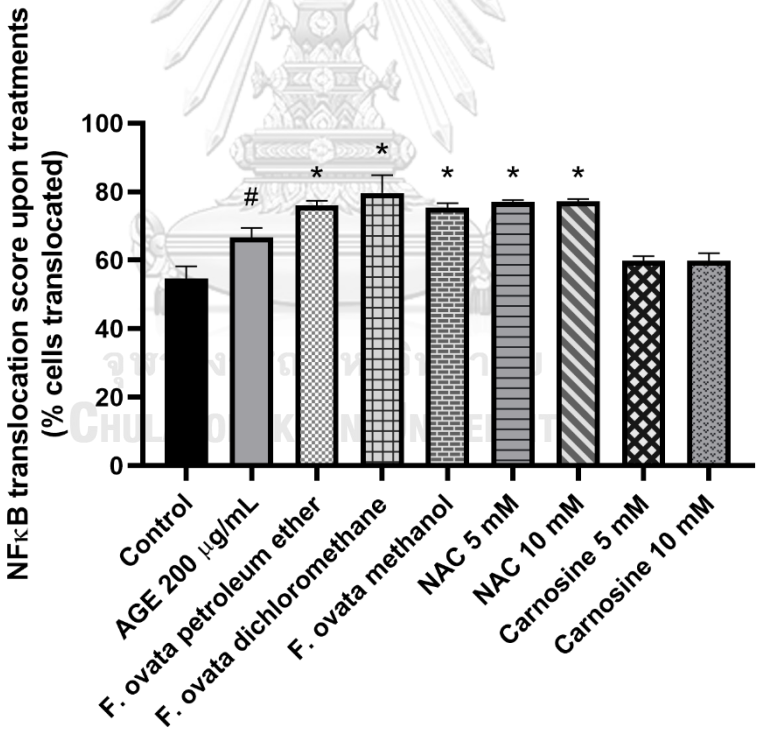


Figure 25: The effect of F. ovata extracts, NAC, and Carnosine on NF**K**B translocation under AGE treatment condition

Effect of *F. ovata* extracts, NAC, and Carnosine on MAPK activation

In this part, SH-SY5Y cells were pretreated with either *F. ovata* extracts, NAC, or Carnosine for 3 hours, then treated with 200 μg/mL AGEs-BSA for an hour. We found that the 100 μg/mL of F. ovata extracts from dichloromethane and methanol significantly decreased ERK/MAPK activation. NAC treatment at 10 mM and Carnosine at 2.5, 5 and 10 mM were also revealed a significantly reduction of MAPK activation (Figure 26).

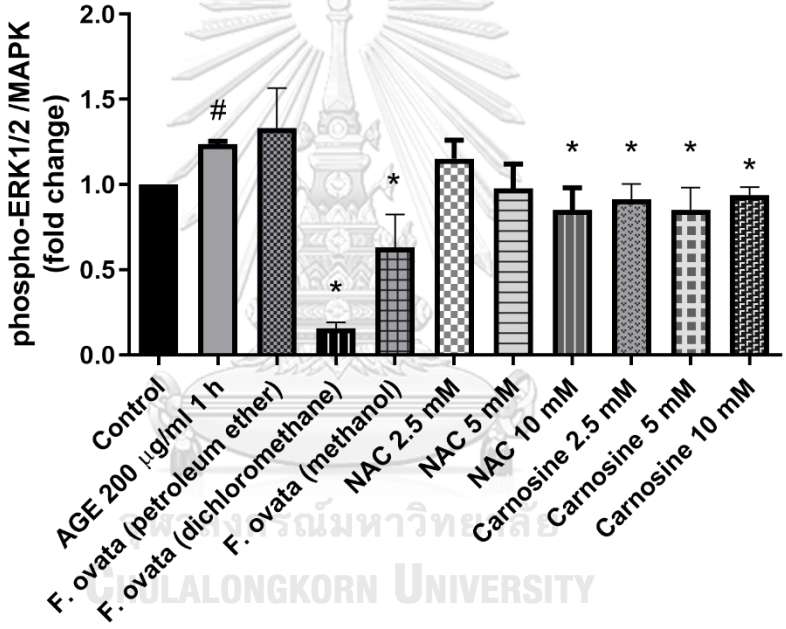


Figure 26: The effect of F. ovata extracts, NAC, and Carnosine on MAPK activation under AGE treatment

Effect of *F. ovata* extracts, NAC, and carnosine on apoptotic event

In this part, SH-SY5Y cells were pretreated with either *F. ovata* extracts, NAC, or Carnosine for 3 hours, then treated with 400 μg/mL AGEs-BSA for 24 hours. We

found that the 100 μg/mL of F. ovata extracts from petroleum ether and methanol significantly decreased cell apoptosis. NAC and Carnosine treatment ranging from 2.5, 5 and 10 mM were also revealed a significantly reduction of apoptotic cells compared to the cells undergoing AGEs activation alone (Figure 27).

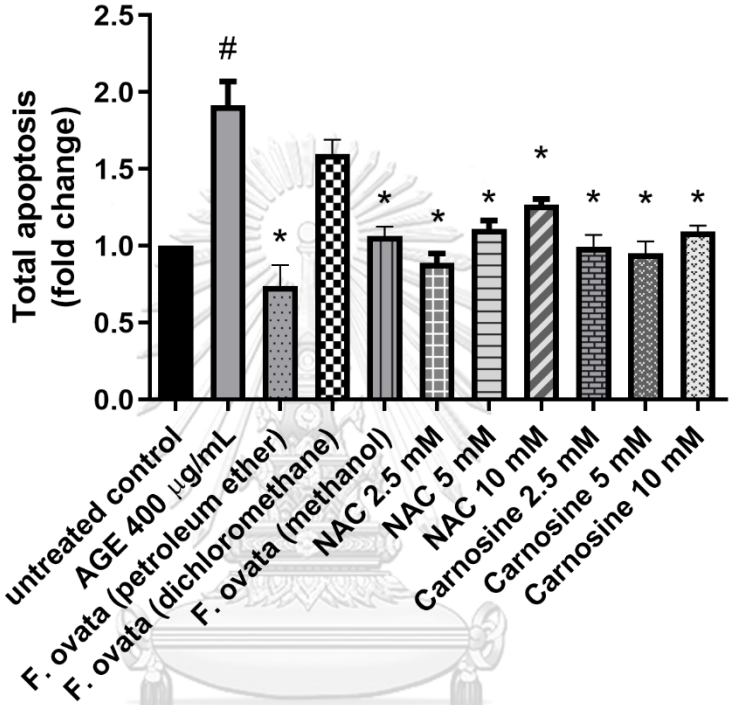


Figure 27: The effect of F. ovata, NAC, and Carnosine on total apoptotic events under AGE treatment

CHAPTER V
CONCLUSION AND DISCUSSION

Alzheimer's disease is the most prevalent form of dementia in the elderly. The disease is an irreversible and progressive neurodegenerative disorder without effective treatment. Some underlying pathological mechanisms of Alzheimer's disease have been conceptualized including amyloid cascade pathway, excitotoxicity, alteration in oxidative biology, inflammation and mitochondrial dysfunction. Neuroinflammation and oxidative stress play crucial role in the Alzheimer's disease pathogenesis. The injuries caused by oxidative stress occur as a result of the imbalance between pro-oxidants and anti-oxidants leading to the accumulation of cytotoxic compounds and their counterpart such as reactive oxygen species (ROS). The receptor for advanced glycation end products (RAGE) is multi-ligand receptor which not only binds advanced glycation end-products (AGEs), but also binds amyloid-β (Aβ). RAGE can mediate transport of Aβ across the blood-brain barrier (BBB) lead to Aβ deposition in the brain. Through the RAGE/ligand interaction, both AGEs and Aβ elicit the production of ROS and pro-inflammatory cellular signaling which are also believed to occur early in Alzheimer's disease pathology. Upon exposure of the optimized AGEs, RAGE expression is expected to increase (Figure 28a). In response to this activation, accumulation of ROS is predictable (Figure 28b). The elevation of such ROS is expected to triggers the activation of nuclear factor-kappa B (NF-**K**B) which is a primary transcription factor that plays an important role in regulating cellular responses (Figure 28c). In addition, increase in program cell death is likely to follow this activation (Figure 28d). As a consequence, these cascades lead

to neuronal degeneration (Figure 28e). Alzheimer's disease is considered to be one of the neurodegenerative disease which have been reported to be involved in the above mentioned pathway.

N-Acetyl Cysteine (NAC), a dietary supplement, which is a precursor to the amino acid cysteine, was found in this study to be able to decrease MAPK activation and cell apoptosis in AGE- induced oxidative stress SH-SY5Y ($p<0.05$) (Figure 28f). NAC plays two important metabolic roles. First, exposure to NAC leads to a large increase in intracellular glutathione (GSH) production which participates in the general antioxidant activities of the body (52). Second, NAC plays a crucial role as a modulator of the glutamatergic system by supplying cysteine (53). GSH is cysteine derivative which exert its role as a redox scavenger. Previous reports have been demonstrated that taking GSH or cysteine orally is toxic and not effectively transported into the cells (54, 65). In contrast to NAC, a precursor to the amino acid cysteine can be ingested orally and acts as a precursor for the GSH synthesis (54, 65). Additionally, NAC itself shows a detoxifying property as a scavenger for ROS, particularly the reduction of the hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). This is partly due to its thiol group (66, 67). Recent reports have suggested that NAC may significantly augment the clinical efficacy on neurological disorders including Alzheimer's disease treatment (71-77). These effects are perhaps due to the above mentioned functions.

Carnosine (β-alanyl-L-histidine) is a natural dipeptide which probably only present in animals especially, in skeletal muscle and the brain. Even though, its physiological role is not completely understood, several studies indicate that the carnosine may exert its protective effect on oxidative damage-related disorders

including Alzheimer's disease (90-92). Carnosine level was found to be lower in individuals with Alzheimer's disease compared to normal control (91). On the other hand, carnosinase activity, a carnosine proteolysis was significantly higher in aging brain (93). Study in Alzheimer animal model has indicated that carnosine decrease intraneuronal Aβ deposition, show a positive trend in rescuing long-term memory deficits (94). Furthermore, supplemented carnosine was found to prevent cognitive decline by decreasing RAGE expression and microglia activation in Alzheimer mice model fed with high fat diet (95). Study in another type of dementia involving excessive zinc also found to be protected by carnosine (96, 97). Our result indicated that carnosine at the lowest concentration of 2.5 mM can significantly alleviate the MAPK activation and cell apoptosis which was increased upon AGE induction ($p<0.05$) (Figure 28f). Considering carnosine as an antioxidant, a metal-chelator, an anti-glycation agent, as well as inhibitor of Aβ toxicity, this compound is perhaps a relevant drug that could enhance clinical efficacy in Alzheimer's disease treatment. Hence, these two compounds may significantly augment the clinical efficacy on Alzheimer's disease treatment through its detoxifying property.

NF-**K**B is a transcription factor which involved in various physiological processes, including immune responses, cell proliferation and growth, synaptic plasticity and cell survival. Studies of NF-**K**B in CNS have been reported that among several regions and involving cells only B lymphocytes and neurons are known to have a high constitutive level of NF-**K**B activity. This constitutive form of NF-**K**B is believed to reflect the high metabolic activity of neurons and associated synaptic activity. There are several evidences suggesting that NF-**K**B is involved in learning and memory. By using several approaches to inhibit the activation of NF-**K**B or knockout

mice, the results revealed that NF-**K**B inhibition causing learning deficit, impaired spatial learning and memory. Moreover, previous evidence suggests that activation of NF-**K**B activity is required for neuronal survival. Activation of NF-**K**B has been reported in several neurological diseases including AD. NF-**K**B activation could be detected around all plaque types in AD subjects. It has been suggested that activation of NF-**K**B in neurons promotes their survival, whereas activation of NF-**K**B in glial cells is detrimental to neurons (101-103). Our results revealed that *F. ovata* extracts and NAC significantly increase activation of NF-**K**B ($p<0.05$) (Figure 28g). Hence, these compounds perhaps act as neuroprotective agent by increasing signal activation through this pathway.

Fimbristylis ovata (Burm.f.) Kern, a plant which its application has been reported in various diseases including anti-Candida, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, antibacterial, antioxidant, cytotoxic and apoptotic, anti-pyretic and analgesic activities (99). The plant belongs to the same family has been previously reported to be a neuroprotective substance against various pathophysiological mechanisms including excitotoxicity, inflammation and apoptosis (100). Previous studies of *F. ovata* extract which has been firstly reported in cultured monocytes and epithelial cells showed that this extract significantly decreases those inflammatory cytokines under oxidative stress induction (7). Our results also revealed the same effect as an antioxidant by decreasing intracellular superoxide radical production (petroleum ether and methanol fractions) ($p<0.05$) (Figure 28i). Moreover, we found that *F. ovata* reduced MAPK activation (dichloromethane and methanol) and enhanced the cell survival (petroleum ether

and methanol) ($p<0.05$) (Figure 28j). Hence this extract might as well exert its protective effects in neuronal cells undergoing oxidative stress condition by AGEs.

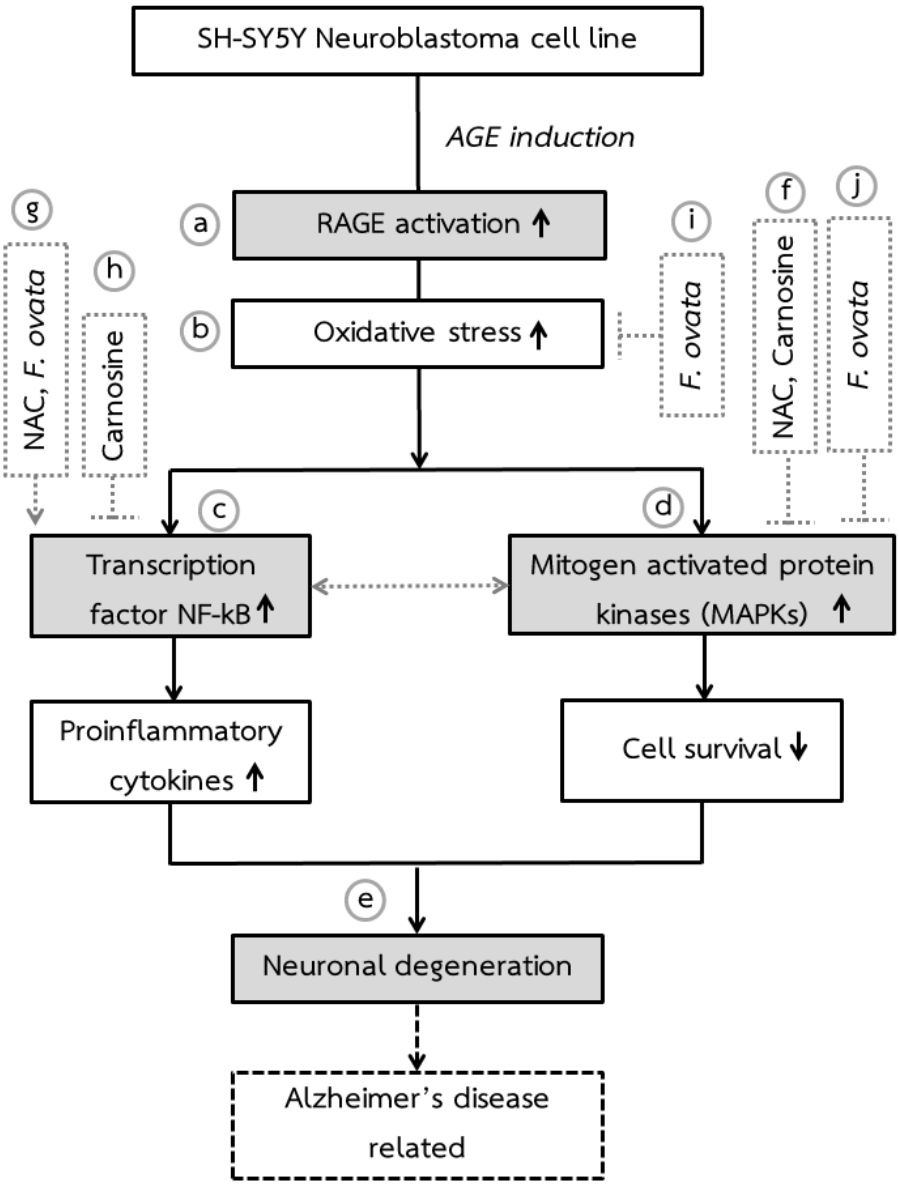


Figure 28: The schematic diagram represents the conclusion of this work

REFERENCES

1. Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet. 2006;368(9533):387-403.
2. ศิริพันธุ์ สาสัตย์ และคณะ. รายงานผลการวิจัยฉบับสมบูรณ์ เรื่อง โครงการการศึกษาสถานดูแลผู้สูงอายุระยะยาวในประเทศไทย. กรุงเทพฯ: สถาบันวิจัยสาธารณสุขไทย มูลนิธิสถาบันวิจัยและพัฒนาผู้สูงอายุไทย; 2552.
3. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends in pharmacological sciences. 1991;12(10):383-8.
4. Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, et al. Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science. 1996;274(5284):99-102.
5. Galasko D, Montine TJ. Biomarkers of oxidative damage and inflammation in Alzheimer's disease. Biomark Med. 2010;4(1):27-36.
6. Taupin P. A dual activity of ROS and oxidative stress on adult neurogenesis and Alzheimer's disease. Cent Nerv Syst Agents Med Chem. 2010;10(1):16-21.
7. Sukjamnong S, Santiyanont R. Effect of Fimbristylis ovata on receptor for advanced glycation end-products, proinflammatory cytokines, and cell adhesion molecule level and gene expression in U937 and bEnd.3 cell lines. Genet Mol Res. 2015;14(2):3984-94.
8. Prince M, Bryce R, Albanese E, Wimo A, Ribeiro W, Ferri CP. The global prevalence of dementia: a systematic review and metaanalysis. Alzheimer's & dementia : the journal of the Alzheimer's Association. 2013;9(1):63-75 e2.
9. Markesbery WR. Oxidative stress hypothesis in Alzheimer's disease. Free radical biology & medicine. 1997;23(1):134-47.
10. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiological reviews. 2001;81(2):741-66.
11. Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. Cell. 2005;120(4):545-55.
12. Pratico D. Oxidative stress hypothesis in Alzheimer's disease: a reappraisal. Trends in pharmacological sciences. 2008;29(12):609-15.
13. Dong XX, Wang Y, Qin ZH. Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta pharmacologica Sinica. 2009;30(4):379-87.
14. Padurariu M, Ciobica A, Lefter R, Serban IL, Stefanescu C, Chirita R. The oxidative stress hypothesis in Alzheimer's disease. Psychiatria Danubina. 2013;25(4):401-9.

15. Mattson MP. Pathways towards and away from Alzheimer's disease. Nature. 2004;430(7000):631-9.
16. Querfurth HW, LaFerla FM. Alzheimer's Disease. New England Journal of Medicine. 2010;362(4):329-44.
17. Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. Cardiovascular research. 1998;37(3):586-600.
18. Gkogkolou P, Bohm M. Advanced glycation end products: Key players in skin aging? Dermato-endocrinology. 2012;4(3):259-70.
19. Sugimoto K, Yasujima M, Yagihashi S. Role of advanced glycation end products in diabetic neuropathy. Current pharmaceutical design. 2008;14(10):953-61.
20. Ko SY, Ko HA, Chu KH, Shieh TM, Chi TC, Chen HI, et al. The Possible Mechanism of Advanced Glycation End Products (AGEs) for Alzheimer's Disease. PloS one. 2015;10(11):e0143345.
21. Soman S, Raju R, Sandhya VK, Advani J, Khan AA, Harsha HC, et al. A multicellular signal transduction network of AGE/RAGE signaling. Journal of cell communication and signaling. 2013;7(1):19-23.
22. Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, et al. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature. 1996;382(6593):685-91.
23. Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H. Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. The Journal of biological chemistry. 2000;275(51):40096-105.
24. Yamagishi S. Role of advanced glycation end products (AGEs) and receptor for AGEs (RAGE) in vascular damage in diabetes. Experimental gerontology. 2011;46(4):217-24.
25. Sattler R, Tymianski M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Molecular neurobiology. 2001;24(1-3):107-29.
26. Hastings TG. The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. Journal of bioenergetics and biomembranes. 2009;41(6):469-72.
27. Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R, et al. Advanced glycation end products contribute to amyloidosis in Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(11):4766-70.

28. Munch G, Mayer S, Michaelis J, Hipkiss AR, Riederer P, Muller R, et al. Influence of advanced glycation end-products and AGE-inhibitors on nucleation-dependent polymerization of beta-amyloid peptide. Biochimica et biophysica acta. 1997;1360(1):17-29.
29. Wautier JL, Zoukourian C, Chappey O, Wautier MP, Guillausseau PJ, Cao R, et al. Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy. Soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats. The Journal of clinical investigation. 1996;97(1):238-43.
30. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell. 1999;97(7):889-901.
31. Yan SS, Wu ZY, Zhang HP, Furtado G, Chen X, Yan SF, et al. Suppression of experimental autoimmune encephalomyelitis by selective blockade of encephalitogenic T-cell infiltration of the central nervous system. Nature medicine. 2003;9(3):287-93.
32. Deane R, Wu Z, Zlokovic BV. RAGE (yin) versus LRP (yang) balance regulates alzheimer amyloid beta-peptide clearance through transport across the blood-brain barrier. Stroke; a journal of cerebral circulation. 2004;35(11 Suppl 1):2628-31.
33. Chen X, Walker DG, Schmidt AM, Arancio O, Lue LF, Yan SD. RAGE: a potential target for Abeta-mediated cellular perturbation in Alzheimer's disease. Current molecular medicine. 2007;7(8):735-42.
34. Toth C, Martinez J, Zochodne DW. RAGE, diabetes, and the nervous system. Current molecular medicine. 2007;7(8):766-76.
35. Maczurek A, Shanmugam K, Munch G. Inflammation and the redox-sensitive AGE-RAGE pathway as a therapeutic target in Alzheimer's disease. Annals of the New York Academy of Sciences. 2008;1126:147-51.
36. Qin J, Goswami R, Dawson S, Dawson G. Expression of the receptor for advanced glycation end products in oligodendrocytes in response to oxidative stress. Journal of neuroscience research. 2008;86(11):2414-22.
37. Ilzecka J. Serum-soluble receptor for advanced glycation end product levels in patients with amyotrophic lateral sclerosis. Acta neurologica Scandinavica. 2009;120(2):119-22.
38. Li J, Schmidt AM. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. The Journal of biological chemistry. 1997;272(26):16498-506.

39. Mackic JB, Stins M, McComb JG, Calero M, Ghiso J, Kim KS, et al. Human blood-brain barrier receptors for Alzheimer's amyloid-beta 1- 40. Asymmetrical binding, endocytosis, and transcytosis at the apical side of brain microvascular endothelial cell monolayer. The Journal of clinical investigation. 1998;102(4):734-43.
40. Deane R, Du Yan S, Submamaryan RK, LaRue B, Jovanovic S, Hogg E, et al. RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. Nature medicine. 2003;9(7):907-13.
41. Donahue JE, Flaherty SL, Johanson CE, Duncan JA, 3rd, Silverberg GD, Miller MC, et al. RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. Acta neuropathologica. 2006;112(4):405-15.
42. Miller MC, Tavares R, Johanson CE, Hovanesian V, Donahue JE, Gonzalez L, et al. Hippocampal RAGE immunoreactivity in early and advanced Alzheimer's disease. Brain research. 2008;1230:273-80.
43. Brownlee M. Advanced protein glycosylation in diabetes and aging. Annual review of medicine. 1995;46:223-34.
44. Takeda A, Yasuda T, Miyata T, Goto Y, Wakai M, Watanabe M, et al. Advanced glycation end products co-localized with astrocytes and microglial cells in Alzheimer's disease brain. Acta neuropathologica. 1998;95(6):555-8.
45. Sasaki N, Fukatsu R, Tsuzuki K, Hayashi Y, Yoshida T, Fujii N, et al. Advanced glycation end products in Alzheimer's disease and other neurodegenerative diseases. The American journal of pathology. 1998;153(4):1149-55.
46. Mruthinti S, Sood A, Humphrey CL, Swamy-Mruthinti S, Buccafusco JJ. The induction of surface beta-amyloid binding proteins and enhanced cytotoxicity in cultured PC-12 and IMR-32 cells by advanced glycation end products. Neuroscience. 2006;142(2):463-73.
47. Rao AM, Drake MR, Stipanuk MH. Role of the transsulfuration pathway and of gamma-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes. The Journal of nutrition. 1990;120(8):837-45.
48. Finkelstein JD. The metabolism of homocysteine: pathways and regulation. Eur J Pediatr. 1998;157(2):S40-S4.
49. Holdiness MR. Clinical pharmacokinetics of N-acetylcysteine. Clinical pharmacokinetics. 1991;20(2):123-34.
50. Cotgreave IA, Moldeus P. Methodologies for the analysis of reduced and oxidized N-acetylcysteine in biological systems. Biopharmaceutics & drug disposition. 1987;8(4):365-75.

51. Olsson B, Johansson M, Gabrielsson J, Bolme P. Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. European journal of clinical pharmacology. 1988;34(1):77-82.
52. Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. Journal of amino acids. 2012;2012:736837.
53. Bridges R, Lutgen V, Lobner D, Baker DA. Thinking outside the cleft to understand synaptic activity: contribution of the cystine-glutamate antiporter (System xc-) to normal and pathological glutamatergic signaling. Pharmacological reviews. 2012;64(3):780-802.
54. Sen CK. Nutritional biochemistry of cellular glutathione. The Journal of nutritional biochemistry. 1997;8(12):660-72.
55. Sato H, Tamba M, Okuno S, Sato K, Keino-Masu K, Masu M, et al. Distribution of cystine/glutamate exchange transporter, system x(c)-, in the mouse brain. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002;22(18):8028-33.
56. Kau KS, Madayag A, Mantsch JR, Grier MD, Abdulhameed O, Baker DA. Blunted cystine-glutamate antiporter function in the nucleus accumbens promotes cocaine-induced drug seeking. Neuroscience. 2008;155(2):530-7.
57. Moran MM, McFarland K, Melendez RI, Kalivas PW, Seamans JK. Cystine/glutamate exchange regulates metabotropic glutamate receptor presynaptic inhibition of excitatory transmission and vulnerability to cocaine seeking. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005;25(27):6389-93.
58. Kantrowitz JT, Javitt DC. N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: the final common pathway on the road to schizophrenia? Brain research bulletin. 2010;83(3-4):108-21.
59. Lewerenz J, Hewett SJ, Huang Y, Lambros M, Gout PW, Kalivas PW, et al. The cystine/glutamate antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic opportunities. Antioxidants & redox signaling. 2013;18(5):522-55.
60. Rudy CC, Hunsberger HC, Weitzner DS, Reed MN. The role of the tripartite glutamatergic synapse in the pathophysiology of Alzheimer's disease. Aging and disease. 2015;6(2):131-48.
61. Looney JM, Childs HM. THE LACTIC ACID AND GLUTATHIONE CONTENT OF THE BLOOD OF SCHIZOPHRENIC PATIENTS. The Journal of clinical investigation. 1934;13(6):963-8.

62. Altschule MD, Siegel EP, Henneman DH. Blood glutathione level in mental disease before and after treatment. AMA archives of neurology and psychiatry. 1952;67(1):64-8.
63. Altuntas I, Aksoy H, Coskun I, Caykoylu A, Akcay F. Erythrocyte superoxide dismutase and glutathione peroxidase activities, and malondialdehyde and reduced glutathione levels in schizophrenic patients. Clinical chemistry and laboratory medicine : CCLM / FESCC. 2000;38(12):1277-81.
64. Bilici M, Efe H, Koroglu MA, Uydu HA, Bekaroglu M, Deger O. Antioxidative enzyme activities and lipid peroxidation in major depression: alterations by antidepressant treatments. Journal of affective disorders. 2001;64(1):43-51.
65. Dilger RN, Baker DH. Oral N-acetyl-L-cysteine is a safe and effective precursor of cysteine. Journal of animal science. 2007;85(7):1712-8.
66. Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free radical biology & medicine. 1989;6(6):593-7.
67. Agnihotri N, Mishra PC. Mechanism of scavenging action of N-acetylcysteine for the OH radical: a quantum computational study. The journal of physical chemistry B. 2009;113(35):12096-104.
68. Yan CY, Ferrari G, Greene LA. N-acetylcysteine-promoted survival of PC12 cells is glutathione-independent but transcription-dependent. The Journal of biological chemistry. 1995;270(45):26827-32.
69. Moreira PI, Harris PL, Zhu X, Santos MS, Oliveira CR, Smith MA, et al. Lipoic acid and N-acetyl cysteine decrease mitochondrial-related oxidative stress in Alzheimer disease patient fibroblasts. Journal of Alzheimer's disease : JAD. 2007;12(2):195-206.
70. Brera B, Serrano A, de Ceballos ML. beta-amyloid peptides are cytotoxic to astrocytes in culture: a role for oxidative stress. Neurobiology of disease. 2000;7(4):395-405.
71. Adair JC, Knoefel JE, Morgan N. Controlled trial of N-acetylcysteine for patients with probable Alzheimer's disease. Neurology. 2001;57(8):1515-7.
72. Robinson RA, Joshi G, Huang Q, Sultana R, Baker AS, Cai J, et al. Proteomic analysis of brain proteins in APP/PS-1 human double mutant knock-in mice with increasing amyloid beta-peptide deposition: insights into the effects of in vivo treatment with N-acetylcysteine as a potential therapeutic intervention in mild cognitive impairment and Alzheimer's disease. Proteomics. 2011;11(21):4243-56.

73. Hsiao YH, Kuo JR, Chen SH, Gean PW. Amelioration of social isolation-triggered onset of early Alzheimer's disease-related cognitive deficit by N-acetylcysteine in a transgenic mouse model. Neurobiology of disease. 2012;45(3):1111-20.
74. Pocernich CB, Butterfield DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease. Biochimica et biophysica acta. 2012;1822(5):625-30.
75. Unnithan AS, Jiang Y, Rumble JL, Pulugulla SH, Posimo JM, Gleixner AM, et al. N-acetyl cysteine prevents synergistic, severe toxicity from two hits of oxidative stress. Neuroscience letters. 2014;560:71-6.
76. Bavarsad Shahripour R, Harrigan MR, Alexandrov AV. N-acetylcysteine (NAC) in neurological disorders: mechanisms of action and therapeutic opportunities. Brain and behavior. 2014;4(2):108-22.
77. Gamba P, Guglielmotto M, Testa G, Monteleone D, Zerbinati C, Gargiulo S, et al. Up-regulation of beta-amyloidogenesis in neuron-like human cells by both 24- and 27-hydroxycholesterol: protective effect of N-acetyl-cysteine. Aging cell. 2014;13(3):561-72.
78. Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. Physiological reviews. 2013;93(4):1803-45.
79. Boldyrev AA, Dupin AM, Bunin A, Babizhaev MA, Severin SE. The antioxidative properties of carnosine, a natural histidine containing dipeptide. Biochemistry international. 1987;15(6):1105-13.
80. Boldyrev AA, Dupin AM, Pindel EV, Severin SE. Antioxidative properties of histidine-containing dipeptides from skeletal muscles of vertebrates. Comparative biochemistry and physiology B, Comparative biochemistry. 1988;89(2):245-50.
81. Boldyrev AA, Dupin AM, Batrukova MA, Bavykina NI, Korshunova GA, Shvachkin Yu P. A comparative study of synthetic carnosine analogs as antioxidants. Comparative biochemistry and physiology B, Comparative biochemistry. 1989;94(2):237-40.
82. Liu WH, Liu TC, Yin MC. Beneficial effects of histidine and carnosine on ethanol-induced chronic liver injury. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2008;46(5):1503-9.
83. Ozel Turkcu U, Bilgihan A, Biberoglu G, Mertoglu Caglar O. Carnosine supplementation protects rat brain tissue against ethanol-induced oxidative stress. Molecular and cellular biochemistry. 2010;339(1-2):55-61.
84. Dursun N, Taskin E, Ozturk F. Protection against adriamycin-induced cardiomyopathy by carnosine in rats: role of endogenous antioxidants. Biological trace element research. 2011;143(1):412-24.
85. Hipkiss AR, Michaelis J, Syrris P. Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. FEBS letters. 1995;371(1):81-5.

86. Hipkiss AR, Chana H. Carnosine protects proteins against methylglyoxal-mediated modifications. Biochemical and biophysical research communications. 1998;248(1):28-32.
87. Aldini G, Facino RM, Beretta G, Carini M. Carnosine and related dipeptides as quenchers of reactive carbonyl species: from structural studies to therapeutic perspectives. BioFactors (Oxford, England). 2005;24(1-4):77-87.
88. Cheng J, Wang F, Yu DF, Wu PF, Chen JG. The cytotoxic mechanism of malondialdehyde and protective effect of carnosine via protein cross-linking/mitochondrial dysfunction/reactive oxygen species/MAPK pathway in neurons. European journal of pharmacology. 2011;650(1):184-94.
89. Bellia F, Vecchio G, Rizzarelli E. Carnosinases, their substrates and diseases. Molecules (Basel, Switzerland). 2014;19(2):2299-329.
90. Carini M, Aldini G, Beretta G, Arlandini E, Facino RM. Acrolein-sequestering ability of endogenous dipeptides: characterization of carnosine and homocarnosine/acrolein adducts by electrospray ionization tandem mass spectrometry. Journal of mass spectrometry : JMS. 2003;38(9):996-1006.
91. Fonteh AN, Harrington RJ, Tsai A, Liao P, Harrington MG. Free amino acid and dipeptide changes in the body fluids from Alzheimer's disease subjects. Amino acids. 2007;32(2):213-24.
92. Hipkiss AR. Could carnosine or related structures suppress Alzheimer's disease? Journal of Alzheimer's disease : JAD. 2007;11(2):229-40.
93. Bellia F, Calabrese V, Guarino F, Cavallaro M, Cornelius C, De Pinto V, et al. Carnosinase levels in aging brain: redox state induction and cellular stress response. Antioxidants & redox signaling. 2009;11(11):2759-75.
94. Corona C, Frazzini V, Silvestri E, Lattanzio R, La Sorda R, Piantelli M, et al. Effects of dietary supplementation of carnosine on mitochondrial dysfunction, amyloid pathology, and cognitive deficits in 3xTg-AD mice. PloS one. 2011;6(3):e17971.
95. Herculano B, Tamura M, Ohba A, Shimatani M, Kutsuna N, Hisatsune T. beta-alanyl-L-histidine rescues cognitive deficits caused by feeding a high fat diet in a transgenic mouse model of Alzheimer's disease. Journal of Alzheimer's disease : JAD. 2013;33(4):983-97.
96. Mizuno D, Kawahara M. The molecular mechanisms of zinc neurotoxicity and the pathogenesis of vascular type senile dementia. International journal of molecular sciences. 2013;14(11):22067-81.

97. Kawahara M, Mizuno D, Koyama H, Konoha K, Ohkawara S, Sadakane Y. Disruption of zinc homeostasis and the pathogenesis of senile dementia. Metallomics : integrated biometal science. 2014;6(2):209-19.
98. Sharma OP. Plant Taxonomy: Tata McGraw-Hill Publ.; 1993.
99. Sivapalan SRS. Medicinal uses and Pharmacological activities of Cyperus rotundus Linn – A Review2013. 2250-3153 p.
100. Sunil AG, Kesavanarayanan KS, Kalaivani P, Sathiya S, Ranju V, Priya RJ, et al. Total oligomeric flavonoids of Cyperus rotundus ameliorates neurological deficits, excitotoxicity and behavioral alterations induced by cerebral ischemic–reperfusion injury in rats. Brain research bulletin. 2011;84(6):394-405.
101. Camandola S, Mattson MP. NF-**K**B as a therapeutic target in neurodegenerative diseases. Expert Opinion on Therapeutic Targets. 2007;11(2):123-32.
102. Mattson MP, Camandola S. NF-kappaB in neuronal plasticity and neurodegenerative disorders. The Journal of clinical investigation. 2001;107(3):247-54.
103. Lanzillotta A, Porrini V, Bellucci A, Benarese M, Branca C, Parrella E, et al. NF-**K**B in Innate Neuroprotection and Age-Related Neurodegenerative Diseases. Frontiers in neurology. 2015;6:98-.

VITA

NAME	SUPHASARANG SIRIRATTANAKUL
DATE OF BIRTH	5 November 1986
PLACE OF BIRTH	Yasothon
INSTITUTIONS ATTENDED	Chulalongkorn University
HOME ADDRESS	79/58 Mitraphap Rd., Amphoe Muang, Khon Kaen, Thailand 40000