ผลของสารสกัดพืชสมุนไพรต่อกลไกภาวะเครียดออกซิเดชัน และการอักเสบทางประสาทในเซลล์
ประสาทเพาะเลี้ยง:รูปแบบการป้องกันการเกิดโรคอัลไซเมอร์

นางสาววริษา อมรฤทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
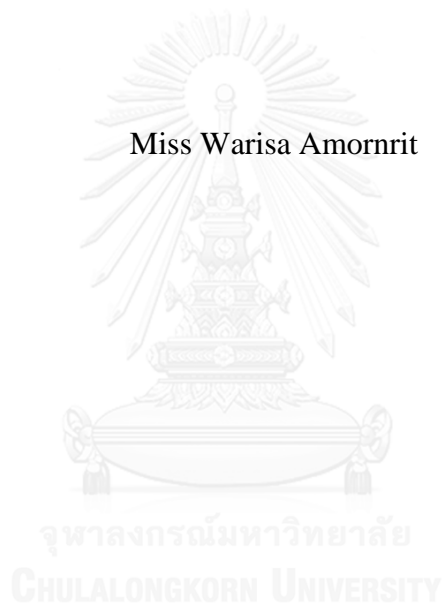สาขาวิชาชีวเคมีคลินิกและอณูทางการแพทย์ ภาควิชาเคมีคลินิก
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EFFECTS OF HERBAL PLANT EXTRACTS ON OXIDATIVE STRESS AND
NEUROINFLAMMATORY MECHANISMS IN CULTURED NEURONAL CELLS
: A MODEL FOR ALZHEIMER'S DISEASE PREVENTION

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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วริษา อมรฤทธิ์ : ผลของสารสกัดพืชสมุนไพรต่อกลไกภาวะเครียดออกซิเดชัน และการอักเสบทางประสาท ในเซลล์ประสาทเพาะเลี้ยง:รูปแบบการป้องกันการเกิดโรคอัลไซเมอร์ (EFFECTS OF HERBAL PLANT EXTRACTS ON OXIDATIVE STRESS AND NEUROINFLAMMATORY MECHANISMS IN CULTURED NEURONAL CELLS: A MODEL FOR ALZHEIMER'S DISEASE PREVENTION) อ.ที่ ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. รัชนา ศานติยานนท์, 208 หน้า.

การแพทย์แผนโบราณได้มีการนำพืชสมุนไพรหลายชนิดที่ประกอบด้วยสารต่างๆซึ่งมีคุณสมบัติมากมายมาใช้ ในการรักษาโรค ผักโขมเป็นพืชสมุนไพรที่เปี่ยมด้วยสารต้านอนุมูลอิสระ มีหน้าที่ป้องกันไม่ให้อนุมูลอิสระก่อตัวขึ้นได้ จึงเป็นพืชสมุนไพรที่มีสรรพคุณทางการแพทย์ โรคต่างๆที่มาจากความผิดปกติของระบบประสาท เช่น โรคอัลไซเมอร์ เกิดจากภาวะเครียดออกซิเดชันจากการที่มีอนุมูลอิสระมากเกินและภาวะอักเสบทางประสาท ส่งผลให้กลไกการทำหน้าที่ ของระบบประสาทผิดปกติ รวมถึงการสูญเสียเซลล์ประสาท นอกจากนี้สารพิษที่เกิดจากขบวนการไกลเคชั่นคือ Advanced glycation endproducts หรือ AGEs เมื่อจับกับตัวรับของมัน (AGEs receptor, RAGE) ในร่างกายจะทำให้เกิด อนุมูลอิสระที่ทำให้เซลล์บริเวณนั้นตายหรือเสื่อมสมรรถภาพในการทำงาน ส่งผลกระทบต่อเซลล์สมองก่อให้เกิดโรคอัล ไซเมอร์ได้เช่นกัน ในปัจจุบันยังไม่มีการศึกษาถึงฤทธิ์ของสารสกัดจากผักโขมต่อการปกป้องเซลล์ประสาทจากการถูก ทำลายด้วย AGEs ที่ก่อให้เกิดพยาธิสภาพของโรคอัลไซเมอร์ การศึกษานี้จึงได้ตรวจสอบการออกฤทธิ์ของสารสกัดใน ส่วนของปิโตรเลียมอีเทอร์ ไดคลอโรมีเทน และ เมทานอลจากผักโขมไทย และผักโขมสวน พบว่าสารสกัดจากผักโขมทั้ง สองชนิดที่มาจากตัวทำละลายเมทานอลมีความสามารถในการต้านอนุมูลอิสระสูงที่สุด เมื่อทำการทดสอบฤทธิ์ของสาร สังเคราะห์ AGEs ต่อการเหนี่ยวนำให้เซลล์ประสาทเพาะเลี้ยง (SH-SY5Y) เกิดภาวะเครียดออกซิเดชัน พบว่า AGEs สังเคราะห์ทำให้เกิดอนุมูลอิสระภายในเซลล์เพิ่มขึ้น และสามารถเพิ่มการแสดงออกของยีนเอนไซม์ต่อต้านออกซิเดชัน HMOX-1 ซึ่งส่งผลทำให้จำนวนเซลล์ประสาทมีชีวิตลดลงและเพิ่มความเป็นพิษภายในเซลล์ นอกจากนี้สารสังเคราะห์ AGEs ยังสามารถเพิ่มการแสดงออกของยีน RAGE ซึ่งเป็นตัวรับของมัน แล้วส่งผลต่อไปยังการเพิ่มการแสดงออกของยีน ในวิถี NF-kB เพิ่มการแสดงออกของยีนเอนไซม์ BACE1 และ PS1 ที่ใช้ในการผลิตเส้นใยแอมีลอยด์ บีตา (Aβ peptide) ซึ่งเป็นพยาธิสภาพของโรคอัลไซเมอร์ และมีการแสดงออกของยีนไซโตไคน์ที่กระตุ้นให้เกิดการอักเสบ TNF-α, IL-1 และ IL-6 ในระดับสูง เมื่อศึกษาฤทธิ์ของสารสกัดต่อการปกป้องเซลล์ประสาทจากการถูกทำลายด้วย AGEs พบว่าสาร สกัดผักโขมทั้งสองชนิดสามารถลดภาวะเครียดออกซิเดชันที่เกิดภายในเซลล์ได้ และสามารถลดความเป็นพิษของสาร สังเคราะห์ AGEs ทำให้จำนวนเซลล์ประสาทมีชีวิตเพิ่มขึ้น นอกจากนี้สารสกัดผักโขมยังมีผลในการลดการแสดงออกของ ยีน HMOX-1 ยีน RAGE รวมถึงยีนต่างๆ ในวิถี NF-kB และลดการแสดงออกของยีนเอนไซม์ที่ใช้ในการผลิตเส้นใยแอมี ลอยด์ บีตา และไซโตไคน์ที่กระตุ้นให้เกิดการอักเสบอีกด้วย จากผลการศึกษาทั้งหมดบ่งชี้ว่าสารสกัดสมุนไพรจากผักโขม ทั้งสองชนิดนี้นอกจากจะมีผลปกป้องเซลล์ประสาทจากการทำลายด้วย AGEs แล้ว ยังมีฤทธิ์ต้านการอักเสบและยังยั้งการ เกิดเส้นใยแอมีลอยด์ บีตา โดยฤทธิ์ของสารสกัดเหล่านี้มีความเชื่อมโยงกับฤทธิ์ในการยับยั้งวิถี RAGE/NF-kB ในเซลล์ ผลการศึกษานี้จึงสนับสนุนการใช้ประโยชน์จากสารสกัดผักโขมในการรักษาภาวะบกพร่องในการรับรู้ที่เกิดจากโรคอัลไซ เมอร์ และอาจใช้เป็นแนวทางใหม่สำหรับการชะลอหรือหยุดการดำเนินของโรคอัลไซเมอร์ได้

ภาควิชา   เคมีคลินิก   ลายมือชื่อนิสิต _____

สาขาวิชา   ชีวเคมีคลินิกและอณูทางการแพทย์   ลายมือชื่อ อ.ที่ปรึกษาหลัก _____

ปีการศึกษา 2558

5377404337 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE

KEYWORDS: ADVANCED GLYCATION END-PRODUCTS (AGES) / OXIDATIVE STRESS / ALZHEIMER'S DISEASE / AMARANTHUS / SECRETASE / NEUROINFLAMMATION

WARISA AMORNRIT: EFFECTS OF HERBAL PLANT EXTRACTS ON OXIDATIVE STRESS AND NEUROINFLAMMATORY MECHANISMS IN CULTURED NEURONAL CELLS: A MODEL FOR ALZHEIMER'S DISEASE PREVENTION. ADVISOR: ASSOC. PROF. RACHANA SANTIYANONT, Ph.D., 208 pp.

Traditional medicine employs a wide range of native herbs containing a variety of substances which are used to treat several disorders. Amaranthus plants or spinach are rich in antioxidant compounds which play a role in scavenging free radicals and are known to possess medicinal properties. Oxidative stress caused by aberrant production of reactive oxygen species (ROS) and neuroinflammation represents important mechanisms for neuronal dysfunction and cell loss of many neurodegenerative diseases such as Alzheimer's disease (AD). Advanced glycation endproducts (AGEs) can lead to the pathological changes of AD by interaction with their receptor (RAGE) elicits the formation of ROS that are believed to occur early in AD pathology. So far no work has been reported on neuroprotective effect of Amaranthus extract on AGEs-induced AD pathology. In the present study, petroleum ether, dichloromethane and methanol were used to extract leaves of *Amaranthus lividus* and *Amaranthus tricolor*; the extracts were analyzed for antioxidant activity which was found to be the highest in the methanol fraction of both kinds of the plants. Human neuroblastoma cell lines, SH-SY5Y, were induced to oxidative damage upon incubation with AGEs as shown by an increase in oxidative stress as well as a significantly upregulated oxidative gene, HMOX-1 resulting in reducing cell viability and increasing cell toxicity in a dose dependent manner. AGEs could induce RAGE and the consequent activation of NF-κB genes expression and were able to upregulate BACE1, PS1 expression that involved in amyloid beta production, pathologic hallmark of AD, and gene expression of pro-inflammatory cytokines, TNF-α, IL-1 and IL-6. Upon incubation with *A. lividus* and *A. tricolor* extracts, they were effective at reducing oxidative stress and were dose dependently capable to attenuate the neuron toxicity caused by AGEs treatment. Interestingly, the extracts significantly decreased the expression of the HMOX-1, RAGE and NF-κB genes. Moreover, the results showed BACE1, PS1 and proinflammatory cytokine genes expression were significantly downregulated when AGEs-induced cells were treated with the plant extracts. The present data suggest that *A. lividus* and *A. tricolor* extracts not only have neuroprotective effect against AGEs-induced oxidative damage but also have anti-inflammatory activity by reducing pro-inflammatory cytokine gene expression and attenuate Alzheimer-like pathophysiological changes by down-regulating the key enzyme for amyloid beta production. The neuroprotective effects of these plants may be associated with their inhibitory actions via the RAGE/NF-kB pathway. The present data support the utilization of these plants for beneficial effect on the cognitive performance from AD and may provide a new to the possibility of using the herbal extracts for potential therapy of AD.

Department:	Clinical Chemistry	Student's Signature ....
Field of Study:	Clinical Biochemistry and Molecular Medicine	Advisor's Signature ....
Academic Year:	2015	

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CONTENTS

CHAPTER I

INTRODUCTION

Background and rationale

Alzheimer's disease (AD) is one of the most common age-related disorders [1, 2]. Among the characterized neuropathology of AD are amyloid plaques, neurofibrillary tangles and neuronal loss. Amyloid plaques are mainly composed of amyloid beta (Aβ) peptide which is extracellular deposits primarily composed of Aβ 40 and 42 amino acid residues. The Aβ peptide is derived from sequential proteolytic cleavage of the transmembrane amyloid precursor protein (APP) by β- and γ-secretase. In contrast, α-secretase cleaves APP within the Aβ domain, thus preventing Aβ generation [3-5]. AD can be classified into sporadic and familial forms, however, more than 90% of patients with AD appear to be sporadic [6]. The pathogenesis of sporadic AD has not yet to been identified, it is likely the consequence of several, different age-related factors. One of these risk factors is advanced glycation endproducts (AGEs), which are formed by the reaction of sugars with amino acid side chains followed by oxidations, dehydrations and rearrangements [7, 8]. Recent studies have suggested that AGEs are present in the core of senile plaques and in their vicinity [9] and they also induce inflammatory responses [10, 11]. Moreover, AGEs have been demonstrated to cause oxidative stress and cytotoxicity in neuronal cells [12-16]. Several studies have shown that oxidative stress increase in the early stages of AD, suggesting that this is an early event in the development of AD pathogenesis [17].

AGEs-mediated damage is through reactive oxygen species (ROS), particularly superoxide and hydrogen peroxide [18, 19]. Another mechanism through the interaction of AGEs with their receptor, RAGE (receptor for advanced glycation end products), trigger the generation of ROS, thereby increasing oxidative stress [20]. The mechanisms underlying the oxidative stress induced increasing of Aβ generation are unclear at present. Recent reports indicate that oxidative stress can upregulate the expression of β-secretase (BACE1) and γ-secretase complex [21, 22]. These observations suggest that oxidative stress is integral to AD pathogenesis. Engagement of the RAGE by AGEs also activates the signal transduction pathways of AGEs downstream of RAGE are transcription factors such as NF-κB which binds to the promoter sites of many pro-inflammatory cytokines and subsequently induce the expression of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF)-α [23]. Upon the onset of inflammatory process, the overexpression of cytokines in neuroinflammation produces many reactions in a vicious circle that cause dysfunction and neuronal death [23, 24].

The cause and progression of AD have not yet been clearly identified and the associated pathologies are multiple. Treatment strategies are also potentially multiple such as reducing protein aggregation pathology, preventing oxidative damage, countering specific neurotransmitter abnormalities, and promoting neuroplasticity [25].

At present, there is no cure for AD, available treatments offer only alleviation of the symptoms [1]. Owing to the limited effectiveness and potential side effects of the current AD medications, there is increasing interest in using herbal medicine as an alternative or complementary therapy for AD. However, pathological mechanisms of AGEs mediated oxidative stress leading to Aβ production and neuroinflammatory believed to be responsible for damage and death of neurons that occurs in AD have not been studied. Moreover, herbal plant extracts with relevant effects on their mechanisms of action that deal with the fundamental pathology of the disease would be interesting to investigate. Therefore, it is very interesting to study the protective effect of herbal plant extracts on AGEs-induced oxidative stress in neuroinflammatory mechanisms and the Aβ production on those *in vitro* model. Since AGEs mediated the production of oxidative stress is through the activation of RAGE, the first aim of the present study was to investigate whether oxidative stress induced by AGEs affected the expression of proinflammatory cytokines including IL-1, IL-6 and TNF-α and secretases enzyme cleavage of the APP processing including β-secreatase (BACE1), α-secretase (ADAM10) and presenilin1 (PS1) of γ-secretase complex by using human neuroblastoma SH-SY5Y cells exposed to AGEs which induced oxidative stress through RAGE. The second aim was also extend to examine the protective effect of plant extracts on oxidative stress and neuroinflammation-dependent damages in cultured neuronal cells induced by AGEs. Our results would provide the molecular evidences of anti-oxidative stress, anti-inflammatory as well as the prevention of Aβ production of plant extracts which eventually may be developed as therapeutic strategies for AD. The present study shall contribute to the possibility of using the herbal plant extracts for potential therapy of AD.

Review of related literature

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

1. **Advanced glycation endproducts (AGEs)**

   Over the past few decades, the discovery of AGEs comes from the investigation of brown-colored end products after the reaction of glucose with the amino acid peptides by L.C. Maillard in the early 1900s [26]. Thus, it comes to be known as the Maillard reaction. The spontaneous discovery of AGEs goes back to the food chemists' speculation the importance of the Maillard reaction in explaining brown color formation and loss of protein quality in cooked and stored foods. However, realization of the importance of Maillard-like reactions *in vivo* begin in the 1970s-1980s [27, 28]. The increasing evidences suggest that this process might be important in the complications of diabetes. There is until around 1980s and 1990s that researchers begin recognizing the significance of AGEs on various complications of diabetes and aging [29, 30]. Today, AGEs is in general believed that the formation of AGEs *in vivo* affects especially long lived proteins, resulting in alteration of protein structure and function which often involved in organ and vessel dysfunction [31, 32]. A recent study has reported the forming AGEs-derived free radicals cause protein fragmentation and oxidation of nucleic acids and lipids [33]. In addition, the amino groups of adenine and

guanine bases in DNA are also susceptible to glycation and AGEs formation, probably by reactive intracellular sugars [33]. AGEs also induce lipid peroxidation by a direct reaction between glucose and amino groups on phospholipids [34]. Moreover, AGEs are strong inducers of RAGE signaling [35]. Interaction of AGEs with RAGE causes oxidative stress and activation of several signalling pathways [36]. Due to AGEs can lead to further protein modifications and activation of a various signaling pathways, an elevated focus on AGEs-associated diseases such as in the development of complications of diabetes mellitus, as well as in the pathogenesis of cardiovascular, renal, and neurodegenerative diseases are greatly increased [37-40].

1.1 Formation of advanced glycation endproducts

AGEs are formed by a broad class of non-enzymatic products of reactions between proteins or lipids and reducing sugars. Glycation refers to a non-enzymatic addition of a protein. The protein glycation is initiated by a nucleophilic addition reaction between a carbonyl group of a reducing sugar or aldehyde with a free amino group, sulfhydryl, and guanidinyl functional groups in a protein to form a freely reversible Schiff base (Figure 1). Reducing sugars such as glucose, fructose, galactose, mannose, ribose are inherently reactive toward nucleophilic nitrogen bases in protein underlies the Maillard reaction [7]. This reaction is relatively fast over a period of hours, and highly reversible. Once formed a reversible Schiff base can be rearranged to a more stable ketoamine or a covalently-bound Amadori product as shown in Figure 1. In the Schiff base, the aldehydic carbon-oxygen double bond of the sugar is converted to a carbon-nitrogen double bond with the amine [7]. This rearrangement of the labile Sciiff base depends on the amount of sugars, free amino groups of the proteins as well as an alkaline pH value. Amadori rearrangements proceed at an acidic pH value and have a slower reaction rate which occurs over a period of days but once formed are practically irreversible [36]. The Amadori rearrangement depends on a histidine side-chain or another lysine amino group from the amino group on which the Schiff base has formed, due to localized acid-base catalysis. Protein glycation is a spontaneous reaction. The glycated proteins can undergo further reactions by break down via its enol form to reactive, free a-dicarbonyl glyoxal compounds which is a key intermediates in the formation of AGEs such as 3-deoxyglucosones (3-DG), methylglyoxal, and glyoxal, giving rise to poorly characterized structures called advanced glycation endproducts (AGEs) as shown in Figure 1 [7].
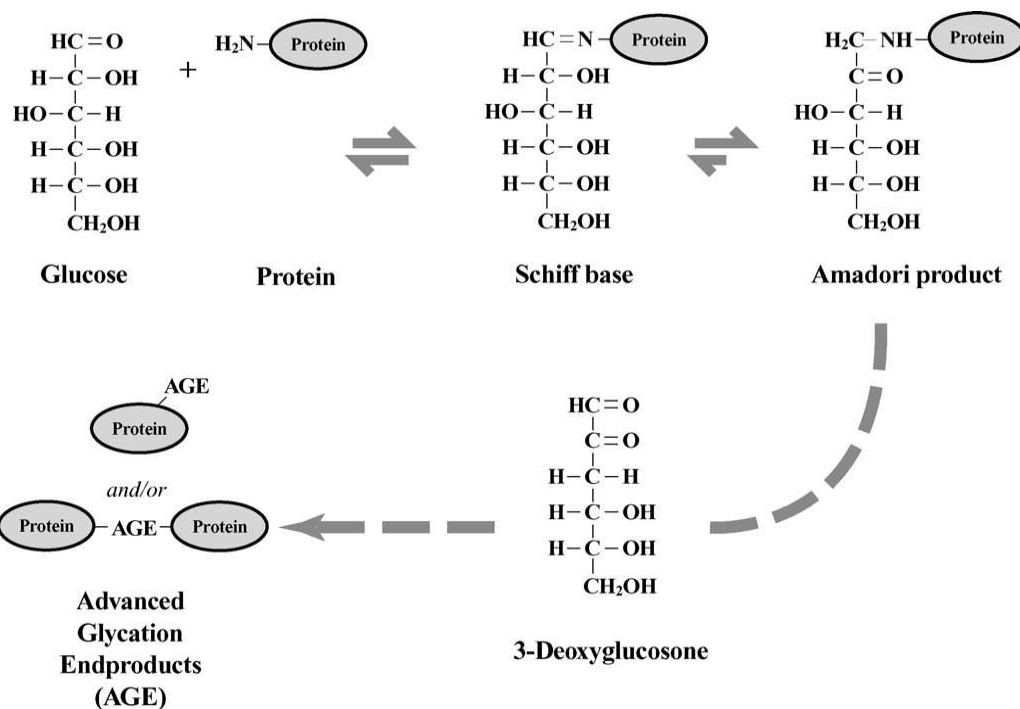
Figure 1. Formation of advanced glycation endproducts (AGEs). Glycation of a protein by glucose and the subsequent formation of AGEs. The initial reaction between glucose and protein amino groups forms a reversible Schiff base that rearranges to a ketoamine or Amadori product. These Amadori products undergo further reactions to form AGEs [8].

Since AGEs are complex, AGEs-forming processes are incompletely understood. Due to the different AGEs-precursors, not all AGEs have been identified. The products include heterogenous molecules that cause protein cross-linking, exhibit browning and generate fluorescence as well as various non-cross linked structures. AGEs can be divided into three categories [7, 8]:

A.) Fluorescent cross-linking AGEs

Along with brown color, fluorescence is one of the qualitative properties classically used to estimate AGEs formation. The fluorescent AGEs crosslinking accounts for only one percent or less of the total crosslinking structures formed under physiological conditions. Many of the AGEs such as pentosidine, pentodilysine, crossline, vesperlysine A, B and C are detected and isolated base on their fluorescent properties.

B.) Non-fluorescent cross-linking AGEs

The non-fluorescent AGEs cross-links are thought to account for the major AGEs structures responsible for protein-protein crosslinking *in vivo*. Known and suspected if non- fluorescence AGEs cross-links of physiological relevance such as pyrraline imine, glucosepa, imidazolium dilysine, aminoimidazoli imine, alkyl formyl glycosyl pyrrole (AFGP) and arginine-lysine imidazole (ALI).

C.) Non-cross-linking AGEs

Besides the crosslinking AGEs, which have profound effects on protein structure and function, a number of non-cross-linking AGEs have been found under physiological conditions. These AGEs may have deleterious effects as precursors of crosslinks or as biological receptor ligands inducing a variety of adverse cellular and tissue changes. Some noncrosslinking AGEs such as pyrraline, 1-carboxyalkyl group which attached to a free amino group of an amino acid residue; N^ε-(carboxymethyl)lysine (CML) and N^ε-(1-carboxyethyl)lysine (CEL), imidazolone A and B.

AGEs formation and accumulation are considered a source of oxidative stress. As mention above, AGEs formation depends on the concentration and reactivity of glucose, the availability of free amino groups and amount of AGEs-precursors. Monosaccharides, like glucose, can undergo auto-oxidation and generate hydroxyl radical (OH•) (The black dot indicates a radical) [41, 42]. The labile Schiff-base products and Amadori products themselves cause ROS production [41]. The monosaccharides normally exist in equilibrium with their enediol, which can undergo autoxidation in the presence of transition metals to form an enediol radical. The glucose becomes oxidise itself to a dicarbonyl ketoaldehye via its enediol radical whereas the radical reduces molecular oxygen to produce the superoxide radical (O_2•). This dicarbonyl ketoaldehyde reacts with a group of proteins amino to form a ketoimine. The ketoimines are similar to, although more reactive than, Amadori products and capable of forming AGEs. These steps are catalysed by transition metals and the generated superoxide radical which can be converted to the hydroxyl radical via the Fenton reaction [8]. This is referred to as autoxidative glycation and is shown in Figure 2.
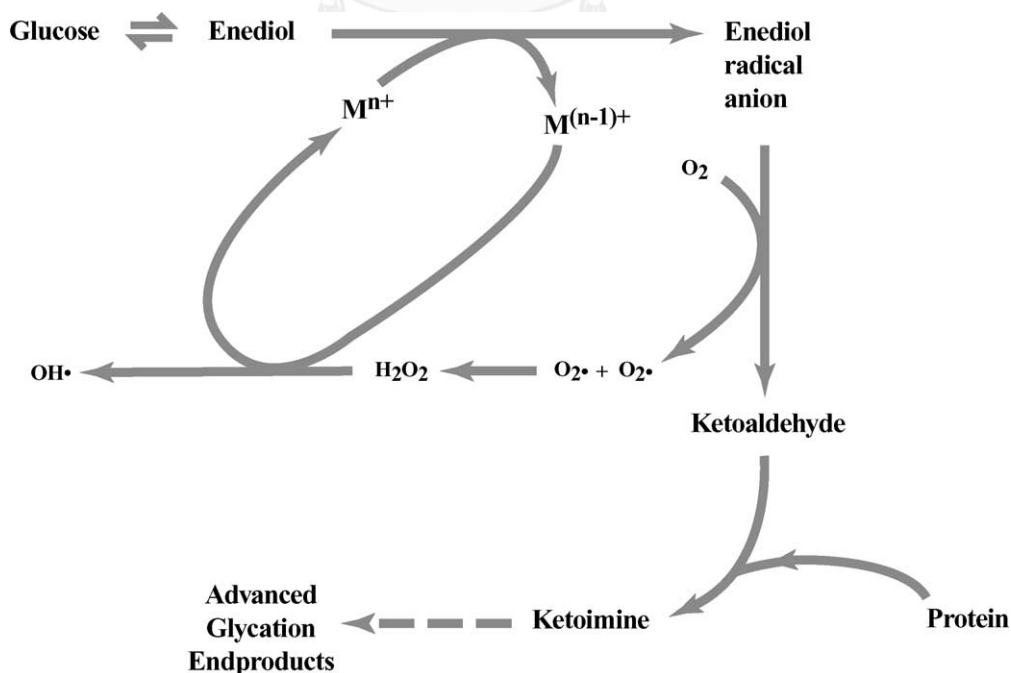
Figure 2. Autoxidative glycation of glucose [8].

In addition, the Amadori products are converted to protein dicarbonyl compounds via a protein enediol generating the superoxide radical in the presence of transition metals and molecular oxygen. As shown in Figure 3, the autoxidation of Amadori products to AGEs termed glycoxidation is used to describe autoxidation of Amadori product to a protein dicarbonyl compounds. These steps are catalysed by transition metals and the generated superoxide radicals can be converted to the highly reactive hydroxyl radical via the Fenton reaction. The protein dicarbonyl compounds can participate in AGEs formation and referred to as glycoxidation products [8].
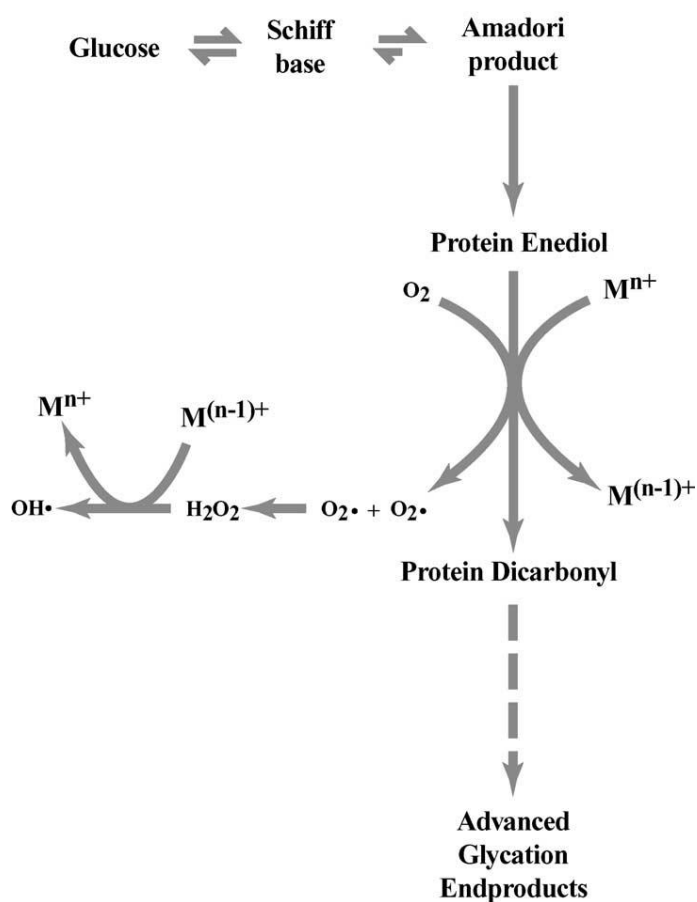
Figure 3. Glycoxidation of Amadori products [8].

AGEs have been shown to be more toxic than a harmless post-translational protein modification; direct toxic effects have been found at the cellular and molecular level via mechanisms of AGEs-induced reactive oxygen species (ROS). As previously described, formation of oxygen free radicals is associated with the autoxidative glycation of glucose and glycoxidation of Amadori products. This can lead to a site-specific attack on the proteins with consequent protein damage and lipid peroxidation [43]. Thus, AGEs could exert cytotoxic effects on cells. The previous study has shown that AGEs derived from chicken egg albumin-AGEs and bovine serum albumin (BSA)–AGEs cause significant decrease the cell viability in a dose-dependent manner [12]. In addition, the incubation of BSA–AGEs in human glial cell lines has shown to induction of ROS formation and induction of apoptosis cell death [44]. Furthermore, the study

has demonstrated that AGEs continually increase the ratio of oxidized to reduced glutathione in neuroblastoma cells, suggesting that AGEs-mediated free radical may deplete reduced glutathione [13]. Therefore, there is involvement between ROS-meditated AGEs production and the amount of ROS as well as the antioxidant property of the cells.

Moreover, AGEs formation leads to the activation of different signaling pathways mediated by a series of cell surface receptors. AGEs can bind to several other cellular receptors such as the receptor for advanced glycation endproducts (RAGE), the MSR type II, OST-48, 80K-H, galectin-3 and CD36 that are involved in the removal and detoxification of AGEs [36]. However, the most studied AGEs-receptor is RAGE.

1.2 Receptor for advanced glycation endproducts (RAGE)

RAGE is a multiligand receptor and is a member of the immunoglobulin superfamily, encoded in the class III region of the major histocompatibility complex. RAGE is composed of three extracellular domains, which include one V type domain that responsible for ligand binding properties, two C-type immunoglobulin domains. The cytoplasmic tail composed of fourth transmembrane domain anchors RAGE in the membrane and is connected to a highly charged fifth intracellular domain that mediates interaction with cytosolic transduction molecules for intracellular signaling [8, 13, 35, 36]. RAGE expresses on several cell types including smooth muscle cells, macrophages, endothelial cells [8], T-lymphocytes [45], dendritic cells [46], fibroblasts [47], neuronal cells, glia cells, chondrocytes [48], keratinocytes [49]. Chronic stress in many inflammation-related pathological states such as vascular disease, cancer, neurodegeneration and diabetes also promotes a broad spectrum of biological processes through RAGE expression and multiple intracellular signaling molecules, including a transcription factor nuclear factor-kappaB (NF-κB), mitogen-activated protein (MAP) kinases, and adhesion molecules, focusing the host inflammatory and reformative response [50, 51]. RAGE has been considered a pattern recognition receptor (PRR) that recognizes a several distinct families of RAGE ligands. They include the high mobility group family proteins (HMGB) including the prototypic HMGB1/amphoterin, members of the S100/calgranulin protein family, matrix proteins such as collagen I and IV, Aβ peptide, LPS and AGEs [35, 36]. Ligand accumulation and engagement in turn upregulates RAGE expression [52]. It is known that RAGE acts as a signal transduction receptor. After the ligand binding to the RAGE, it follows by activation of different signaling pathways vary with individual RAGE ligands [36]. For example, S100-calgranulins which are a group of pro-inflammatory cytokines, S100B activates RAGE through phosphatidylinositol 3-kinase (PI3K) /AKT and NF-κB, whereas S100A6 activates through c-Jun N-terminal kinases (JNK) signal transduction pathways [53]. In chronic inflammatory demyelinating polyneuropathy and vasculitic polyneuropathy, binding of RAGE to AGEs activates the NF-κB pathway suggests that the RAGE pathway may also play a role in the upregulation of inflammation in this setting [54]. As mentioned above, AGEs have been considered to be a strong inducer of RAGE signaling and AGEs-RAGE currently known to involve in many signaling pathways. In the following section is described some of them.

1.3 AGE–RAGE interaction

Binding of the ligands AGEs to RAGE can trigger a range of signaling pathways including ERK1/2 MAPK, p38 MAPK, SAPK/JNK MAPK, rho GTPases, PI3K and the JAK/STAT pathway, as well as the downstream activation of NF-κB or interferon-stimulated response elements (ISRE) followed by an increased expression of a number of cytokines and growth factors [20, 55-59]. In addition, recent data has been shown that the AGEs-RAGE interaction results in the persistent NF-κB activation via a protein synthesis-dependent pathway and sustains translocation of NF-κB (p50/p65) from the cytoplasm into the nucleus. This result confirms long-lasting sustained activation of observed in diabetes patients [60]. The RAGE interaction with AGEs also triggers generation of reactive oxygen species (ROS), at least in part via activation of NADPH oxidase leading to produce reactive nitrogen species (RNS) products of nitric oxide (NO) produced by nitric oxidesynthase (NOS) and superoxide anion ($O_2\bullet$) produced by NADPH oxidase [61]. The increased of ROS and/or RNS promotes the activation of transcription factor NF-κB, resulting in further upregulation of inducible nitric oxide synthase (iNOS) and additional increase of formation of a peroxynitrite ($ONOO^-$), a nitrating and oxidizing agent, leading to inactivation of functional proteins [36]. Moreover, recent data has revealed that RNS are involved in AGEs formation. They have shown that incubation of AGEs with peroxynitrite results in an increase of AGEs generation [62]. Thus, ROS-meditated AGEs production can induce the development of further reactive oxygen and nitrogen species production and leads to vicious cycle of oxidative stress and further protein damage. As mention above, AGEs-RAGE interaction may activate a range of signaling pathways, however, Jak/Stat pathway can be driven and is connected to many different pathways. The increasing evidences have suggested a mechanism for AGEs-mediated Jak–Stat activation. The incubation macrophages with AGEs results in an increase Jak-2 and Stat-1 phosphorylation and eventually induce of inflammatory responses [63]. Thus, AGE-RAGE interaction can regulate different transcription factors via activation of Jak and Stat signaling.

As mentioned in the section above, AGE-RAGE plays an important role in AGEs-mediated signaling, inflammatory response and ROS-related NF-κB activation. The current study has highlighted a key target of RAGE signaling is the transcription factor NF-κB due to the promoter region of RAGE has been reported that contains functional binding elements for NF-κB [64]. Since translocation of cytosolic NF-κB into nucleus is the considerable process for the initiation of transcription for a number of the NF-κB dependent genes, including intercellular adhesion molecules, growth factors, cytokines, and RAGE itself [55, 65, 66]. The accumulated studies has attempted to document evidence showing the interactions between AGEs and RAGE initiate many biological responses including induction of oxidative stress on endothelial cells [20], expression of matrix-metallopeptidase13 (MMP-13, collagen III) and ADAMST4/5 (cleavage of proteoglycan aggrecan) responsible for degradation of collagen type II on macrophage cells [63], induction of oxidative stress, endothelin-1, tissue factor, thrombomodulin, adhesion molecules and generation of pro-inflammatory cytokines on macrophage cells [67], and chemotaxis of mononuclear phagocytes [68] via NF-kB pathway. These findings have been demonstrated a potential link between the AGEs-ligand and AGEs receptor. The previous study has provided information regarding the involvement of RAGE in regulation of NF-κB activity. They demonstrate that AGEs-induced ROS formation via activation of the p21[ras], p42/44 MAPK phosphorylation,

and increased NF-κB binding and transcriptional activity that result in enhanced expression of RAGE [69]. The intracellular signalling pathways following activation of RAGE by AGEs are outlined in Figure 4.
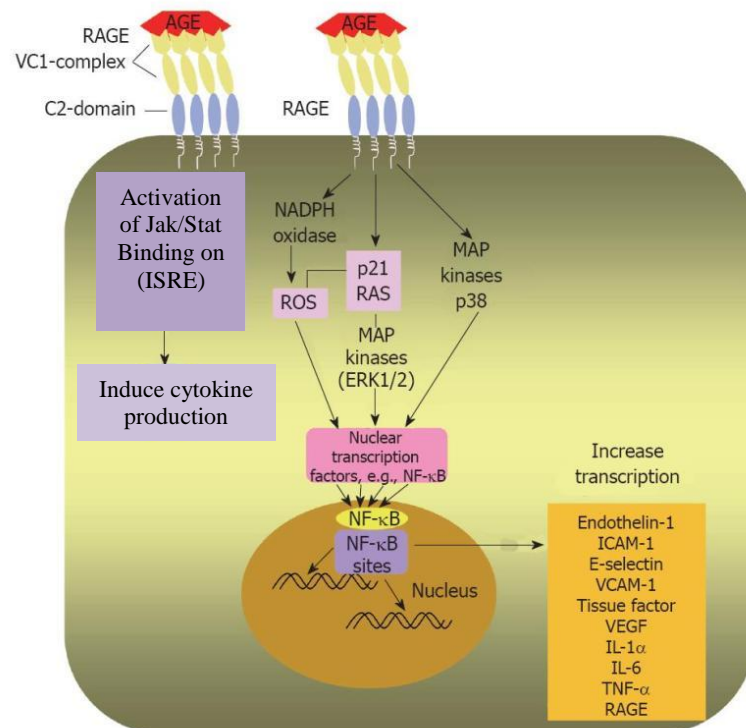
Figure 4. AGEs-RAGE mediated signaling. The interaction stimulates a various number of signaling cascades, including Jak/Stat, NADPH oxidase, mitogen activated protein kinase (MAPK), such as p38, and extracellular regulated (ERK)-1/2 (JNK). The AGEs-mediated signaling via RAGE leads to the activation of transcription factors, such as NF-kB followed by an increased expression of cytokines or growth factors (modified from Zegab H., et al., 2012) [70].

2. <u>Oxidative stress</u>

Oxidative stress is defined as imbalanced between overproduction or incorporation of free radicals from environment to living system and the ability to detoxify the free radicals by antioxidant system of body [71, 72]. Since oxidative stress contributes to proteins and DNA injury leading to inflammation, tissue damage and subsequent cellular apoptosis [73], cells have antioxidant mechanisms to prevent the oxidative stress [74]. Free radicals are molecules containing unpaired electrons in their outer orbit. Electrons normally are in pairs in specific orbitals in molecules. Free radicals, which contain unpaired electrons in any orbital, are usually unstable toward losing or picking up an extra electron, so that all electrons in the atom or molecule are paired. The unpaired electron needs to encounter another molecule and seek to find another electron to pair its unpaired electron. The free radicals often pull an electron off from a neighboring molecule, causing the affected molecule to become a free radical itself. Then this newly free radical pulls an electron off from the next molecule leading

to a chemical chain reaction of radical production [75]. Such an event causes damage to the biomolecules in the cells; lipids, proteins and DNA eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases as well as neurodegenerative diseases [71, 75, 76]. Previously, the free radical is only concerned with free radicals such as superoxide (O_2^-), however currently theory has been descried to include oxidative damage from other reactive oxygen species (ROS) and reactive nitrogen species (RNS) [77].

Since oxygen is essential for all living cells in any kinds of cells. It involves in many biochemical activities of cells such as signal transduction including; apoptosis, gene expression and the activation of cell signaling cascades; host cell defense mechanisms and regulation of soluble guanylate cyclase activity, however it is potentially dangerous if in excess. Thus, there is a tight regulation of complex system that controls and monitors the usage and uptake of this vital molecule [71]. ROS are a number of reactive molecules and free radicals derived from molecular oxygen. It comprises hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide anions (O_2^-) and the highly reactive hydroxyl and monoxide radicals (OH•, NO•) molecule [71]. There are many cellular sources of ROS within a cell (Figure 5). These sources can be broadly divided into two main categories. Firstly, there are those biological processes that release ROS as a byproduct, or a waste product, of various other necessary reactions. Secondly, there are those processes that generate ROS intentionally, either in molecular synthesis or breakdown, as part of a signal transduction pathway, or as part of a cell defense mechanism [74]. In the first category, most ROS are generated as by-products during glucose break down in mitochondria through oxidative phosphorylation. Since the reactions occur during oxidative phosphorylation processes frequently lose electrons during mitochondrial electron transport chain. These electrons react with molecular oxygen to produce ROS. In the second category of ROS sources are many enzymes that generate ROS for several functions. Most ROS are generated by the phagocytic NADPH oxidase, which can produce superoxide by using NADPH to reduce molecular oxygen. This superoxide is typically used as a cell defense mechanism against infectious pathogens due to it can be converted in phagosomal compartments by superoxide dismutase and myeloperoxidase to hypochlorous acid, which is a potent microbicidal compound [78]. During this process, there frequently leak of ROS from the phagosome and they enter the cytosol, contributing to the oxidative stress of the cell. However, there are many different potential intracellular sources of ROS generated by the cell to function specifically within signaling pathways (Figure 5), and much of which are capable of influencing, or being influenced by, NF-κB activity [74].

Intracellular ROS Sources

NADPH Oxidases

Cytokine & Growth Factor Receptors

Mitochondrial Electron Transport Chain Leakage

Metabolic processes
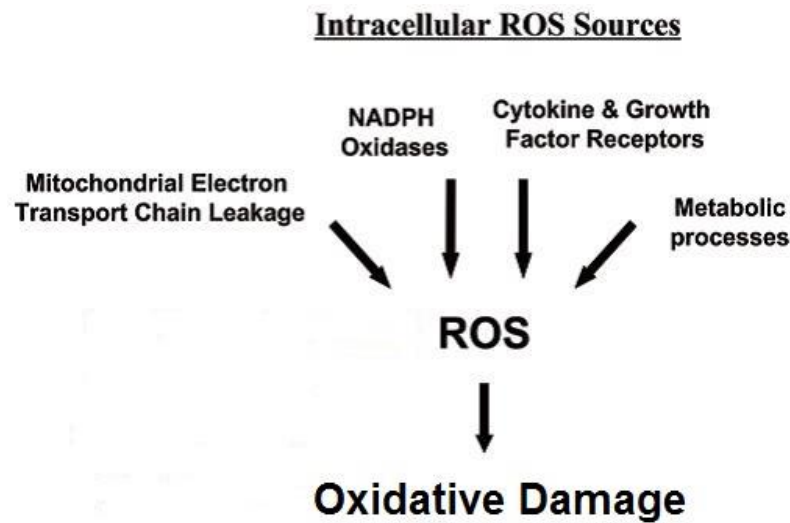
ROS

Oxidative Damage

Figure 5. Intracellular sources of ROS. The mitochondria is a major source of ROS. In addition, ROS are also produced by NADPH oxidases, sometimes in response to cytokines and other growth factor receptors, which may also use other pathways to produce ROS for utilize in their signaling pathways [74].

The cellular system is required activated molecular oxygen-mediated ROS generation for metabolic system in order to interact with organic molecules. In addition, the cellular system has evolved a number of metallo-enzymes that facilitate ROS generation upon interaction of redox metals with molecular oxygen using various catalytic pathways. Since these ROS are toxic to cells, under normal circumstances, cells have efficient regulating system for tight check of complex system that regulates the ROS generation [79]. Actually, some metals have a strong catalytic power to generate highly reactive radicals. Since this discovery, the iron catalyzed hydrogen peroxide (H_2O_2) has been called Fenton's reaction [80]. *In vivo* Fenton chemistry is initiated by the by-products of aerobic respiration, such as hydrogen peroxide (H_2O_2). H_2O_2 can oxidize Fe^{2+} to Fe^{3+} forming a highly reactive hydroxyl radical (OH•) and a hydroxide ion (OH^-) in the process. Fe^{3+} is then reduced back to Fe^{2+} by another molecule of H_2O_2, forming a hydroperoxyl radical (HOO•) and a proton (H^+) as it shows in the following equations:

1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO• + OH^-$
2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO• + H^+$

2.1 Influence of ROS on NF-κB activation

ROS can generate by a variety of cellular processes. Accumulated evidences have demonstrated that ROS interacts with NF-κB signaling pathways in many ways. While certain transcription of NF-κB dependent genes play a major role in regulating the amount of ROS in the cell, and in turn, ROS have various inhibitory or stimulatory roles in NF-κB signaling. Here we review the regulation of ROS levels by NF-κB targets and various ways in which ROS have been proposed to impact NF-κB signaling pathways.

2.1.1 NF-κB

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells, is a protein complex in a family of transcription factors that is of central importance in control transcription of genes that are involved in regulating cell growth, differentiation, development, and apoptosis [81, 82]. NF-κB is found in almost all animal cell types and plays a key role in regulating the in cellular responses to a number of stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL and bacterial or viral antigens [83-85]. The mammalian NF-κB proteins consist of five different related family members sharing a Rel homology domain (RHD) in their N-terminus that is essential for DNA binding and dimerization. A subfamily of NF-κB proteins, including RelA (also known as p65), RelB, and cRel, have a C-terminal transcription activation domain (TAD) that serves to positively regulate gene expression. The two other mammalian NF-κB proteins include NF-κB1 and NF-κB2 proteins that are synthesized as larger precursors, p105, and p100, which have a C-terminal ankyrin repeats that inhibit DNA binding until partially processed by proteasome to generate the mature NF-κB subunits, p50 and p52, respectively [83-85]. In addition, these p50 and p52 proteins lacking a TAD therefore have no intrinsic ability to activate transcription unless paired as a heterodimer with one of the Rel proteins. All NF-κB proteins are capable of homodimerization or heterodimerization with the other NF-κB proteins with the exception of RelB, which can only form heterodimers. The NF-κB dimer combinations in various homo- and hetero-dimer configurations bind to 10-base pair κB sites with varying affinities in distinct DNA sequences, and thus they have arisen to regulate the DNA binding activity of various NF-κB homo- and hetero- dimers [81, 82, 86]. In general, there are two main signaling pathways of activating NF-κB lead to the activation of NF-κB target genes. These are referred to as the classical and alternative pathways. These two pathways usually be distinguished by whether the p50 product of p105 (classical) or p52 product of p100 (alternative) is involved [81, 82, 87, 88]. In general, NF-κB activation is principally regulated by the IκB proteins that are generally inhibitory of DNA binding due to these proteins can bind to NF-κB proteins and mask their DNA binding domains. The activity of the IκBs is controlled through phosphorylation by upstream IκB kinases (IKKs). IKK is consisting of IKKα and IKKβ, which are the catalytic kinases, and IKKγ which acts as a regulatory subunit NEMO (also known as NEMO). When activated by signals in the classical pathway, the p105 (nfκb1 gene product) is constitutively processed by the proteosome into p50, which is held inactive as a heterodimer with RelA (or c-Rel) by its interaction with the inhibitory IκB proteins. Then the phosphorylation of the IκB kinase on two serine residues located in an IκB regulatory domain leading to the ubiqutinatation of these protein normally IκBα, which then leads them to degrade by the proteosome and this unmasks the DNA binding activity of the p50/RelA heterodimer and also allows it to translocate to the nucleus where it can bind to κB sites and activate gene transcription [81, 82, 87, 88]. On the other hand, some select set of cell-differentiating or developmental stimuli activate the alternative NF-κB pathway to induce RelB/p52 dimer in the nucleus. This NF-κB activation is stimulated by specific TNF receptor family members that signal through the recruitment of TRAF2 and TRAF3 by several ligands such as LTβR, CD40, CD27, CD30, BAFF-R, and RANK [89]. The upstream kinase in this pathway is the NF-κB-inducing kinase (NIK). Continual degradation of NIK in resting cells prevents constitutive activation of the alternative NF-κB pathway [90]. Degradation of NIK has

occurred by a complex between TNF receptor family such as TRAF3, TRAF2, and cIAPs 1 and 2, which ubiquitinates NIK, targeting it for proteosomal degradation, whereas the degradation of TRAF2 or TRAF3 by receptor-stimulated processes can prevent NIK degradation resulting in NIK stability [91, 92]. Stabilization of NIK results in the stimulation of downstream signaling cascades. The activation of the NIK upon receptor ligation led to the phosphorylation and subsequent proteasomal processing of the NF-κB2 precursor protein p100 into mature p52 subunit in an IKKα dependent manner. Then p52 dimerizes with RelB to appear as a nuclear RelB/p52 DNA binding activity and regulate a distinct class of genes [93]. Thus p100 processing is a critical step in the alternative NF-κB signal pathway.

As described in the above section that ROS are toxic in cells at certain levels, due to accumulated ROS induce the oxidative stress that can exert deleterious reaction with proteins, lipids, and nucleic acids. The oxidative stress can therefore trigger cell death depending on the severity. Nevertheless, when too much cellular damage has occurred, the body has to develop beneficial mechanisms to prevent or slow the damage. In general, the expression of NF-κB target genes typically promotes cellular survival. Thus it is not surprising that ROS can modulate an NF-κB response and that NF-κB target genes may possible to attenuate ROS to promote survival.

2.1.1.1 Antioxidant NF-κB targets

The increased expression of NF-κB target antioxidants is one of the main signaling pathways in which NF-κB activation effects ROS levels (Figure 6). Nowadays, there are many potential antioxidant targets that may contribute to protection from ROS. Manganese superoxide dismutase (MnSOD, or SOD2) is a mitochondrial enzyme which considered as the most famous of NF-κB targets with antioxidant activity, due to it can protect cells from oxidative stress, causing the dismutation of $O_2\bullet$ into H_2O_2 [94]. Likewise, its cytoplasmic relative, copper-zinc superoxide dismutase (Cu, Zn-SOD, or SOD1) has been shown to be an NF-κB target that can catalyze a similar reaction by converting $O_2\bullet$ into H_2O_2 [95]. Ferritin heavy chain (FHC) is the second-most well known NF-κB target that protects cells from oxidative damage [96]. FHC is an iron storage protein it thus does not directly scavenge ROS, but protects the cell from oxidative damage by preventing iron-mediated generation of highly reactive $OH\bullet$ radicals from Fenton reaction. As well as FHC may synergize with MnSOD to prevent the cell of ROS by preventing the generation of more highly reactive radicals $O_2\bullet$ and $OH\bullet$ and promoting the breakdown of H_2O_2 into water by peroxidases and catalases [97]. Catalase has been shown to be a NF-κB target in at least one study and they suggest that catalase could be the target of inhibitory p50 homodimers [98]. Thioredoxin 1 and 2 (Trx1 and Trx2), two of the most important cellular antioxidants in the cell, have been shown to be regulated by NF-κB. Thioredoxins have 2-cysteine active site that reacts with ROS so that they can reduce oxidized proteins and protection from oxidative stress. In addition, they also serve as hydrogen donors to the thioredoxin-dependent peroxide reductases [74]. There is evidence for the upregulation of glutathione s-transferase pi (GST-pi) is induced by oxidative stress through NF-κB [74]. GST-pi is a phase II enzyme that catalyzes the reaction of the GSH thiolate to toxic compounds, thus allowing highly reactive radicals to be eliminated through excretion machinery [74]. Metallothionein-3 (MT3) has been reported to be an NF-κB target in keratinocytes and fibroblasts [74]. MT3 has cysteine-

rich proteins which can bind to many different metals resulting in regulating metal toxicity [99]. NAD(P)H dehydrogenase (quinone)1 (NQO1) is an NF-κB target that is activated in response to the DNA crosslinking agent such as mitomycin C [100]. Since NQO1 is a cytoplasmic 2-electron reductase that reduces quinones to hydroquinones, it thus prevents the one electron reduction of quinones to produce radical species [101]. Heme oxygenase (decycling) 1 (HO-1 or HMOX1) have been shown to be upregulated by NF-κB in response to oxidative stress and hypoxia [102-104]. It is indirectly scavenge ROS. Since HMOX1 catalyzes heme degradation resulting in the formation of carbon monoxide and biliverdin which is subsequently reduced to bilirubin by biliverdin reductase. The bilirubin is a potent antioxidant, it is thought that HMOX1 is therefore protective from oxidative stress. Glutathione peroxidase-1 (Gpx1) is one of the most important members of antioxidant proteins that abundantly exist in cytoplasm. It has been reported to be an important NF-κB target in response to oxidative stress. It can concert H_2O_2 into water using glutathione as a substrate [105].

2.1.1.2 Pro-ROS NF-κB targets

Since NF-κB is also important in inflammation, some enzymes that promote the production of ROS are also regulated as its targets, especially in cells of the immune system. There are pro-oxidant NF-κB targets that have been studied in the previous reports. For example, NADPH oxidase NOX2 (gp91 phox) has been reported to be in dependent and induced by NF-κB [106]. NADPH oxidase is an enzyme that mainly produces ROS. NADPH oxidase uses NADPH to produce superoxide, which is used for immune defensing and for cell signaling. Xanthine oxidase/dehydrogenase (XOR, or Xanthine Oxidoreductase) is also regulated by NF-κB [107]. XOR typically catalyzes the interconversion of xanthine and urate using NAD+ and water as cofactors. Due to this enzyme has very low specificity, it thus transfer of electrons to O_2 instead of NAD+ results in the generation of superoxide and hydrogen peroxide [108]. The inducible nitric oxide synthase, or iNOS is also an NF-κB target [109]. This enzyme actually produces a RNS such as NO. However, NO is often produced where it can react with superoxide leading to formation of the more toxic highly reactive peroxynitrite ($ONOO^-$). Peroxynitrite can cause potentiate ROS cellular damage, including damage to DNA and can activate cell death pathways [110]. Cyclooxygenase-2 (COX-2 or prostaglandin G/H synthase 2) is a well-known NF-κB target involved in inflammation [111]. It converts arachidonic acid into prostaglandin H2 (PGH2) by a free radical mechanism involving a tyrosyl radical generated by cooperation from a heme prosthethic group. During the second step of the reaction of generation of PGH2, superoxide is also generated [112]. Thus, superoxide is a by-product of this reaction and may contribute to oxidative stress. In addition to COX-2 that generate ROS during arachidonic acid metabolism, other enzymes have also been reported to be NF-κB targets including 12-lipoxygenase (LOX-12, or ALOX12) and arachidonate 5-lipoxygenase (LOX-5, or ALOX5) [74]. Cytochrome p450 enzymes are phase I enzymes involving to detoxify toxic compounds. They have well known to produce ROS when uncoupled, particularly H_2O_2 and hydroxyl radicals. Previous studies have reported that Cyp2E1, Cyp2C11, and Cyp7b have NF-κB promoter elements. Cyp2E1 and Cyp2C11 have been reported to be down-regulated NF-κB in response to pro-inflammatory cytokines, whereas Cyp7b has shown to be up-regulated. In addition, both Cyp2E1 and Cyp2C11 are known to produce ROS via uncoupled reactions [74].
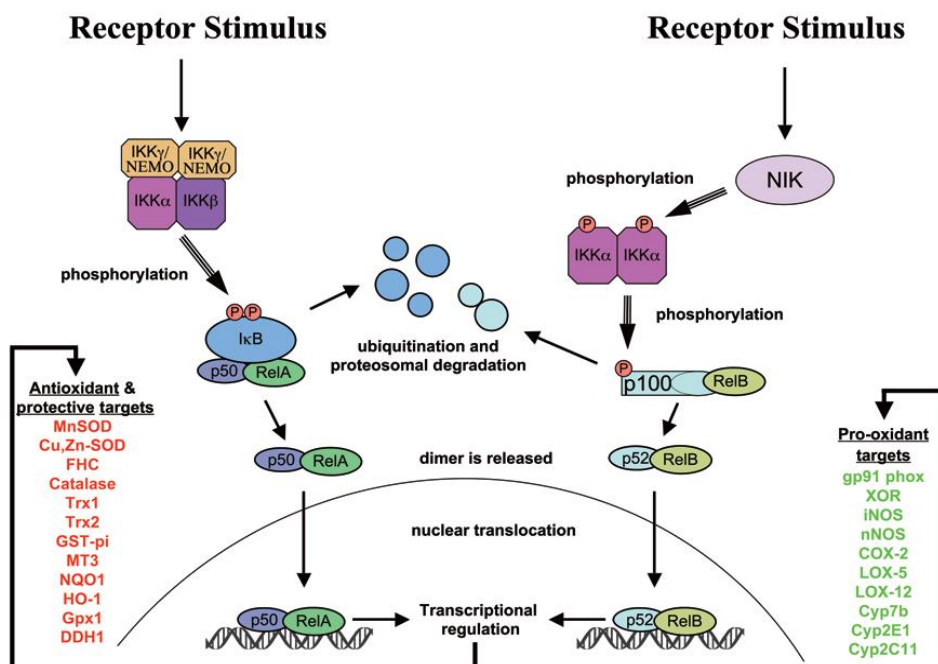
Figure 6. Activation of NF-κB and regulation of downstream transcriptional antioxidant and pro-oxidant targets [74].

ROS have been reported to both activate and repress NF-κB signaling. The studies have shown that ROS often stimulate the NF-κB pathway in the cytoplasm, conversely, some studies have shown that ROS inhibit NF-κB activity in the nucleus [113]. For instance, ROS has been shown to repress NF-κB signaling by overexpression of the antioxidant protein TRX1 resulting to diminish NF-κB activation by inhibiting IκB degradation [114], whereas others studies have shown that TRX1 translocation to the nucleus during TNF or PMA stimulation serves to enhance NF-κB DNA binding [115, 116]. However, a high degree of complexity characterizes ROS interactions with NF-κB pathways due to the use of different methodology and at numerous places which are also attributed to the study of different upstream pathways and cell type-specific differences. In addition, ROS direct oxidation of NF-κB has also shown to inhibit its DNA binding ability [117]. Due to cysteine of p50 is sensitive to oxidation by ROS [118]. Therefore its oxidation inhibits DNA binding resulting in less transcriptional activity [119]. Another indirect way of ROS influences on DNA binding of NF-κB proteins is involved with the Ser-276 phosphorylation by ROS. Phosphorylated Ser-276 is necessary for the interaction of RelA with its binding site to regulate transcription [120]. PKAc mediates phosphorylation of Ser-276 is induced by ROS [121]. Thus Ser-276 phosphorylation of RelA through a PKAc-mediated mechanism is thought to be dependent on ROS and this event contributes to its DNA binding activity [122]. Having examined effect of ROS regulation on some of the upstream NF-κB, IκBα allow to know that ROS regulate NF-κB activation through alternative phosphorylation of IκBα [123, 124]. The phosphorylated IκBα is bound by the p85α regulatory subunit of PI3K, thus unmasking NF-κB and allowing it to translocate to the nucleus for induce transcription, in this case without the degradation of IκBα may not be necessary. On

the other hand, ROS can inactivate the proteasome resulting in IκBα stability leads to inhibition of NF-κB activation [125]. IKK is another primary target for ROS in influencing NF-κB signaling. IKK is inactivation through oxidation of Cys-179 of IKKβ by ROS, thus inactivating its kinase activity leads to less in NF-κB signaling [126]. Conversely, some studies have shown that ROS can activate IKKs in some cell types. The study has demonstrated that phosphorylation of IKK subunits was potentiated by H_2O_2 through inhibition of an IKK phosphatase [127]. In addition, the kinases upstream of IKK could be possibly regulated by ROS. Since MEKK1, which is a redox-sensitive kinase, is inactivated by ROS, is suggested to play a role in NF-κB activation [128]. Akt, the upstream kinase of IKK is believed to be regulated both positively and negatively by ROS. Akt positively influences IKKβ- mediated NF-κB activation. Oxidation of Akt by ROS inactivates the kinase activity of this enzyme, suggesting that ROS could prevent IKK activation by inactivation Akt [129]. However, not only Akt itself is regulated by ROS, but PTEN, an upstream inhibitor of Akt activation, is also oxidized by ROS, thus inactivating its phosphatase activity [130]. Thus, Akt can be regulated both positively and negatively by ROS.

3. **Alzheimer's disease (AD)**

Alzheimer's disease (AD) was first identified in 1907 by a Bavarian psychiatrist named Alois Alzheimer, but for many decades after Alzheimer's original description, it has been recognized as the most common cause of dementia [1, 2]. For many decades after Alzheimer's original description, the observation of postmortem examination of the AD demonstrate the striking ultrastructural changes underlying the two classical lesions to qualify as the neuropathologic hallmarks of the AD: senile (neuritic) plaques and neurofibrillary tangles as shown in Figure 7 [131, 132]. Senile plaques are extracellular deposits of fibrils and amorphous aggregates of amyloid β-peptide (Aβ), while neurofibrillary tangles are intracellular fibrillar aggregates of the microtubule-associated protein tau which is hyperphosphorylation and oxidative modifications. Plaques and tangles are present mainly detected in AD brain regions involved in learning and memory and emotional behaviors such as the cortex, hippocampus, basal forebrain and amygdala (Figure 8) [4]. AD brains also exhibit reduced numbers of synapses, and damaged neuritis, reactive gliosis, microglial activation, neuroinflammation and dramatic shrinkage from cell loss and widespread debris from dead and dying neurons [131, 133, 134]. These abnormalities lead to brain tissue shrink due to degenerate in brain region responsible for regulate memory and acquired skills causing of AD is a well known neurodegenerative disease which is the most common cause of dementia [135]. Dementia is also caused by other diseases and conditions such as vascular dementia and Parkinson's disease (PD) dementia. Different causes of dementia are associated with distinct symptom patterns and brain abnormalities, however it can be normally characterized by a decline in memory, language, problem-solving and other cognitive skills that affects a person's ability to perform everyday activities and eventually ultimately fatal in the final stages of the disease [1, 2]. AD is one of the most common neurodegenerative diseases, with more than 44 million cases worldwide and a number that is projected to increase. As AD progresses over the years, the number of cases is expected to nearly double every 20 years and, reach 65.7 million in 2030, and 115.4 million in 2050 [136]. AD prevalence dramatically increases with

age beyond the age of 65. Indeed, AD incidence increases from 1% between the ages of 60 and 70 to 6% to 8% at the age of 85 years or older [137]. In the United States, an estimated 1-in-9 Americans over 65 have AD; by mid-century, the number of people living with AD will be projected to grow by nearly 10 million. Interestingly, Americans is now developing AD in every 67 seconds, making AD the sixth leading cause of death in the United States [136].
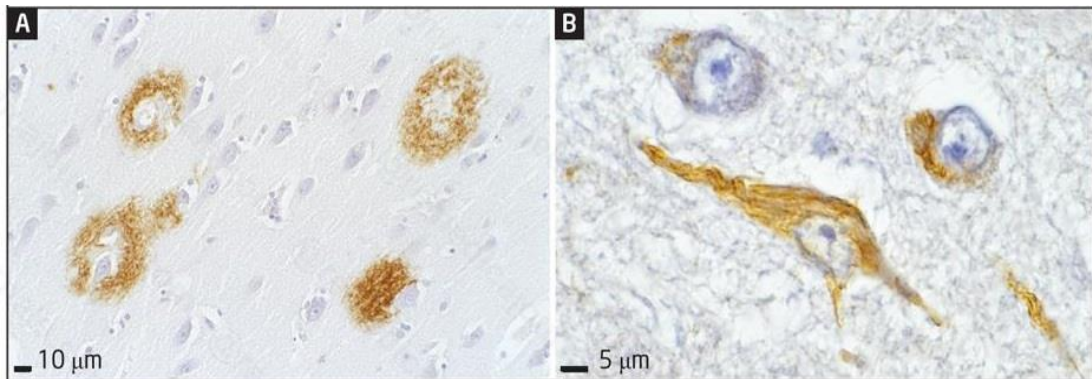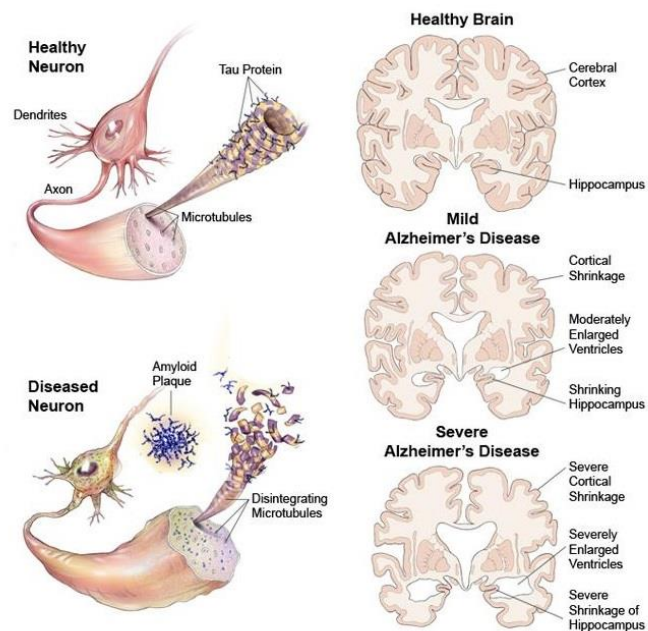

Figure 7. Alzheimer's disease pathology. A). Amyloid plaques identified with anti-Aβ antibody (40x) and B). Neurofibrillary tangles identified with anti-neurofilament antibody (100x) in hippocampus [138].
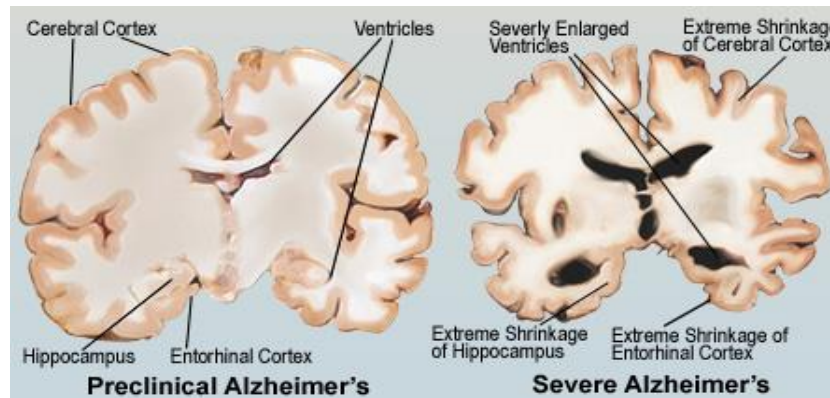
A)

B)

Figure 8. Comparison of a normal aged brain and the brain of a person with Alzheimer's disease progression. A). Drawn illustration of tau protein and senile plaque involved in Alzheimer's disease and the brain of a normal and person with Alzheimer's disease progession [139] and B). Cross-sectional anatomy of the brain of a normal and person with Alzheimer's disease progession showing brain tissue shrinks but the ventricles, chambers within the brain that contain cerebrospinal fluid are noticeably enlarged [140].

3.1 Alzheimer's disease forms

AD can be divided into familial and sporadic forms. The disease is refered familial when more than one person in a family is affected, on the other hand, sporadic considers to AD cases when no other cases have been seen in close family members. However, more than 75% of patients with AD appear to be sporadic, with the rest is familial [141]. AD is further classified into 2 subtypes based on age of onset including early-onset AD and late-onset AD. Early-onset AD accounts for approximately 1% to 6% or less than 10% of all AD cases and is defined as AD with an age at onset before age 65 years. Late-onset forms denotes with an age at onset later than 65 years. However, almost all cases of sporadic AD are late-onset AD which is the most common form of AD. Both early and late onset AD may occur in people with a positive family history of AD. More than 90% of familial AD is late-onset, while the rest, less than 10% of all AD cases, are familial early-onset [6, 141]. Overall, more than 90% of patients with AD appear to be sporadic and to have a later age at onset of 60 to 65 years of age [142].

3.2 Clinical Symptoms

AD symptoms vary among individuals. The most common initial symptom as dementia begins with a gradual decline an ability to remember new information. This decline may be due to the first neuron in part of the brain involved in forming new memories have been damaged and no longer function normally and eventually die [136]. An inability to retain recently acquired information is typically the initial presentation, whereas memory for remote events is relatively spared until later [6]. As the neuronal damage eventually affects parts of the brain that enable a person to carry out basic bodily functions, the following are common symptoms of AD: memory loss that disrupts daily life, challenges in planning or solving problems, difficulty completing familiar tasks at home and/or at work, confusion with time or place, trouble

understanding visual images and spatial relationships, problems with words in speaking or writing, misplacing things and losing the ability to retrace steps, decreased or poor judgment, withdrawal from work or social activities and changes in mood and personality, including apathy and depression [1].

The pace at which symptoms advance from mild to moderate to severe varies from person to person. With disease progression, impairment in neurons in other parts of the brain that enable a cognition; language, abstract reasoning, and executive function or decision, people in this stage typically is associated with difficulty at work or in social situations or household activities [1]. Affected people need help for basic activities of daily living such as bathing, dressing, eating and using the bathroom, lose their ability to communicate, fail to recognize loved ones and become bed-bound and dependent on around-the-clock care. AD is ultimately death commonly occurs from general inanition because affected people have difficulty moving, they are more vulnerable to infections, including malnutrition and pneumonia [143].

3.3 Diagnosis

Currently, there is no single, simple test exists to diagnose AD. The diagnosis is made by an individual's primary care physician based on clinical history, neurological examination and neuropsychological tests [1]. A criteria for diagnosing is available to help make a diagnosis are following: obtaining a medical and family history including psychiatric history and history of cognitive and behavioral changes from individual, asking a family member or other person close to the individual to provide input about changes in thinking skills or behavior, looking for input from a specialist such as a neurologist, conducting cognitive tests and physical and neurologic examinations and having the individual undergo a magnetic resonance imaging (MRI) scan which use to the exclusion of other neurodegenerative disorders associated with dementia, such as tumor associated dementia. Exclusion of AD from other forms of dementia is usually done through clinical history and neurological examination. In addition, other systemic causes of dementia need to be done, especially cognitive impairment that is treatable such as dementia due to chronic drug intoxication, chronic central nervous system infection, thyroid disease, vitamin, B12 and thiamine deficiencies [144]. However, the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for diagnosing dementia requires the loss of 2 or more of the following: memory, language, calculation, orientation, or judgment before making a diagnosis of probable AD [145]. Another commonly used criteria, the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDSADRDA) work group requires the loss of at least 2 cognitive domains, absence of other systemic disorders, progressive worsening of memory for the diagnosis of probable AD [146]. A definitive diagnosis of AD requires a clinical assessment of probable AD as well as postmortem examination of the brain which must contain sufficient numbers of 2 histopathological features: neurofibrillary tangles and amyloid plaques to qualify as AD [131, 132]. The major component of amyloid plaques, found in AD brain, is Aβ40 and 42 amino acids [147, 148]. Neurons bearing neurofibrillary tangles are another frequent finding in AD brains [149], however, neurofibrillary tangles can be found in other disorders such as frontotemporal dementia and progressive supranuclear palsy. Moreover, these tangles are not necessarily associated with the cognitive dysfunction and memory impairment

that is typical of AD [148]. Plaques and tangles can also be detected in cognitively normal age-matched controls, although the number of the plaques and the distribution of neurofibrillary tangles are more greatly in patients with AD [150].

3.4 Risk factors for developing Alzheimer's disease

Generally AD is believed to develop as a result of multiple factors rather than a single cause. We review only known risk factors for AD, however other factors that may affect risk of AD has been being studied.

- Age: Age is found to be the highest risk factor for AD. As AD prevalence dramatically increases with age beyond the age of 65. The incidence increases from 1% between the ages of 60 and 70 to 6% to 8% at the age of 85 years or older [137]. Age is the greatest risk factor, nevertheless AD is not normally occurrence in aging and age alone is not sufficient to cause the disease.

- Apolipoprotein E (ApoE) 4 gene: The ApoE gene divides into the 2, 3 and 4 form. People inherit one form of the ApoE gene from each parent. Detecting the ApoE 4 form has been found to increase risk to develop AD compared with other forms [151]. In addition, the onset of the disease occurs at a younger age in those with ApoE 4 form [1]. For the detail of ApoE, see in the below section.

- Family history: The increased risk for AD associated with having a family history of AD. People who have a parent, brother or sister with AD have been more likely to develop the disease than those who do not have a first-degree relative with AD [151]. However, family history of AD is not necessary for developing the disease. Since the disease is multiple factors, heredity (genetics) and environmental and lifestyle factors, or both, may play a role in develop AD.

- Mild cognitive impairment (MCI): MCI is a condition in which an individual has a little but a noticeable changing in thinking abilities but do not affect an ability to carry out everyday activities. Revised criteria and guidelines for diagnosis of AD suggest that in some cases MCI is actually an early stage of AD or other dementias [1]. People with MCI, especially involving memory problems, are more likely to develop AD and other dementias, however MCI does not always lead to dementia. In some cases, people with MCI revert to normal cognition or remains stable.

- Cardiovascular disease risk factors: Many factors that increase the risk of cardiovascular disease are also associated with a higher risk of dementia including smoking, obesity in midlife, diabetes, midlife hypertension and midlife high cholesterol [1]. It is well known that the brain is closely linked to the heart and blood vessels. The brain is nourished if the body is rich networks of blood vessels. A healthy heart ensures that enough blood is pumped through these blood vessels, and healthy blood vessels also ensure that the brain is supplied with the oxygen- and nutrition to function normally. Therefore, factors that protect the heart may also protect the brain and reduce the risk of developing AD and other dementias. Emerging evidence suggests physical activity and consuming a good diet (low in saturated fats and rich in vegetables and fruits) may be associated with reduced AD and other dementias [152].

- Education: It has been found that people with fewer years of formal education are at higher risk for AD and other dementias than those with more years of formal education [153]. Having more years of education may help to build a cognitive reserve that enables to increase the connections between neurons in the brain and enables the brain

to compensate for the early onset of the disease by using alternate routes of neuron-to-neuron communication to complete a cognitive task [154].

- Social and cognitive engagement: Evidenced studies suggest that remaining socially and mentally active throughout life may support brain health and possibly reduce the risk of AD and other dementias [155]. The socially and mentally active may help build cognitive reserve, but the exact mechanism by which this may occur is unknown. More research is needed to better understand how social and cognitive engagement effect biological processes to reduce risk of AD.

- Traumatic brain injury (TBI): TBI is the disruption of normal brain function caused by a blow or jolt to the head or penetration of the skull by a foreign object. Moderate TBI is defined as a head injury resulting in loss of consciousness or post-traumatic amnesia that lasts more than 30 minutes while severe TBI is defined a loss of consciousness or post-traumatic amnesia lasts more than 24 hours. It has been found that moderate and severe TBIs increase the risk of developing AD and other dementias [156]. Moderate TBI is associated with twice the risk and severe TBI is associated with 4.5 times the risk of developing AD and other dementias compared with no head injuries [157]. Individuals who have repeated head injuries such as boxers, football players and combat veterans, are at higher risk of dementia, cognitive impairment and neurodegenerative disease than individuals who have not experienced head injury [1].

3.5 Risk factor for Alzheimer's disease pathogenesis

A.) Acetylcholine deficiency

For a quarter of a century, the pathogenesis of AD has been linked to a deficiency in the brain neurotransmitters. The earliest hypothesis is that AD is caused by a loss of the cholinergic markers choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) found in AD brains. Acetylcholine (ACh) is one of neurotransmitters if it found in cells called cholinergic neurons. These neurons are located in a number of areas in the brain and spinal cord that are involved in a variety of functions including cognitive processing and motor function. When ACh is released from the cholinergic neurons, a receptor on a neighboring neuron bind to it for passing signals along from cell to cell [158]. The loss of cholinergic neurons and the existence of a correlation between the loss of ChAT and decline in mental status scores lead to the cholinergic hypothesis of cognitive symptoms in AD [158]. The cholinergic neurons affected in AD are those located in an area called the nucleus basalis of Meynert (NbM) of the basal forebrain. The NbM involves in connecting the cortex and amygdala together and these areas are involved in learning, memory, attention and emotional regulation [159]. Cholinesterases or ChE are an ubiquitous enzyme of serine hydrolases that hydrolyze choline esters with various efficiency. In vertebrates, two forms of ChE encoded by two distinct genes; AChE and butyrylcholinesterase (BuChE) [160]. The main function of AChE is the rapid hydrolysis of ACh at cholinergic synapses [161], while the function of BuChE is still unclear. Over the years, increasing evidences for the relationship between ACh dysfunction and AD have been demonstrated [162]. It has been found that most of the AChE is found to be associated with neuritic plaques in which it is co-localized with Aβ presented in AD brains [163]. In addition, several studies have shown that AChE promotes aggregation of Aβ peptides and amyloid formation [164]. These results suggest that AChE has the ability to promote assembly of Aβ monomer into amyloid plaques. Although in AD brains there is the appearance

of amyloid plaques, there is no direct connection between the number and location of the plaques and cholinergic neuron loss. Interestingly, the loss has been linked to the toxicity of Aβ that can damage the cholinergic neurons resulting in reducing the production and release of ACh as well as it can interfere with the actions of a nerve growth factor that is involved in maintaining the structure and function of cholinergic neurons [162]. Thus it has been suggested that an ACh deficiency might not be a direct cause of AD, but could be a result of AD-related brain damage. Furthermore, the cholinergic hypothesis has not maintained widespread support, largely because medications that based on AChE inhibitors have not been very effective for AD. The drugs can only inhibit the release of the AChE that breaks down Ach, but do not stop the destruction of cholinergic cells; so although they may temporarily improve symtoms of AD.

B.) Amyloid β-peptide (Aβ) accumulation

Amyloid β-peptide (Aβ) is the main peptide prominent in the brain plaques characteristic of AD and various other metabolites are derived from amyloid precursor protein (APP) by proteolytic cleavage resulting in increased production and accumulation in the brain of Aβ [165]. APP is widely expressed in cells throughout the body where the amount produced is influenced by the developmental and physiological state of the cells. The transmembrane protein APP has been known to play a role in neuronal growth, survival and postinjury repair [166, 167]. Three different enzyme activities involved in cleavage of APP at the α-, β- and γ-secretase sites are being identified. *In vivo*, APP is processed by one of two major pathways [168]. Most is processed through the non-amyloidogenic pathway, which precludes Aβ production. As shown in Figure 9, APP is preferentially processed by the first enzymatic cleavage, α-secretase, of which putative candidates belonging to the family of a disintegrin and metalloprotease (ADAM) have been identified and ADAM9 and ADAM10 are candidates [4]. Cleavage of APP by α-secretase occurs within the Aβ domain, thus preventing the Aβ generation and divides APP into a large soluble α-APP domain (sAPPα) and a smaller C-terminal fragment (C83) [4, 168, 169]. It has been shown that the increase production of sAPPα leads to increase in response to electrical activity and activation of muscarinic acetylcholine receptors, suggesting that neuronal activity increases α-secretase cleavage of APP [4]. Furthermore, C83 can undergo an additional cleavage mediated by γ-secretase to release the P3 fragment (which is non-toxic) [170]. On the other hand, APP that is not cleaved by the non-amyloidogenic pathway becomes a substrate for amyloidogenic pathway. Amyloidogenic APP processing involves sequential cleavages by β -secretase (β -site APP-cleaving enzyme 1; BACE1) and γ-secretase at the N- and C-termini of Aβ domain, respectively. The N-terminus of APP is processed by β-secretase enzyme (BACE1) yields a ectodomain soluble form of APP (sAPPβ) and retaining a 99 amino acid C-terminal fragment of APP (C99) within the membrane. The first amino acid of C99 is the first amino acid of Aβ peptide. The C99 can be internalized and further cleaved 38–43 amino acids from the N-terminus to release Aβ, by the γ-secretase complex, which consists of four different proteins; presenilin 1 (PS1) or PS2, nicastrin, anterior pharynx defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2). Since the active site of γ-secretase requires the aspartyl protease activity of PS1 conferred by aspartate residues in adjacent transmembrane domains of the C- and N-terminal cleavage fragments of PS1. In addition, nicastrin, Pen-2 and Aph-

1 have been shown to be the important components of γ-secretase and each can modify enzyme activity in specific ways and in response to physiological stimuli [4, 168, 169]. This cleavage predominantly produces Aβ1-40, and the more amyloidogenic Aβ1-42 at a ratio of 10:1 [171]. In addition, cleavage of C99 by γ-secretase releases an APP intracellular domain (AICD) that can translocate to the nucleus where it may regulate gene expression including the induction of apoptotic genes [172].
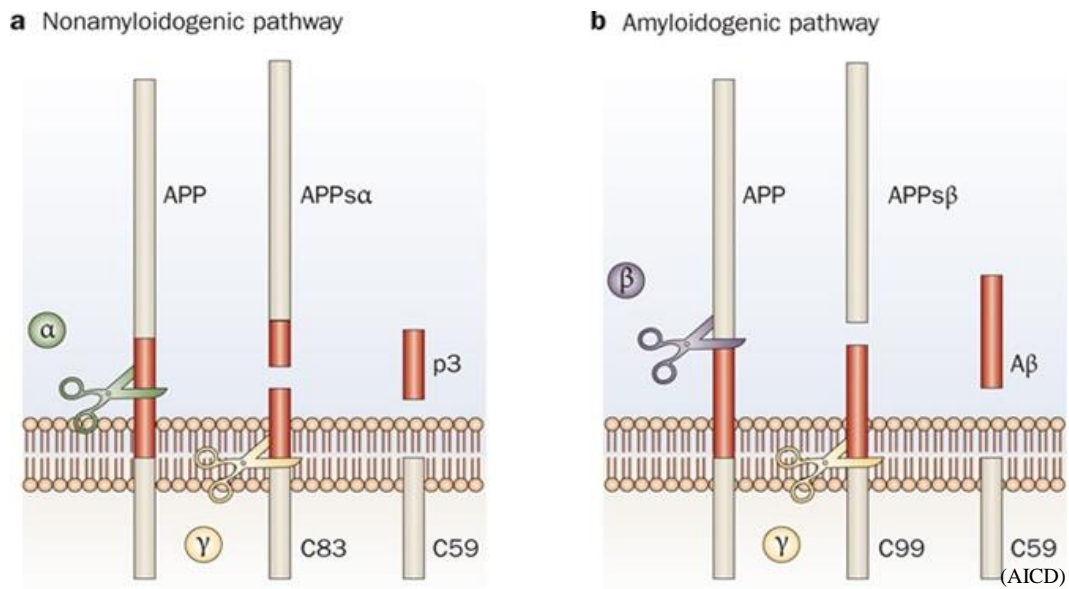

Figure 9. Schematic representation of APP processing. Mature APP is metabolized by 2 competing pathways: A). Nonamyloidogenic pathway which APP is firstly cleaved by α-secretase within the Aβ sequence, which releases the sAPPα and C83 ectodomains. Further processing of the resulting carboxyl terminal by γ-secretase results in the release of the p3 fragment. B). Amyloidogenic pathway which APP is initiated when β-secretase cleaves APP at the amino terminus of the Aβ peptide and releases the sAPPβ and C99 ectodomains. Further processing of the resulting carboxy-terminal fragment by γ-secretase results in the generating Aβ (modified from Strooper B.D., et al., 2010) [165].

Diverse lines of evidence supporting the amyloid hypothesis that APP and Aβ contribute to the pathogenesis of AD is extensive and has recently been reviewed. The evidence supporting this hypothesis reveals that autosomal dominant early-onset familial AD where the genetic mutations in APP, PS1, or PS2 can alter APP processing and increase the production or self Aβ aggregation. The defective gene causes an increase in the production of the long 42 amino acid form of Aβ (Aβ42) in patients, in cultured cells and in transgenic mice [173, 174] as well as a promoting aggregation and accumulation of Aβ in the brain [175]. The causes of altered APP metabolism and Aβ deposition in sporadic form of AD are not understood, but it may include age-related increases in oxidative stress, impaired energy metabolism and perturbed cellular ion homeostasis. Indeed, Aβ has been detected in some humans without symptoms of AD. However, the latter are almost diffuse Aβ forms and not associated with surrounding neuritic and glial pathology and may be analogous to early fatty streaks of cholesterol. Moreover, the degree of symtoms in AD also found to be correlate biochemically assay

of Aβ, the concentration of soluble Aβ species rather than with histologically determined plaque counts [176]. Since the initial experiments indicate that the neurotoxic effects of synthetic fragments of Aβ protein cause dead of cultured neuronal cell line [177], led to a series of studies that have demonstrated the chemical and cell biological effects on the synaptic dysfunction and death of neurons in AD. In addition, aggregates of Aβ have shown to activate microglia and induce the production of inflammatory mediators such as NO, ROS, TNF-α, Interleukins (IL); IL-1β, IL-6, IL-18, and prostaglandins (PGE2), that contribute to the death of neurons [178, 179], suggesting that Aβ can be directly toxic to neurons and also greatly increases their vulnerability to oxidative and metabolic stress, and activation of inflammatory systems leading to progression of AD. Moreover, several lines of evidence suggest that Aβ is cause of tau-dependent aberrant excitatory network activity and synaptic depression, which may lead through hippocampal remodeling and eventually cognitive dysfunction [176, 180]. In considerable part, the neurotoxic species of Aβ and its effects on neuronal function remains controversial. However, several lines of evidence have recently demonstrated that Aβ may be most toxic when it is in the form of soluble oligomers but not monomers or insoluble amyloid fibrils [181, 182]. The soluble oligomers of Aβ may be responsible for synaptic dysfunction in the brains of AD. The studies have revealed that Aβ oligomers impair synaptic ion and glucose transporters as well as impair synaptic plasticity [183, 184]. In addition, neurons may be susceptible to the adverse effects of soluble Aβ oligomers as suggested by the ability of them to induce oxidative stress and disrupting cellular calcium homeostasis [183]. Moreover, microinjection of naturally secreted human Aβ into living rats of culture medium revealed that Aβ oligomers in the absence of monomers and amyloid fibrils inhibit long-term potentiation in the hippocampus leading to memory deficits [176].

C.) Tau hyperphosphorylation and neurofilament accumulation

In addition to Aβ accumulation, neurofibrillary tangles (NFTs) are another pathological marker of AD. These tangles composed of tau aggregates that are biochemically similar to or, in some cases, indistinguishable from those in AD have been described in other neurodegenerative diseases such as Progressive supranuclear palsy [185], Pick's disease [186], and parkinsonism linked to chromosome 17 [187]. The electron microscopy detection of bundles of abnormal fibers that occupied neurons in the brain regions typically affected in AD reveals that these fibers consist of paired helical filaments. Extensive immunocytochemical and biochemical analysis of these tangles reveal that they are composed of the microtubule-associated hyperphosphorylated tau protein. A variety of kinases have been shown to be capable of phosphorylating tau at various sites [3]. Normally tau is an abundant soluble protein in axons and promotes the assembly and stability of neuronal microtubules. The binding of tau to microtubules can be regulated by phosphorylation which results in release of tau from microtubules thereby decreasing stability but increasing the dynamic nature of the microtubule network. In the disease state, tau becomes hyperphosphorylated at many serine-threonine sites throughout the protein and it accumulates as NFTs in nerve cells. The hyperphosphorylated tau is insoluble and has low affinity for microtubules and self-assembles [188]. Nevertheless, it has not become clear whether direct events such as up-regulation or aberrant activation of tau kinases, down-regulation of phosphatases, mutations of the tau gene, covalent modifications of tau proteins and

indirect events such as Aβ-mediated toxicity, oxidative stress and inflammation are responsible for the hyperphosphorylation of tau *in vivo* that leads to its apparent dissociation from microtubules and aggregation into insoluble paired helical filaments [189]. Several recent findings have suggested that various enzymes, such as glycogen synthase kinase 3β (GSK-3β), cyclin-dependent protein kinase-5 (cdk5), protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII), casein kinase-1 (CK-1), mitogen activated protein (MAP) kinase ERK 1/2 and stress activated protein kinases (SAPKs), involve in tau phosphorylation [190, 191]. Accumulating evidence suggests that loss of microtubule-stabilization would lead to destroy the structure and regulatory functions of the cell's cytoskeleton which collapses the neuron's transport system thus contribute to malfunction in biochemical communication between neurons leading to synaptic dysfunction and later in the neurodegeneration [190, 192, 193]. Since the main function of tau is to bind and stabilize microtubules to normally facilitate or enhance excitatory neurotransmission by regulating the distribution of synaptic activity-related signaling molecules [194]. Several recent findings have suggested the aggregated tau proteins found in AD brains causes memory deficits through a loss-of-function mechanism [194]. Studies in tau overexpression in cell culture and tau transgenic animal models suggest that tau becomes enriched in dendritic spines where it can interfere with neurotransmission and contribute to synaptic dysfunction [195]. Interestingly, recent data have found that NFTs are not sufficient to cause cognitive decline or neuronal death. Santa C., et al. have shown that there is hyperphosphorylated tau accumulation even when tau levels are reduced by transgene suppression, possibly because tangles may act as a sink for tau intermediates that are predicted to have formed as part of the pathogenic process. Thus they suggest that the tau intermediates are the neurotoxic species. However, tau intermediates have not yet been currently identified, but similar intermediates composed of other proteins such as huntingtin or Aβ protein have been suggested to be neurotoxic elements in other diseases [196]. The evidence in line with this study has found that the hyperphosphorylated tau can cause neurodegeneration without forming large aggregates [197].

3.6 Genetics of Alzheimer's disease

AD is a complex disease and a number of interactions among multiple genetic, epigenetic, and environmental factors have been discovered that may increase the risk of developing the disease. The most well-established linking between AD and genetics is in familial early onset AD. The following genes have been identified that account for a significant number of onset AD cases.

3.6.1. Genes associated with familial early onset AD

3.6.1.1. The APP (amyloid precursor protein) gene encodes the amyloid precursor protein which is normally cleaved to form Aβ. Aβ is derived from its large precursor protein, APP, by sequential proteolytic cleavages. APP is a type-I integral-membrane protein that resembles a signal-transduction receptor. It is expressed in many tissues but concentrated in the synapses of neurons. The APP gene, located on chromosome 21, is alternatively spliced yielding 3 major isoforms based according to their length in amino acids; APP695, APP751 and APP770 and expressed differentially by tissue type [3]. The APP 751 or 770 are widely expressed in nonneuronal cells throughout the body in both the peripheral and central nervous system (CNS) and also occur in neurons,

whereby the 3 isoforms that are most relevant to AD are restricted to the neurons express even higher levels is APP 695 which occurs at very low abundance in non-neuronal cells [198]. Its primary function is not known, however a number of possible functions have been described to APP holoproteins and/or their major secreted derivative. APP is important for biological processes due to deletion of the APP gene in mice results in neither early mortality nor appreciable morbidity shown in cerebral gliosis and changing in locomotor behavior occurs later in adult life [199]. Consistantly, neurons cultured at birth have diminished viability and retarded neurite outgrowth [200]. The secreted derivative of APPs has been implicated in acting as an autocrine factor [201] and a neuroprotective and perhaps neuritotrophic factor for neural plasticity and as a regulator of synapse formation [3]. In addition, the secreted APP isoforms are involves in cell-cell and cell-substrate adhesive properties in culture [202]. The APP holoprotein has also been suggested to function in cell-cell interactions when inserted at the plasma membrane, based on *in vitro* studies [203]. Since there is a number of different APP missense mutations have been identified which are available in the NCBI database and the Alzheimer Disease Mutation Database (www.molgen.ua.ac.be/ADMutations). Mutations in APP affect APP processing leading to alter production of different Aβ peptides that is more likely to form plaques [175]. Mutations in APP account for 10%-15% of familial early-onset AD cases and do not occur within the majority of sporadic cases with AD [141, 204].

3.6.1.2. The PSEN (presenilin) genes encode proteins that function in the cleavage of Aβ precursor or APP protein. The linkage studies have established the presence of AD locus on a chromosome 14 as the locus of a mutations responsible for inherited AD in several family, called presenilin-1 (PS1) gene as well as mutations in a gene on chromosome 1 with high homology to PS1, named presenilin-2 (PS2), have then been shown to cause a few cases of inherited AD [6]. PS1 is a protein that is predicted to traverse the membrane 6 to 10 times so that the amino and carboxyl termini are both oriented toward the cytoplasm, while PS2 consists of 9 transmembrane domains and has a large loop structure between the sixth and seventh domains [6]. Presenilins are major components of the atypical aspartyl protease complexes that are responsible for the γ-secretase cleavage of APP. Since PS1 can form the catalytic core of the γ-secretase complex, similarly, PS2 has also been described as a component of γ-secretase [205]. However, PS2 can be expressed in a variety of tissues, including the brain, where it is expressed primarily in neurons [206]. This complex can cleave many type-I transmembrane proteins in the hydrophobic environment of the phospholipid bilayer of the membrane such as APP [205, 207], Notch [3], ErbB4 [208] and E- and N-cadherin [209, 210]. APP clevage at the γ-secretase site has been reported to be differentially affected by specific presenilin mutations. Although, the functions and biological importance of presenilin splice variants are poorly understood, but it appears that differential expression of presenilin isoforms may lead to differential regulation of the proteolytic processing of the APP [6]. Mutations in both PS1 and PS2 result in inappropriate APP processing leading to alter production of different Aβ peptides; this seems to occur through increased Aβ42 production, decreased Aβ40 production, or alternatively, a combination of increased Aβ42 production and decreased Aβ40 production thereby affect their relative ratios [174, 211-213]. To date, there have been 212 PS1 mutations identified which are available in the NCBI database

(http://www.molgen.ua.ac.be/ADmutations). The majority of these mutations are missense mutations. Indeed, the missense mutations in PS1 which cause amino acid substitutions throughout the PS1 are thought to account for 18% to 50% of for all cases of familial early-onset AD, while mutations in PS2 are thought to account for less than 5% [141, 204]. In contrast to the mutations in the PS1 gene, mutations in PS2 are a much rarer cause of familial AD. To date, 28 PS2 mutations have been reported which are listed through the NCBI database (http://www.molgen.ua.ac.be/ADmutations). In addition, the age of onset of PS2-affected families appear to differ from the PS1-affected families is generally older (45–88 years) than for some family members with PS1 mutations (25–65 years). Furthermore, the age of onset is highly variable among PS2-affected members of the same family; whereas in families with PS1 mutations, the age of onset is generally quite similar among affected family members, and it is even similar among people from different families with the same mutation [214]. PS2 mutations have been reported to affect APP processing by increase the ratio of $A\beta42$ to $A\beta40$, suggesting that presenilins possible to modify the way in which γ-secretase clevage APP [211].

Familial early-onset AD is inherited in an autosomal dominant manner which defined as an inheritance of one mutant allele of these genes usually develops the disease. In addition, children of an affected parent have a 50% chance of inheriting the mutation and results in development of the disease [141, 204]. Notely, it is important to consider that mutations in APP, PS1 and PS2 do not account for all cases of familial early-onset AD, so there are likely other genes not yet described that play an important role in familial early-onset AD.

3.6.2. Genes associated with risk in sporadic AD

Sporadic late-onset AD is the majority of all AD cases. This form can be occured by a number of gene mutations together with aging, the most important known nongenetic risk factor for late-onset AD and exposure to potential environmental risk factors for late-onset AD include head injury, low educational levels, hyperlipidemia, hypertension, homocysteinemia, diabetes mellitus, and obesity [215]. Sporadic late-onset AD has thus far been consistently, across numerous studies, associated with only 1 gene, the apolipoprotein E (ApoE). Combinations of ApoE with one or more of these environmental risk factors may further increase the risks for late-onset AD and age-related cognitive decline.

3.6.2.1. Apolipoprotein E (ApoE) gene is located on chromosome 19 and is in a cluster with other apolipoprotein genes including APOC1, APOC2, and APOC4 [6]. ApoE in human is a polymorphic protein with three common isoforms; ApoE2, ApoE3, and ApoE4 that are differ from one another by single-amino acid substitutions [216]. ApoE plays an important roles in neurobiology including neurite remodeling in which apoE3 can stimulate neurite outgrowth while apoE4 inhibits it [216, 217] and modulation of glutamate receptor function and synaptic plasticity in which ApoE3 can stimulate receptor recycling while ApoE4 inhibit this process in neurons [218]. For ApoE4, it has been demonstrated that it has susceptibility to proteolysis [216, 217] can induce impairments in neurite outgrowth, and mitochondrial functions and associate with astrocytic dysfunction [215]. In addition, it is known that ApoE plays an important role in the distribution and metabolism of lipids within many organs and cell types [216]

especially in neurons after neuronal degeneration and it can redistribute them for proliferation, membrane repair, or remyelination of new axons [216, 217]. ApoE can bind to a specific receptor for endocytosis to rapidly remove the major Apo of the chylomicron in the brain; this process is established for the normal catabolism of triglyceride-rich lipoprotein constituents [219]. It is known that ApoE which derived from different cellular sources has distinct roles in both physiological and pathophysiological pathways [216, 217]. Accumulated evidences suggest that neurons-derived ApoE have isoform specific effects on mitochondrial function, tau phosphorylation, lysosomal and neuronal integrity, androgen receptor levels and cognitive functions [215]. Interestingly, this type of ApoE can bind aggregated Aβ in which ApoE4 being much more effective than the ApoE3, suggesting that ApoE4 enhances the deposition of the Aβ peptide [220]. *In vitro* and *in vivo* studies have demonstrated that both ApoE3 and ApoE4 can form stable complexes with Aβ peptides, but ApoE4 forming complexes more rapidly and effectively [221].

The ApoE gene has been associated with both familial late-onset and sporadic late-onset AD in numerous studies, however ApoE4 has been genetically linked to both forms of AD [222, 223]. ApoE4 has clearly demonstrated to increase Aβ accumulation, amyloid plaque formation and tangle pathology in both human and transgenic mouse models [224-228]. The mechanism by which ApoE4 protein leads to increased Aβ deposition has not been elucidated. To date, no evidence has been emerged that Aβ production is increased in cells that coexpress APP with the ApoE4. However, ApoE4 seems to enhance the steady-state levels of Aβ peptides, probably by decreasing its clearance from the brain in some mechanisms [227]. The function of ApoE4 in AD progression is elusive. Studies have reported an association between gene dose of ApoE4, age of onset and cognitive decline [229]. The increased number of ApoE4 alleles presented in the AD patients appear to increase risk of developing AD in after age 65 [229]. On the contrary, ApoE4 allele appears to associate with earlier age of onset of AD [230]. The frequency of the ApoE4 allele has been found to vary between ethnic groups, however, more than 50% of people with AD carry at least one ApoE4 allele. In addition, it has been reported that ApoE4 mutation is incompletely penetrant which imply that the mutation does not always increase risk of developing the disease, and there are likely other mutations that contribute to the development of sporadic late-onset AD [141].

3.7 AGEs role in pathology in Alzheimer's disease

There is increasing evidence demonstrate AGEs may be involved in the pathogenesis of AD in a different way. In humans, AGEs are intracellular cytoplasmic localized in pyramidal (glutaminergic) neurons, exhibiting a granular, perikaryonal distribution [231]. In addition, evidence support for susceptibilities between AD and AGEs comes from a non-enzymatic reaction of glucose to form AGEs on long-lived protein deposits. Distribution of AGEs has been investigated in intracellular NFTs [232, 233] and extracellular senile plaques [234, 235]. Due to AGEs are able to irreversibly crosslink long-lived proteins which are characteristic hallmarks of AD both amyloid plaques and NFTs, several studies have demonstrated that the higher AGEs level in AD tissue is reflected by increased AGEs-Aβ levels in the CSF of AD patients [236]. AGEs are able to mediate crosslinking for forming of Aβ aggregation [237]. AGEs play a role in stabilizing monomer nucleation as well as polymerization of Aβ to larger

aggregates and eventually the amyloid plaques. The neurotoxic effect of Aβ appears to correlate with the aggregation state and the rate of amyloid plaque formation is likely to be important for the progression of AD [238]. This suggests that AGEs may indeed represent a driving force in the acceleration of Aβ deposition and senile plaque formation. AGEs have been detected colocalized in NFTs [232, 233]. It has been reported that microtubule associated protein (MAP)-tau which is a major component of NFTs is preferentially to be subject to intracellular glycation at its tubulin binding site, suggesting that glycation may be one of the modifications hampering the binding of tau to tubulin in AD [239]. The immunohistochemical co-localization of AGEs with NFTs is supported by the relevance of glycation as an additional pathological modification of MAP-tau *in vitro* [240]. Moreover, incubation of glycated MAP-tau into cells has generated oxygen-free radicals resulting in disturbing neuronal function, including up-regulation of APP and release of Aβ [241]. Taken together, these results suggest that glycation in tau may play a role in stabilizing paired helical filaments (PHF) aggregation, leading to tangle formation in AD.

Since AGEs signaling has recently emerged as an important factor in the pathophysiological events of AD. It has been reported that AGEs activate the transcription factor NF-κB, AP-1 as well as the mitogenic pathway involving protein kinases that involved in the pathophysiological events in AD [242-244]. Studies in the neurons and astroglia of brain sections from patients with AD have revealed that AGEs are able to activation of the p65 NF-κB subunit, whereby this activation is shown to be in the close vicinity of early senile plaques [245]. Furthermore, AGEs can cause neurotoxicity by inducing the release of cytokines and free radicals via the NADPH-oxidase/superoxide/NF-κB-pathway [246]. On the other hand, AGEs have shown to activate p21 (ras) as well as the MAP-kinases Erk 1 and ERK2 via RAGE-dependent pathway in different cell lines [247]. The small G-protein p21 or ras is a critical regulator of cell proliferation and differentiation. The downstream members of the p21 (ras) pathway are the mitogen-activated protein kinase kinase (MAPK kinase) and the mitogen-activated protein kinase (MAP-kinase or ERK). Since it is kinase which is able to phosphorylate MAP-tau, thereby stabilizing PHF aggregation, leading to tangle formation that are similar found in AD. Increased expression of the small G-protein p21 has also been demonstrated within neuritic plaques as well as in neurons and glial cells closely associated with plaques [248]. In addition, the cyclin-dependent kinase inhibitor p16, a regulator of the orderly progression through the cell cycle, and a cyclin-dependent kinase-4 (cdk-4), a critical regulator of the cell cycle, have been shown to be increase in the pyramidal neurons of the hippocampus in the AD brains [249]. As p16 is normally unfound in differentiated neurons, it seems paradoxical that the increased of these proteins in AD re-entry and progression through the cell cycle, which is likely deleterious in terminally differentiated neurons and may contribute to biochemical abnormalities, particularly axonal sprouting and hyperphosphorylation of tau as well as the neuronal degeneration characteristic of the pathology of AD [250]. Therefore, the activation of the MAP kinase cascade by AGEs is important in the processes of neurodegeneration and aberrant repair. As mentioned above is an explaination of the elevated level of AGEs and AGEs cross-linked proteins in the brain of AD patients. In addition, the factors that may involve in promoting AGEs formation in AD are following [247]:

-The intracellular increased AGEs reactive carbonyl compounds such as methylglyoxal in AD patients could be a consequence of inhibition of mitochondrial respiration, resulting from disturbed glucose metabolism.

-The increased in unchelated transition metals such as copper and iron have been observed loosely bound to amyloid plaques, leading to acceleration of the oxidation of glycated proteins and subsequent increase in highly reactive glycoxidation products. These elements do not only increase oxidation of sugars and Amadori products, but also induce the aggregation of Aβ peptides by bringing the monomers in close proximity for AGEs-induced crosslinking.

-The depletion of the antiglycation substance such as pool the histidine dipeptides including carnosine and anserine.

-The defective Aβ clearance mechanism which increases the half-life of these peptides therefore enhancing its effect on AGEs formation. Furthermore, AGEs may contribute to the inability of microglia to clear amyloid plaques by introducing crosslinks to Aβ associated proteins which makes it difficult to uptake and degrade by inhibiting lysosomal proteases such as cathepsin D.

3.8 Oxidative stress in Alzheimer's disease

In the brain, cells especially neurons, are highly vulnerable to the detrimental effects of oxidative stress. Due to their high metabolic rate, rich composition of fatty acids likely to peroxidation, high levels of intracellular transition metals for catalyzing the formation of reactive radicals whereas low levels of antioxidants, and reduced capability to regenerate [251]. In addition, it has been reported that ROS are particularly active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, which are unique to the brain and serve as sources of oxidative stress. Oxidative damage is a major pathogenic feature of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and HIV-1-associated dementia (HAD). The oxygen free radicals have also found in brain regions affected by these diseases for example the hippocampus in AD patients, the substantia nigra and caudate putamen in PD patients, and spinal fluids in ALS and HAD patients [252, 253]. Importantly, it has been revealed that antioxidant mechanisms on neurons are operative for up-regulation antioxidant defenses, which suggest a balance for oxidant damage in the diseases. However, when free radicals exceed the antioxidants controls, a cascade for oxidative stress damage arises and affects the diseases [254]. The increased free radicals attack neurons, which are post-mitotic cells and thereby particularly sensitive to free radicals. These free radicals have the capacity to attack proteins, polysaccharides, lipid bilayers, and DNA causing cellular oxidative damage leading to neuronal damage [255]. Oxidative stress has been shown to be one of the earliest events of AD, with participates as an important mediator in the onset, progression and pathogenesis of the disease. The generation of ROS and its consequent cellular damage contributes to the hallmark AD pathology seen in susceptible neurons. However, the exact mechanisms by which oxidative stress involved in AD remain elusive, here we provide an overview of the potential causes of oxidative stress in AD.

Since oxidative stress is the imbalance of free radicals production and antioxidative defense, it has been shown that the antioxidant cell defense system increases with age but the rate of ROS generation exceeds the antioxidant ability, leading to an oxidative stress. Previous study has shown that AD tissues have an imbalance in radical detoxifying enzymes; the decrease of the ratio of superoxide dismutase to catalase, leading to an accumulation of hydrogen peroxide, which subsequent products of reactive hydroxyl radicals in the presence of free iron [256, 257]. In addition to the imbalance in radical detoxifying enzymes, the decline of glutathione level and the antioxidant enzyme activities have been observed in AD and shown to correlate with the severity of the disease [258, 259]. Furthermore, it has been demonstrated that the increase levels of protein carbonyls and 3-nitrotyrosine, which are resulted from protein oxidation [260] and the increase in the nucleic acid oxidation which is detected as elevated levels of 8-hydroxyl-2-dexoyguanosine in DNA and 8-hydroxyguanosine in RNA have been found in AD [261]. Hydroxyl radical-mediated DNA damage often results in DNA strand breaks. This DNA strand cleavage by endonucleases is a part of apoptosis, therefore it is likely that oxidative damage to DNA is responsible for apoptosis in AD. Lipid peroxidation by-products, such as malondialdehyde (MDA), 4-hydroxynonenal, and F2-isoprostanes, are also abundantly increased in brain regions and cerebrospinal fluid (CSF) of patients with AD [262, 263]. Evidence continues to mount that the induction of heme oxygenase-1 (HMOX-1), an indicator of the oxidative stress response in cells, demonstrated increased in AD brains and this increase is tightly correlated with regions of NFTs pathology [264, 265]. Moreover, it has been found that glycation of tau-pathology in AD also induces an oxidative response in neuroblastoma cells shown in induction of HMOX-1, translocation of NF-κB and peroxidation of membrane lipids [20, 241, 266]. Importantly, accumulated data studying in MCI, which is proposed as an intermediate state between normal aging and dementia, has indicated that the oxidative stress damage in AD may occur preceding the onset of the disease. These results suggest that oxidative stress may be one of the earliest alterations that occur during the initiation and development of AD.

3.8.1 Oxidative stress and Aβ-induced toxicity

Accumulating evidence has shown that the presence of oxidative stress is a characteristic of AD brains in addition to the established pathology of amyloid plaques. As mention above that Aβ peptides is formed upon proteolytic processing of APP by β- and γ-secretases, resulting in the release of Aβ peptides. Increased production and/or decreased clearance of Aβ peptides leads to the accumulation of Aβ, which can stimulate several cell signaling pathways, eventually resulting in synaptic degeneration, neuronal loss and decline in cognitive function [267]. There is overwhelming data suggests the implicated oxidative stress in Aβ-induced neurotoxicity. *In vitro* experiments using cell models have shown that Aβ treatment efficiently initiates oxidation of different biomolecules. It induces lipid peroxides [268, 269], increases the levels of hydrogen peroxide [268] and hydroxynonenal (HNE) in neurons [270], damages DNA [271] and inactivates transport enzymes [272]. Consistent with other studies using various AD transgenic mouse models carrying mutants of APP and PS-1, have shown to increase ROS such as hydrogen peroxide and nitric oxide production and elevate oxidative modifications of proteins and lipids which are correlated with the Aβ accumulation, suggesting that Aβ promotes oxidative stress [273-275]. Evidence

continues to reveal that antioxidants are able to attenuate oxidative stress in Aβ-induced neurotoxicity. It has been demonstrated in AD cells and AD transgenic mouse models that natural antioxidants, such as EGb 761, curcumin, and green tea catechins, can exert neuroprotective functions by reducing Aβ-induced ROS generation and attenuating neuronal apoptosis [276, 277]. Furthermore, recently data has indicated that oxidative stress promotes the Aβ production. The elevated oxidative stress by breakdown the antioxidant defense system resulted in significantly increasing Aβ deposition in AD transgenic mice as well as it showed to be associated with the earlier onset and more severe cognitive dysfunction [278]. Consistantly, the improvement of antioxidant system by dietary antioxidants supplementation such as curcumin can lower the elevation of oxidized proteins and decrease brain Aβ levels and Aβ plaque burden [278, 279]. Overexpression of antioxidant enzymes such as manganese superoxide dismutase in AD transgenic mice has shown to decrease protein oxidation and reduce Aβ plaque burden and restore the memory deficit in AD transgenic mice [280], suggesting the enhancement of Aβ production/plaque formation by oxidative stress is important for the initiation and development of AD. In addition to mediating Aβ production, numerous studies have revealed that oxidative stress decreases the activity of α-secretase while promoting the expression and activation of β- and γ-secretase, enzymes for the production of Aβ from APP processing [45, 281-283]. Furthermore, it has been demonstrated that oxidative stress induce β-secretase BACE1 and γ-secretase PS1 expression and the activation has been found to be dependent on the activation of JNK pathway. The activation of JNK signaling cascade [282, 284, 285] and the up-regulation of BACE1 and PS1 expression/activity have been found in AD brains [286, 287]. Therefore the increased oxidative stress in AD brains may initiate the activation of a cascade of redox-sensitive cell signal pathways including JNK, which promotes the expression of BACE1 and PS1, resulting in increasing the production of Aβ. JNK is an important cell signaling cascade which is activated by oxidative stress and has also been implicated in Aβ-induced neuronal apoptosis [288]. Thus, it is possible that oxidative stress may enhance Aβ production as well as mediate Aβ-induced neurotoxicity through the activation of redox-sensitive signaling pathways.

3.8.2 Oxidative stress and tau pathology

As hyperphosphorylated tau protein, the major component of NFTs is another hallmark of AD pathology that correlates with neurodegeneration and cognitive decline. As mention in the above section that abnormal hyperphosphorylation of tau impairs its binding with tubulin leading to microtubule assembly and eventually self-aggregation into filaments. Although less well studies have shown that oxidative stress is linked with tau pathology. The linkage between oxidative stress and tau pathology has been demonstrated in the animal models overexpressing tau protein. Reduction of gene dosage of thioredoxin reductase together with mitochondrial SOD2 has shown to enhance tau-induced neurodegenerative histological abnormalities and neuronal apoptosis. In addition, it has been demonstrated overexpression of these antioxidant enzymes or treatment with vitamin E attenuate tau-induced neuronal cell death [289]. Moreover, the increased levels of ROS have found in cortical neurons derived from a transgenic rat model expressing human tau protein, and this effect could be ameliorated by antioxidant supplementation such as vitamin C [290, 291]. In addition, the study of brains of P301S transgenic mice, transgenic mouse models carrying the human tau gene

with P301S mutations which exhibit an accumulation of hyperphosphorylated tau and develop NFTs and neurodegeneration, have shown elevated oxidative stress including increased protein carbonyl levels in cortex mitochondria, altered the activity and content of mitochondrial enzymes involved in ROS formation and energy metabolism [292]. Consistently, administration of P301S mice with coenzyme Q10, an antioxidant which plays a key component in the electron transport chain, greatly increases complex I activity and reduces lipid peroxidation as well as improves survival and behavioral deficits [293]. Furthermore, growing evidence has also demonstrated that oxidative stress may have a role in the hyperphosphoryaltion and polymerization of tau. In AD brains, elevated oxidation of fatty acids has been detected which is correlated with the polymerization of tau, thus, it is possible to serve as a linker between oxidative stress and the formation of the fibrillar pathology in AD [294]. All of these observations imply that tau-induced neurotoxicity may at least partially mediated by oxidative damage.

3.8.3 Oxidative stress and mitochondria dysfunction in Alzheimer's disease

Mitochondria is unique organelles that essential for a number of cellular functions including production of ATP, calcium homeostasis, and cell survival and death. During mitochondrial activity, ROS production is greatly produced in the cell in mitochondrial electron transport chain, thus mitochondria is particularly vulnerable to oxidative stress [295, 296]. There is strong evidence for the role of ROS and mitochondrial dysfunction involved in the pathogenesis of AD. A large number of studies has indicated a high percentage of mitochondrial and metabolic abnormalities have been identified in AD patients [297, 298]. It has been reported an increased oxidative utilization of glucose in AD patients [299]. In addition, a quantitative morphometric measurements of mitochondria analyzed from biopsies from AD brains has shown a significant reduction whereas the mitochondrial DNA and protein increase in the cytoplasm and in the vacuoles associated with a lysosome suggested as the site of mitochondrial degradation by autophagy [297, 298]. Several factors that involved in mitochondrial dysfunction in AD have been reported following: 1) there is a low vascular blood flow in the brain during chronic hypoxia/hypoperfusion and has been implicated in the development of AD [300]; 2) there is a number of sporadic mutations in the mitochondrial DNA control region associated with deleterious functional consequences for mitochondrial homeostasis that are unique to AD [301]; 3) there is APP processing-mediated Aβ formation occurs in mitochondria which contributes to impede mitochondrial import channel and thus impairing mitochondrial function. The detail is presented on a below paragraph [274, 302]; 4) there is a hyperhomocysteinemia which can inhibit several genes encoding mitochondrial proteins and promotes ROS production, thus homocysteine is an independent risk factor for the development of AD [303]. In addition, the abnormalities of mitochondria lead to mitochondrial dysfunction resulting in less efficiency to produce ATP but more efficiency to produce ROS both characteristics occur in AD [304, 305]. In line with this observation, a defect of several key enzymes of oxidative metabolism such as a-ketoglutarate dehydrogenase complex (KGDHC), pyruvate dehydrogenase complex (PDHC) and cytochrome oxidase (COX) are found in AD patients [304, 306]. Notably, these mitochondrial abnormalities have been found accompanied by oxidative stress. In addition, it has found that oxidative stress induces the oxidative damage marked by 8-hydroxyguanosine and nitrotyrosine, suggesting the defect in mitochondria occurs during the progression of AD [297]. Moreover, the occurrence of oxidative damage of mitochondrial proteins and DNA in

early stages of the disease consistently demonstrates a role of oxidative stress mediated mitochondria dysfuntion in disease progression [17, 307]. Accumulated evidence has demonstrated that Aβ is implicated in mitochondrial dysfunction and contributes to the deficiency of energy metabolism and neuronal death seen in AD. It has been shown that Aβ is localized to mitochondria in AD brains and AD mice model as well as in neuroblastoma cells stably expressing human mutant APP and associated with impaired mitochondrial metabolism and increased mitochondrial ROS production [274]. In fact, the function of mitochondria is dependent on their intact structure. Previously *in vitro* experiment has demonstrated that mitochondria exposure to Aβ could induce oxidative damage to mitochondrial membrane by disruption lipid polarity and protein mobility and inhibiting key enzymes of the mitochondria respiratory chain, leading to increased mitochondrial membrane permeability and cytochrome c released [308, 309]. Indeed, the intracellular APP or A*β* can directly bind to the protein import machinery of mitochondria thus impairing import of mitochondrial proteins, leading to decrease activity of the mitochondrial electron transport chain and eventually in increase ROS production [302]. In addition, these proteins as well as tau bind at distinct sites of the mitochondrial electron transport chain resulting in deregulation of the mitochondrial function and oxidative stress [310]. Moreover, Aβ has also shown to alter cellular protective mechanisms against oxidative damage to mitochondria. Uncoupling proteins (UCPs) are a mitochondrial inner membrane protein that decreases the proton gradient generated in oxidative phosphorylation for diverse physiological functions. The expression and activation of UCPs are considered to be a protective mechanism in response to oxidative stress. Evidence suggests that UCP2 and UCP3 can be activated by ROS, then reduce mitochondrial membrane potential and ATP production, causing mitochondria uncoupling and diminish of ROS generation from mitochondria [311]. This protective mechanism found dysfunctional in AD brains is limited since the expression levels of UCP 2, 4, and 5 have significantly reduced [307]. *In vitro* AD cells treatment with Aβ has demonstrated the activation of cyto-protective mechanisms of UCP2 and UCP4 protein levels in response to the exposure of superoxide, indicating that Aβ may lead to irreversible cellular alterations that render the cell more susceptible to oxidative stress [312]. Moreover, Aβ has shown to hamper mitochondrial import ion channel. It has been demonstrated that superoxide treatment on AD cells results in diminished in UCP2- and UCP4-dependent upregulation of mitochondrial free calcium, suggesting the Aβ is possible to impede of mitochondrial import channel of intracellular calcium leading to an increased cell sensitivity to the loss of calcium homeostasis [312]. On the other hand, oxidative stress has demonstrated to activate signaling pathways that affect the processing of APP as well as the phosphorylation of tau. The studies have revealed that oxidative stress increases the expression of *β*-secretase through activation of JNK and p38 MAP kinase resulting in the generation of A*β* [313]. As well as A*β* has shown to activate NADPH oxidase in primary cultures of cortical neurons thereby causing the generation of ROS within the cells [314]. Therefore, the oxidative stress mediated mitochondria dysfunctions may play an important role in the onset and progression of AD.

3.8.4 Metal homeostasis and oxidative stress in Alzheimer's disease

Most types of oxidative injury in AD result from glycation, protein oxidation, lipid peroxidation and nucleic acid oxidation associated with metal-catalyzed free

radical formation [315]. Transition metals such as copper (Cu), zinc (Zn), and iron (Fe) have an important catalytic roles in many enzymes and are crucial for a diverse of biological processes including brain functions. For Example; Cu and Zn participate in regulating synaptic function [316, 317] while Fe is essential for neuronal processes such as myelination, synaptogenesis, and synaptic plasticity [318]. Meanwhile, these transition metals play essential roles in neural functions, it is well documented that their levels and transportation are strictly regulated. It is not surprising that the aberrant metal homeostasis in the brain is accompanied by severe neurological consequences result in neurotoxic free-radical production. It has been shown that excess Fe or Cu can directly interact with oxygen to produce superoxide ion, hydrogen peroxide, and hydroxyl radical leading to oxidative stress and a cascade of biochemical alterations that eventually cause neuronal cell death [319]. Growing evidence has shown that there is a loss of metal homeostasis in AD patients [319]. The presence in abnormal levels of transition metals within the amyloid deposits indicates the aberrant accumulation of transition metals may be linked with $A\beta$ pathology in AD [320, 321]. The presence of aberrant metal homeostasis has been demonstrated to play a role in several important aspects of AD pathogenesis such as the production and aggregation of $A\beta$ and the oxidative stress mediated by $A\beta$. Indeed, overaccumulation of Fe has been detected in the hippocampus and cerebral cortex colocalizes with AD pathology, senile plaques, and neurofibrillary tangles in AD brains [322]. Since Fe is a transition metal involved in the formation of OH• via the Fenton reaction [322] thereby it is an important cause of oxidative stress in AD. For Cu, it has a catalytic role in many enzymes that require for oxidation-reduction reactions such as in the catalytic site of COX of the mitochondrial electron transport chain, and Cu-Zn superoxide dismutase (SOD) [315]. While Cu mediated oxidative damage has emerged as one of the important factors in AD pathogenesis, the mechanisms by at least two pathways: 1) alterations in ceruloplasmin which is a copper binding protein that plays a role in protective mechanism against oxidative stress. As ceruloplasmin involves in the regulation of the redox state of Fe by converting the ROS catalytic-Fe^{2+} to a less reactive Fe^{3+}, the increased level of ceruloplasmin in brain tissue and cerebrospinal fluid of AD patients may indicate a compensatory response to increased oxidative stress. While ceruloplasmin level in neurons remain unchanged, suggesting that neurons may play a role in metal catalyzed damage [323]; 2) Cu interaction with $A\beta$ contributes to generation of ROS. $A\beta$ can bind Cu^{2+} with high affinity, forming a cuproenzyme-like complex. $A\beta$ is able to reduce Cu^{2+} to Cu^+ by an electron transfer reaction forming positively charged $A\beta$ radical ($A\beta$+•) [324]. Cu^+ then donates two electrons to oxygen, generating H_2O_2 for further Fenton reaction that increases the production of OH• [325]. Similarly, iron-$A\beta$ interaction also results in reduction of Fe^{3+} to Fe^{2+} and the generation of H_2O_2 setting up conditions for Fenton reaction [326]. In addition, the metals also play an important role in the production and aggregation of $A\beta$. It has been shown that both Cu and Zn can bind to $A\beta$ monomers, promoting conformational changes of the $A\beta$ peptide that results in amyloid aggregation [327]. Additionally, studies have shown that metal homeostasis, especially Fe and Cu, is involved in the production and processing of APP. *In vitro* experiment using AD model which overexpresses the mutant form of human APP treatment with iron, has shown to increase the release of $A\beta$42 peptides [328, 329]. In addition, it has been reported an iron-responsive element within the 5'-untranslated region of APP transcript which is

selectively downregulated in response to intracellular Fe chelation thereby increase in Fe levels may lead to upregulation of APP protein translation through binding of this regulatory proteins to the APP resulting in promotion of Aβ from APP processing [330]. These observations suggest the generation of ROS from the aberrant accumulation of metals, the disturbance of metal homeostasis and the interactions of Aβ with metals are key contributors to the oxidative stress in Aβ-mediated neurotoxicity and AD pathogenesis.

3.8.5 Advanced glycation endproducts (AGEs) and oxidative stress in Alzheimer's disease

As describes in the previous section that AGEs result in formation of oxygen-derived free radicals, they represent an important source of the oxidative stress in AD. A number of studies have reported the accumulation of AGEs in the aging brain [331] and the Maillard reaction is implicated in the development several age-related diseases including AD [331-335]. In AD brains, AGEs levels have found to significantly elevate [237, 266, 336]. As mention earlier, AGEs are protein modifications that contribute to the formation of the histopathological and biochemical hallmarks of AD such as amyloid plaques and neurofibrillary tangles. Recent studies have identified increased levels of several specific and non-specific products of Maillard reaction such as pentosidine, carboxymethyl lysine pyrraline and hexitol lysine, in association with neurofibrillary pathology in AD [237, 266, 335-337] indicating the presence of the meta-stable Amadori intermediates generated upon early glycation. This observation suggests that active glycation is still occurring in the disease process which is also demonstrated both by the induction of stress and cytotoxicity and their ability to produce Aβ [15, 338]. Moreover, AGEs and Aβ can bind and activate RAGE result in enhancing the intracellular ROS production and trigger a range of signaling pathways [36].

3.8.6 Neuroinflammation and oxidative stress in Alzheimer's disease

There is more and more evidence that inflammation is involved in the pathogenesis of AD. A number of studies have shown that in pathologically vulnerable regions of the AD brain with the presence of Aβ plaques and neurofibrillary tangles as well as injured neurons can provoke inflammation resulting in increased inflammatory molecules and activated inflammatory cells. Interestingly, the secretion of ROS/RNS by inflammatory cells is a major mechanism for attacking opsonized targets and for activation of inflammatory cells from the resting to activated state. The activated inflammatory cells are capable of expressing a wide range of inflammatory mediators [133, 134]. It has been demonstrated that Aβ directly activates the NADPH oxidase in microglia, which results in a generation of superoxide radicals and increased production of hydrogen peroxide [339, 340]. In addition, the increase expression of NADPH oxidase in microglia have been found in AD brain as well as increased expression of iNOS which involved in production of NO, which in turn reacts with superoxide to form peroxynitrite, that is also detected in astrocytes surrounding plaques in AD brain [341, 342]. Furthermore, activated microglia have the potential to produce large amounts of ROS/RNS by the enzyme myeloperoxidase (MPO). Furthermore, it has been reported that aggregated Aβ induces MPO gene expression in microglia-like cells *in vitro* as well as it has also been detected in activated microglia around amyloid

plaques in the AD brain [343]. MPO catalyzes a reaction between hydrogen peroxide and chloride to form hypochlorous acid, following it can further react with other molecules to generate other ROS such as hydroxyl ions. MPO has also found to catalyze the formation of nitrotyrosine-modified proteins [344] as well as cause AGEs modifications [345]. These observations have been detected in AD pathology [244, 346]. The detail of neuroinflammation is reviewed in the below section.

3.9 Neuroinflammation in Alzheimer's disease

Inflammation is a complex cellular and molecular response to defend against insults; stress, injury or infection [169]. Brain inflammation is a pathological hallmark of AD. It is a process that is closely related to the onset of various neurodegenerative diseases including AD. Results of a number of studies suggest that in pathologically vulnerable regions of the AD brain with the presence of Aβ plaques and neurofibrillary tangles have found the increased inflammatory molecules and activated inflammatory cells [347-353]. However, the classical signs of inflammation such as swelling, heat, and pain are not present in the brain. Brain inflammation is a chronic condition that continuously deteriorates the surrounding tissues. This inflammation normally tends to accumulate slowly, sometimes even asymptomatically during years and eventually can lead to severe tissue deterioration [354]. Neuroinflammation is defined as the brain's response to the insults which is primarily mediated via one of two cell systems: glia of the central nervous system (CNS), and lymphocytes, monocytes, and macrophages of the hematopoietic system resulting in the release of inflammatory mediators (Figure 10) [355-357]. Activated cells are strongly involved in the secretion of proinflammatory mediators such as proinflammatory cytokines, chemokines, macrophage inflammatory proteins, monocyte chemo-attractant proteins, prostaglandins, leukotrienes, thromboxanes, coagulation factors, ROS and other free radicals, nitric oxide, complement factors, proteases, protease inhibitors, pentraxins, and C-reactive protein [358-361]. In addition, chronic inflammation due to the release of the mediators leads to recruitment of monocytes and lymphocytes through the blood brain barrier (BBB) [362] as well as recruitment of activated glial cells, promoting their proliferation, and resulting in further release of more inflammatory factors [363]. This activation results from the inflammatory responses contribute to the progress of AD, thus accelerating the course of the disease.

The involvement of microglia, astrocytes, and neurons responsible for the inflammatory reaction in the onset and progress of neurodegenerative process in AD is becoming increasingly recognized [134]. Glial cells are the primary modulators of inflammation in the central nervous system [355, 356, 364]. They constantly survey their environment and can be activated by a local insult or a response to a systemic insult [365]. Activated glia cells have a progressive pattern of association with Aβ plaques and tangles. They stimulate a chronic inflammatory reaction to clear these insults [366]. The dystrophic neurites, activated microglia, and reactive astrocytes can be seen in these plaques [366-368]. These observations, together with known functions of the involved inflammatory mediators, oxidative stress and neuroinflammation stimulate a chronic inflammatory reaction. It has been reported that aggregated amyloid fibrils and inflammatory mediators secreted by glia cells contribute to neuronal

dystrophy [369]. Chronically activated glia can, furthermore, kill adjacent neurons by releasing highly toxic products such as reactive oxygen intermediates, nitric oxide, proteolytic enzymes and, complementary factors [370]. In addition, it is now well documented that inflammatory mediators and a stress conditions can enhance APP production and the amyloidogenic processing of APP to induce Aβ production as well as also inhibit the non-amyloidogenic APP processing to produce soluble αAPP fraction [371-373]. On the other side, several lines of evidence suggest that Aβ also induces the expression of proinflammatory cytokines in glia cells [374], the activation of the complement cascade [375] and the induction of inflammatory enzyme systems such as iNOS [376] contributing to neuronal dysfunction and cell death.

Glial cells activation quickly leads to the release of both pro- and anti-inflammatory mediators. The final effect of the reaction is dependent on the balance between these opposing responses. When glial cells normally provide neurotrophic factors for neurogenesis that serve as a protective function, on the other hand, they are also activated by a set of stressing events and continuous activation leads to exaggerated release of proinflammatory mediators [377]. Glial cells in the CNS are following:

A). Microglia: Microglia constitutes around 10% of the cells in the nervous system. They are cells that support and protect neuronal functions. They act as the first line of defense against invading pathogens or other brain tissue injuries. Microglia not have only neuroprotective functions such as phagocytic and scavenger functions, but also have neurotoxic effects in the brain [356]. The current evidence suggests that microglia play a central role in the response to pathological lesions of AD such as Aβ plaques [378]. Amyloid peptides and their precursor APP are potent glial activators. Aβ attracts and activates microglia, leading to cluster of microglia around Aβ deposits sites in the brain [379, 380]. Microglia are activated through RAGE and then stimulate a nuclear factor NF-κB dependent pathway and subsequent activation of ERK and MAPK pathways that lead to the production of inflammatory mediators [381]. Activated microglia secreate a variety of pro-inflammatory mediators such as proinflammatory cytokines IL-1, IL-6, and TNF-α, the chemokines IL-8, macrophage inflammatory protein-1α (MIP-1α), and monocyte chemoattractant protein-1 [361, 382, 383]. In some situations, activated microglia reduce Aβ accumulation by increasing its phagocytosis, clearance, and degradation and also secrete a number of soluble factors, such as the glia-derived neurotrophic factor (GDNF), which has been found to be beneficial [384, 385]. Although microglia have neuroprotective functions, neurotoxic mechanisms involving continuous activation of microglia and toxic factors released by microglia may lead to neuroinflammation. Subsequently, the continuous activation of microglia may act as trigger for the progression of AD pathology.

B). Astrocytes: Astrocytes are characteristic star-shaped glial cells in the brain and spinal cord and are the most abundant type of glial cells in the CNS. Astrocytes have various functional capacities including biochemical support of endothelial cells of the BBB, supplying nutrients to nervous tissue, maintenance of extracellular ion balance, and healing the brain and spinal cord following traumatic injury [169, 386]. Astrocytes appear to be involved in the induction of neuroinflammation. Similar to microglia, they also secrete various proinflammatory molecules such as ILs, prostaglandins, leukotrienes, thromboxanes, coagulation factors, complement factors, proteases, and

protease inhibitors [169]. Several researchers have shown that in AD brain tissues, reactive astrocytes have found cluster around Aβ deposits which also produced inflammatory mediators which is accompanied by progressive damage and loss of adjacent neurons [387-389]. Astrocytes themselves are known to be an important mechanism for Aβ clearance and degradation, for providing trophic support to neurons, and for forming a protective barrier between Aβ deposits and neurons [390]. The presence of large numbers of astrocytes associated with Aβ deposits in AD demonstrate that these lesions generate chemotactic molecules that mediate astrocyte recruitment. However, *in vitro* and *in vivo* experiments suggest that inflammatory active astrocytes do not generate significant amounts of these molecules. The presence astrocytes tendency to release proinflammatory molecules are thought to stimulate and even accelerate the progression of AD [169, 386].

C). Neurons: Neurons have traditionally believed to be passive bystanders in neuroinflammation, however, more recent evidence suggests that neurons can generate inflammatory molecules. Neuronal chemokines particularly act as versatile messengers between neurons and glial cells [391, 392]. In addition, neurons are able to serve as a source of complement proteins, pentraxins, C-reactive protein, amyloid P [169], cyclooxygenase (COX)-2-derived prostanoids [393, 394], macrophage colony-stimulating factor (MCSF) [395] and iNOS [396, 397] and therefore may contribute to cause neuronal dysfunction and cell death. In addition, it has been shown that neurons secrete CD22 for inhibiting microglial proinflammatory cytokine production [398]. Neurons have also demonstrated to produce several proinflammatory cytokines such as IL-1 [399-401], IL-6 [400, 402-404] and TNF-α [399, 405-408]. There are a number of reports have indicated changing in levels of IL-1α, IL-1β, IL-6, TNF-α, granulocyte-macrophage colony-stimulating factor (GMSF), IFN-α, the type B of IL-8 receptor (IL-8RB), and the receptor for CSF-1 in AD brain, blood, and CSF [409].

The detailed of interesting cytokines are following:

A). IL-1: IL-1 is a proinflammatory cytokine and found to be an important initiator of the immune response. It plays a key role in the onset and development of a complex hormonal and cellular inflammatory cascade. It has several activities in the brain both under physiological and pathophysiological conditions [386, 410]. Elevated IL-1 is recognized as a critical component of the brain's patterned response to insults, termed neuroinflammation, and of leukocyte recruitment to the CNS. IL-1 secretion has found in response to both Aβ and neuronal damage, and can become a chronic source for chronic immune response [411]. IL-1 plays a role in neuronal degeneration. Recently evidence has revealed that IL-1 induces IL-6 production and stimulus for iNOS expression [412]. In addition, IL-1 enhances microglial activation, astrocyte activation, and additional IL-1 production, thereby establishing a self-propagating cycle [410]. In addition, the recent evidence has suggested IL-1 regulates APP processing and Aβ production leading to chronic, sustained and progressive neuroinflammation found during early AD pathogenesis [389].

B). IL-6: IL-6 is a multifunctional cytokine that plays an important role in host defense with major mediators of inflammation [23]. IL-6 has found to be one of the mediators of inflammation in the neurodegenerative process in AD. IL-6 can promote

glia activation and stimulate the production of acute phase proteins [413-415]. The evidence demonstrates that IL-6 has shown to be increase in the brain of AD patients suggesting a pathological interaction [416, 417]. Moreover, in another research, monocyte-derived dendritic cells from AD patients has been shown to secrete high amounts of IL-6 that confirming the more pronounced of this cytokine in AD patients [418].

C). TNF-α: TNF-α is a potent proinflammatory cytokine. It triggers downstream signaling cascades that control a variety of cellular processes related to cell viability, gene expression, ion homeostasis, and synaptic integrity. In the brain, among other functions, TNF-α serves as a gliotransmitter, that regulates synaptic communication between neurons and has shown to affect both synaptic strength and mediate synaptic scaling for controlling the neural networks. Moreover, it also plays a role in initiating and regulating the cytokine cascade during an inflammatory response [419]. In the healthy brain, levels of TNF-α expression are low, however in inflammatory or disease states, TNF-α along with several other proinflammatory mediators and neurotoxic substances greatly produce in response to pathological stimuli [405]. TNF-α involves in the pathogenesis of several brain diseases and neuroinflammatory disorders, including AD. Increased TNF-α has been reported to cause neuronal dysfunction and mediates amyloid-induced disruption of molecular mechanisms involved in memory function [419]. The studies have revealed that elevated levels of the TNF-α in serum and postmortem brains of patients with AD [386].

It can be concluded that the production of these proinflammatory cytokines by neurons may in fact trigger further neuroinflammatory processes and lead to even greater neuronal damage and to the pathogenesis of AD.
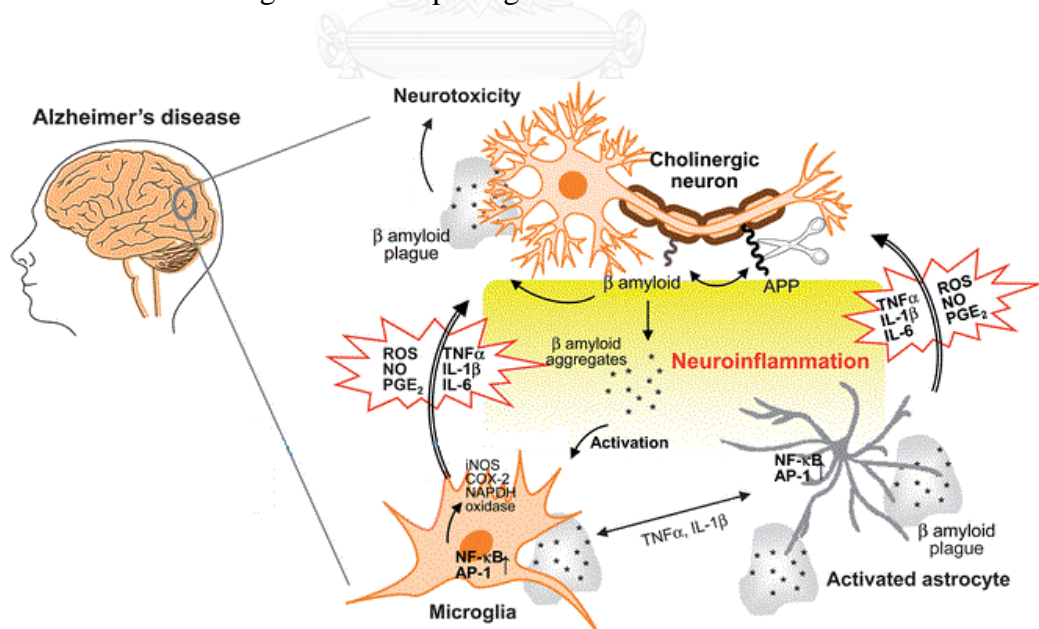

Figure 10. Neuroinflammation-mediated neuronal loss in Alzheimer's disease (adapted from Pan M.H., et al., 2010) [420].

3.10 Treatment of Alzheimer's disease

As AD progresses, the brain cells die and the brain's communication network where transmission of neurotransmitters carry information from one cell to another is damage, causing cognitive symptoms to worsen. To date, there is no cure for AD. AD medications can temporarily slow the worsening of symptoms and improve quality of life for those with AD and their caregivers.

3.10.1. Pharmacologic treatment

While current medications can not stop the damage AD causes to brain cells, they may help lessen or stabilize symptoms for a limited time by affecting certain chemicals involved in carrying messages among the brain's nerve cells. The Food and Drug Administration (FDA) has approved six drugs which are two types of drugs specifically to treat symptoms of AD including a cholinesterase inhibitors and NMDA (N-methyl-D-aspartate)-type glutamate receptors antagonist [421]. Cholinesterase inhibitors inhibit acetylcholine esterase (AChE) to breakdown the levels of the acetylcholine (ACh) neurotransmitter, a chemical messenger that's important for alertness, memory, thought and judgment, which is depleted in AD brains [422]. Therefore, cholinesterase inhibitors are prescribed to treat symptoms related to memory, thinking, language, judgment and other thought processes. There are four drugs of this type: tacrine, donepezil, rivastigmine and galantamine. These medications currently approved to treat AD symptoms in early to moderate stages. A second type of medication, NMDA receptor AD antagonist or memantine is approved by the FDA for treatment of moderate to severe AD. Memantine or antagonize NMDA (N-methyl-D-aspartate)-type glutamate receptors prevents aberrant neuronal stimulation and regulate the activity of glutamate, a messenger chemical involved in learning and memory, which has been found a lot in AD brains [422]. Memantine is prescribed to improve memory, attention, reason, language and the ability to perform simple tasks. In addition, on December 2014, the FDA approved the sixth drug, which combines two existing FDA-approved AD drugs called Namzaric, combines memantine hydrochloride extended-release and donepezil hydrochloride and is for moderate to severe disease [1, 2]. These therapies, however, only relieve symptoms of the disease. In addition, they have also been associated with many side effects such as diarrhea, nausea, vomiting, muscle cramps and leg cramps, decreased heart rate (bradycardia), decreased appetite and weight, increased gastric acid production and abnormal dreams [169].

3.10.2. Non-pharmacologic therapy

Non-pharmacologic therapies are those that employ approaches other than medication such as music therapy. Similar to current pharmacologic therapies, non-pharmacologic therapies have not been shown to alter the course of AD. The aims of this approach is used for maintaining or improving cognitive function, the ability to perform activities of daily living, or overall quality of life such as reducing depression, apathy, wandering, sleep disturbances, agitation and aggression [1, 2]. The recent studies on nonpharmacologic therapies have found that some, such as cognitively stimulating environments (for example, gardening, word games, listening to music and cooking), physical exercise and diets low in calories and low in cholesterol and saturated fats can reduce the risk of AD [4, 423, 424]. A few non-pharmacologic therapies have been tested in animal studies, which provide the strongest evidence of

whether a therapy is effective. In latter epidemiological findings have shown that cognitively stimulating environments, physical exercise and dietary restriction regimens increase the resistance of neurons in the brain to degeneration, enhance neurogenesis and improve learning and memory [423, 425, 426]. Although the possibility that one's risk for AD can be reduced by modifications of diet and lifestyle is considerable interest. Thus non-pharmacologic therapies is needed to be further determined for their effectiveness.

3.10.3. Potential therapeutic strategies

Regarding of factors of AD, sufficient progress in delineating the disease has now been achieved to envision several therapeutic targets for treatment AD. Because the production of Aβ from APP appears to be a pivotal event in AD pathogenesis, there is intense interest in developing drugs that block the β- or γ-secretase enzymes. Inhibitors of BACE1 can reduce Aβ production without major side effects and could be therapeutic in the early phases of the disease [427]. Specific γ-secretase inhibitors have been designed to decrease Aβ production without side effects resulting from blockade of γ-secretase cleavage of Notch and other protein substrates [428]. Another approach to reducing amyloid accumulation in the brain is agents that can bind Aβ monomers for prevention their assembly into potentially cytotoxic oligomers [3]. An alternative and attractive approach would be to use anti-inflammatory drugs that could interfere with inflammatory responses that occur in the AD brain. The epidemiological evidence that consumption of anti-inflammatory agents such as COX-2 inhibitors and aspirin [429] and steroids that decline during normal aging such as estrogen and testosterone [430] for other purposes may be associated with a reduced developing AD. However, drugs that target specific sites in neurodegenerative cascades have only been investigated in cell culture and animal models of AD, and their potential in the clinic remains unclear [431]. Other therapeutic approaches being tested include using a variety of antioxidants, free radical scavengers, calcium channel blockers, metal chelators and modulators of certain signal transduction pathways that might inhibit the deleterious effects from the downstream of the Aβ accumulation [3, 4]. One of the most promising approaches for preventing and treating AD is vaccination to remove Aβ from the brain. This approach is based upon stimulating the immune system to remove Aβ. It has been reported that active immunization with human Aβ42 [432] or passive immunization with Aβ antibodies [433] result in the clearance of Aβ plaques from the brains of APP mutant transgenic mice and also ameliorate memory deficits in the mice [434] but their use in humans may be compromised by adverse reactions occurred in some patients [435]. However, an effective vaccine would have a major impact on the disease, and is currently one of the most exciting areas of AD research. Finally, the ability to identify individuals at risk for AD based upon genetic or environmental factors will allow the application of more aggressive interventions in those individuals.

4. Herbal therapy for the treatment of Alzhemer's disease

Since the greatest aim of AD therapy is to stop or slow down the disease progression. Cholinesterase inhibitors as well as NMDA receptor antagonist medications have a modest clinical effect on the symptoms, but do not prevent the

deterioration of AD [421]. Finding an effective method to treat AD still poses a significant clinical challenge. Other drugs have been studied and used in an attempt to modify the course or improve the symptoms of AD. Since the underlying causes of AD (with the exception of familial AD) have not yet been identified, treatment strategies associated with AD pathologies are potentially multiple and include the following [25]:

- Reducing Aβ and tau pathologies
- Countering specific neurotransmitter abnormalities; mainly cholinergic abnormalities
- Treatment with anti-inflammatory, antioxidant and anti-apoptotic agents
- Promoting neuroplasticity including synaptic, dendritic and neurogenic

Herbal medicine has long been used as therapy for dementia. The longstanding use of traditional Chinese medicine and European herbal medicine up provide an invaluable data on the safety and efficacy of numerous species. Due to their complexity of chemical content and variety of bioactivities, herbal medicine offers the prospect of the kind of "built in" poly-pharmacology that is increasingly apparent for alternative drugs [436]. Melanie J.R., et al., 2011 collected information on therapeutic approaches associated with pathological mechanisms of AD and phytochemicals with relevant mechanistic activities [25] are summarized in Table 1.

Table 1. AD pathologies, therapeutic approaches and phytochemicals with relevant mechanistic activities (adapted from Melanie J.R., et al, 2011) [25].

Pathological mechanism	Treatment strategy	Examples of phytochemicals with relevant activities	
		Phytochemical	Plant(s) source
Widespread of extracellular amyloid deposits	Inhibition of Aβ formation; inhibition of secretases in APP processing particularly β- and γ-secretase inhibitors	Nicotine Oleanolic acid glycosides Tannic acid Curcumin Epigallocatechin-3-gallate Asiatic acid Rosmarinic acid Scyllo-cyclohexanehexol	*Nicotiana* sp. *Pulsatilla koreana* *Quercus* sp. *Curcuma longa* (turmeric) *Camellia sinensis* (tea) *Centella asiatica* Lamiaceae (subfamily Nepetoideae) *Cocos nucifera* (coconut)
Intracellular aggregations of abnormal neurofilament and tau protein	Inhibition of tau aggregation and dispersing; inhibitors of GSK3	Icariin Genistein Polyphenols	*Epimedium brevicornu* *Glycine max* (soya bean) *Vitis vinifera* (grape seed)

Pathological mechanism	Treatment strategy	Examples of phytochemicals with relevant activities	
		Phytochemical	Plant(s) source
		Ginsenoside Rb$_1$	*Panax* sp. (ginseng)
		Delphinidin	Widely distributed anthocyanin
		Epigallocatechin-3-gallate	*Camellia sinensis*
A loss of cell leading to neurotransmitter changes	Countering neurotransmitter abnormalities by anti-apoptosis; trophic factors (anti-excitotoxicity)	Ginkgolide B Baicalein	*Ginkgo biloba* *Scutellaria baicalensis*
		Galantamine	*Galanthus* sp. (snowdrop)
		Piceatannol	*Vitis vinifera* (grape)
		5-*O*-Caffeoylquinic acid	*Coffea arabica* (coffee)
		Procyanidins; procyanidin B$_2$	*Theobroma cacao* (cocoa)
Cholinergic system degeneration resulting in neurotransmitter changes	Countering neurotransmitter abnormalities by increasing synaptic Ach; including AChE and BChE enzyme inhibition	Galantamine (anti-ChE)	*Galanthus* sp.
		Physostigmine (anti-ChE)	*Physostigma venenosum* (calabar bean)
		Huperzine A (anti-ChE)	*Huperzia serrate*
		Conypododiol (anti-ChE)	*Asparagus adscendens*
		Neferine (anti-ChE)	*Nelumbo nucifera*
A cell loss induced by excess glutamate resulting in neurotransmitter changes	Countering neurotransmitter abnormalities by NMDA glutamate receptor antagonism or modulation	Paeoniflorin	*Paeonia* sp. (peony)
		Curcumin	*Curcuma longa*
		Rhynchophylline and Isorhynchophylline	*Uncaria* sp.
		Ginsenosides Rh$_2$ and Rg$_3$	*Panax* sp.
		Pyrolyzates	*Phyllostachys* sp. (bamboo)
		Nobiletin	*Citrus* sp.
Oxidative stress	Antioxidants	Neferine	*Nelumbo nucifera*
		Curcumin	*Curcuma longa*

Pathological mechanism	Treatment strategy	Examples of phytochemicals with relevant activities	
		Phytochemical	Plant(s) source
		Rosmarinic acid	Lamiaceae (subfamily Nepetoideae)
		Epigallocatechin-3-gallate	*Camellia sinensis*
		Resveratrol	*Vitis vinifera*
		Tanshinone IIA	*Salvia miltiorhiza* (Chinese sage)
		Ginsenoside Rb_1	*Panax* sp.
		4-*O*-Methylhonokiol	*Magnolia officinalis*
		Silibinin	*Silybum marianum* (milk thistle)
Inflammatory processes	Inflammatory agents	Polyphenolic compounds	*Vaccinium* sp.
		Neferine	*Nelumbo nucifera*
		Curcumin	*Curcuma longa*
		Xanthorrhizol	*Curcuma* sp.
		Crocin and crocetin	*Crocus sativus* (saffron)
		Daidzein	*Glycine max*
		Celastrol	*Celastrus* sp.
		Fisetin	*Rhus* and *Acacia* sp.
Neuroplasticy leading to loss of synaptic and dendritic neuronal cell processes	Trophic factors	Protopanaxadiol saponins	*Panax* sp.
		Withanolide A, withanosides VI and IV	*Withania somnifera*
		Trigonelline	*Coffea Arabica*
		Secoiridoids	*Gardenia* sp.
		Boswellic acid	*Boswellia serrata* (frankincense)

ACh= acetylcholine; AChE= acetylcholinesterase; BChE = butyrylcholinesterase; ChE = cholinesterase; GSK3= glycogen synthase kinase 3

4.1 Natural compounds with antioxidative properties

A growing body of research has reported a burst of oxidative stress is a characteristic of AD brains in addition to the established pathology of AD [267]. Therefore, much attention has been paid to use antioxidants for inhibition of ROS bursts

for treatments of AD. Antioxidants are exogenous or endogenous molecules those act against any form of oxidative stress and their effects on cellular system. Since the human body can produce oxygen free radicals and other ROS as by products through numerous physiological and biochemical processes, antioxidants defense systems co-evolved along with aerobic metabolism to counteract oxidative damage [437]. Exogenous antioxidants such as glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, and vitamin A and tea polyphenols from our daily diet as well as antioxidant enzymes, e.g. superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase have further supported the antioxidant system with those exert synergistic actions in removing free radicals to prevent oxidative stress [438]. Antioxidants not only neutralize ROS and other kinds of free radicals produced as consequence of oxidative stress but also have attracted the attention of clinicians due to therapeutic potential. In fact, many natural compounds with antioxidant ability such as flavonoids from *Scutellaria baicalensis*, carnosic acid from *Rosmarinus officinalis*, curcuma oil from rhizomes of *Curcuma longa*, ginkgo biloba extract EGb761 from *Ginkgo biloba* leaves, and cinnamophilin from *Cinnamomum philippinense* have exhibited neuroprotective effects [439]. It has been reported that aryl amines and indoles-carotene, polyenes-carotene, polyphenols (flavonoids, stilbenes, and hydroquinone), monophenols (tocopherols(vitamin E),17-estradiol (estrogen) and 5-hydroxytryptamine (serotonin) can direct chemical (nonenzymatic) scavenging of ROS generated free radicals leading to neuroprotection in AD [71, 440]. There are accumulated reports focusing on the precise mechanisms or direct targets of natural antioxidant compounds protecting against oxidative stress. It has been reported that CA, a catechol-type electrophilic compound found in the herb rosemary obtained from *Rosmarinus officinalis* exhibit direct regulatory effects on endogenous antioxidant enzyme systems [441]. Pretreatment with CA has found to induce the expression of a set of antioxidant enzymes, including heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase 1 (NQO1), and c-glutamyl cysteine ligase (c-GCL). Tetrahydroxystilbene glucoside (TSG), an active component of the rhizome extract from *Polygomum multiflorum*, has been reported to attenuate intracellular ROS generation and mitochondrial membrane potential dissipation caused by directly upregulate the expression of sirt1. Sirt1 is a class III histone deacetyltransferase that promotes cell survival and subsequently reduces the expression and activity of iNOS. Sirt1 activation leads to hamper the DNA binding of NF-κB by inhibit the phosphorylation and subsequent degradation of IκB [442]. The enzyme NADPH oxidase is another important target for ROS production. A recent study has been revealed that some natural compounds inhibit NADPH oxidase such as sinomenine which is an alkaloid extracted from a Chinese medicinal plant, *Sinomenium acutum* [443], the ethanol extract of Erutaecarpa [444].

4.2 Natural compounds with anti-inflammatory effects

As brain inflammation is another pathological hallmark of AD [348, 350]. Many hundreds of plants contain well known anti-inflammatory agents. However, the phytochemical compounds of anti-inflammatory agents have been reported to engage a variety of compound such as polyphenols, flavonoids, terpeniods, alkaloids, anthraquinones, saponins, lignans, polysaccharides and peptides [445]. The targets related to inflammation, such as peroxisome proliferator- activated receptor-γ (PPARγ)

and NF-κB, have been reported to mediate inflammation through regulation of gene promoter regions, leading to inflammatory gene transcription [446]. Recent studies have revealed that chlorophyllins A-C exhibit potent PPARγ agonistic effect [447] and prenyloxycinnamic acid derivative 4'-geranyloxyferulic acid extracted from *Acronychia baueri* Schott increases PPARγ activity [448]. In addition, some plant-derived compounds exhibit direct regulation of NF-κB. For instance, incensole acetate and its nonacetylated form, incensole (IN), from *Boswellia Resin*, has shown to inhibit NF-κB activation [449]. Parthenolide from *Tanacetum parthenium* (Feverfew) has also exhibited regulation of NF-κB by directly binding to IκB kinase resulting in reducing of NF-κB activation [450]. Hesperetin which is a flavanone derived from citrus fruits has shown to suppress NF-κB activation in animal models through multiple signal transduction pathways [451]. These observations suggest that nuclear transcription factors may serve as the direct target of natural anti-inflammatory compounds.

There are several studies and documents that indicate a unique role of herbal medicines in the treatment of AD.

Galantamine: It is an alkaloid originally derived from the bulbs and flowers of *Galanthus caucasicus* (Caucasian snowdrop, Voronov's snowdrop), *Galanthus woronowii* (Amaryllidaceae) and also occurs in species of Narcissus (daffodil) and *Leucojum aestivum* [25]. Galantamine is a potent competitive and selective acetylcholinesterase (AChE) inhibitor. Like the synthetic AChE inhibitor donepezil and rivastigmine, galantamine is brain selective [436, 452]. It has shown to increase the concentration and action of acetylcholine (Ach) in the brain thus galantamine is effective in AD associated with cognition and also behavioral symptoms and activities of daily living and at all stages of the disease tested, but not for mild cognitive impairment (MCI) [453]. Galantamine is also an allosteric modulator of nicotinic acetylcholine receptors (nAChRs), potentiating the presynaptic response to Ach [25, 436, 452]. However, alterations in nAChRs may not be responsible for the cognitive improvements in AD patients seen with this drug [454]. Only pooled data from one study has shown increased cognitive performance in galantamine treatment AD patients with increased hippocampal glutamate, which is suggested to result from potentiation of presynaptic nAChRs by galantamine, thus increasing glutamatergic neurotransmission; however, it should be noted that this study had a small sample size and a short follow-up time of just 4 months [455]. To date, in the US, galantamine is a licensed drug for the treatment of symptoms of mild to moderate dementia in AD [25]. In addition, recent study has demonstrated that galantamine has a neuroprotective mechanism against Aβ toxicity through regulation of the calcium signalling mediators calpain and calcineurin, down regulation β-secretase (BACE1) expression thereby inhibit Aβ formation, inhibition Aβ aggregation, prevention Aβ-induced oxidative damage via alteration in the glutathione antioxidant system and inhibition apoptosis by preventing mitochondrial dysfunction and endoplasmic reticulum stress [25]. However, these neuroprotective effects translated into clinical effects is undetermined.

Ginkgo biloba: *Ginkgo biloba* is one of the most extensively studied plants and widely used for complementary therapies due to a variety of the pharmacological actions of *G. biloba* including anti-apoptosis, anti-inflammatory effects, neuroprotection, anxiolysis,

MAO-inhibitory effects, ROS scavenging and antioxidant effects [456-458]. There are far more mechanistic and clinical data on *G. biloba* relevant to dementia with many studies having focused on the standardized extract EGb 761. It has been reported that EGb 761 prevents oxidative damage [458] and improves learning and memory in the aged rats [456]. The researchers have determined the effect EGb 761 compare with the cholinesterase inhibitors AD drugs such as donepezil and rivastigmine. The result has shown that EGb 761 is as effective as any of these commonly prescribed drugs in treating the symptoms of AD patients. Considering the evidence, it is suggested that cholinesterase inhibitors should be used in preference to *G. biloba* in patients with mild to moderate AD [459]. In addition, mechanistic effects relevant to AD are listed in Table 1. However, the clinical studies focusing on the cognitive effects of *G. biloba* in AD are under investigation. The recent clinical data still remain surprisingly ambiguous for AD. In 2002, a Cochrane review demonstrated an improvement in cognition and activities of daily living. Conversely, more recent reviews have indicated no evidence or inconsistent and unreliable evidence for any predictable clinical benefit in dementia [25, 436]. Since the clinical studies have focused on the effects of *G. biloba* extracts rather than the active phytochemicals. In fact, the phytochemical compounds of *G. biloba* have been reported to be involved with cognitive effects take part in a variety of compound such as the terpenoid (bilobalide and ginkgolides) and some flavonoid constituents such as quercetin. In addition, the phytochemical compounds have been reported to engage in a number of mechanistic effects include neuroprotection (ginkgolides A and B, bilobalide), upregulation of NGF (ginkgolide B), neurogenesis and synaptogenesis (bilobalide, quercetin), PAF receptor antagonism (ginkgolides A and B) and vasodilatory effects (quercetin, bilobalide, ginkgolides A, B and C) [25].

Huperzine A: Huperzine A extracted from a particular type of club moss (*Huperzia serrata*). It is a medicinally active plant derived chemical that belongs to the class known as alkaloids. *H. serrata* contains the quinolizidinerelated alkaloids, huperzines A and B [25]. Huperzines A is a potent, reversible and selective inhibitor of acetylcholinesterase [25, 436, 452, 460] and has neurotrophic effect [461]. In addition, it has been shown to protect neurons from oxidative damage induced hydrogen peroxide, Aβ, glutamate and ischaemia [462], regulate the expression of apoptotic proteins, protect mitochondria, and modulate amyloid precursor protein (APP) metabolism [463]. According to the clinical evidence for efficacy on improvement cognition and activities of daily living of huperzine A has been show in China three Chinese double-blind trials [464]. Therefore the drug "Shuangyiping," a tablet formulation of huperzine A, was developed in China and used for symptomatic treatment of AD as well as huperzine A is also marketed in the USA as a dietary supplement as powdered *H. serrata* for memory impairment [436].

Vinpocetine: Vinpocetine is a indole alkaloid vincamine derived from *Vinca minor* (lesser periwinkle) as well as the seeds of various African plants. It is used as a treatment for memory loss and mental impairment due to a variety of its properties [25, 436, 452]. For instance it has been shown to increase cerebral blood flow, reduce cerebral insufficiency due to ischaemia and are associated with improvement in short-term memory [465]. Vinpocetine has also improved spatial memory and modulated cholinergic functions in an animal model of AD [466]. In addition, a synthetic

derivative of the vincamine alkaloid has been reported to stimulate cerebral metabolism, modulate neurotransmitter release and block voltage-gated Na+ channels [465] and protect against NMDA-induce neurotoxicity [467]. Some promising clinical trial data have shown that vinpocetine is effective in dementia. Unfortunately, most of the trials investigating the efficacy of vinpocetine in dementia have been flawed in both in design and reporting. There is insufficient evidence to support the use of vinpocetine in dementia [25, 436, 452]. Further trials are needed to provide more evidence for the therapeutic potential of vinpocetine in dementia.

Lamiaceae: Sage and Lemon Balm
Members of the Lamiaceae family and one of interesting phytochemical components from the Lamiaceae family (subfamily Nepetoideae) is rosmarinic acid have been widely studied for mechanistic effects and some clinical effects relevant to dementia [25, 436, 452, 460]. For Lemon balm (*Melissa officinalis*), this European herbal medicine is reputed to improve memory and to possess calming and antidepressant properties. Neurobiological activities of *M. officinalis* include ACh receptor activity with both nicotinic and muscarinic binding properties; 5-HT1A, 5-HT2A and GABAA receptors of the essential oil [25, 436, 452] and these may be use pharmacologically relevant for cognitive symptoms. The clinical trials have been reported that *M. officinalis* extract improves cognitive impairment and reduces agitation in AD [468]. When administered via aromatherapy, *M. officinalis* oil also reduces agitation in AD patients [469]. It has been suggested that the effects of *M. officinalis* oil on agitation may be associated with physiological effects on GABAA-mediated transmission and a reduction in spontaneous synaptic transmission.

For *Salvia officinalis* L. or *S. lavandulifolia* Vahl. or sage, this European herbal medicine is a longstanding reputation in European herbal encyclopedias for improving memory and circulation. Sage has a range of relevant biological activities that support the traditional uses which include anti-AChE and anti-BuChE activities, anti-inflammatory, anti-estrogenic activities, antioxidant effects, anti-amyloidogenic activity, neuroprotective activity and memory enhancement [25, 436]. The study in healthy young and elderly volunteers has revealed that sage enhances memory cognitive ability, including immediate word recall scores [470, 471]. The clinical trails have also shown that patients with mild to moderate AD receiving *S. officinalis* extract significant benefits in cognitive and behavioral effects [472, 473].

Ginseng: Ginseng is the common name used to describe various species of Panax, usually *Panaxi ginseng*. It has a reputation for a numerous health benefits involved with anti-aging and adaptogenic properties. Active constituents found in most ginseng species are a group of saponins including ginsenosides, polysaccharides, peptides, polyacetylenic alcohols and fatty acids [474]. Main active ingredient in *P. ginseng* is the ginsenosides which can enhance psychomotor and cognitive performance, and has benefit for AD by improving brain cholinergic function, reducing the level of Aβ and repairing damaged neuronal networks [475]. Ginsenosides are triterpene saponins. Over 30 ginsenosides have been identified and classified into two categories: 1). the 20(S)-protopanaxadiol (PPD); Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, and Rs1; 2). the 20(S)-protopanaxatriol (PPT); Re, Rf, Rg1, Rg2, and Rh1 [476]. Mechanistic effects that support the traditional uses have been reported for some of their phytochemical

components, and include inhibition of glutamate- and Aβ-induced cytotoxicity by Rb1, Rh2, Rg2, Rg3 and prevention of Aβ-induced tau phosphorylation by Rb1, neuroprotective effects by Rg3, antagonization of NMDA receptors by Rg3, Rh2, modulation of acetylcholine release and increasing choline acetyl transferase (ChAT) and improving cognition by Rb1, Rg1, reducing Aβ formation by Re, Rg1 and Rg3 [25]. Clinical data relating to dementia are positive but not yet strong. Most trials in AD have shown statistically significant improvement of cognition at the end of the study, when compared with the control group. However, these studies have serious methodological limitations with an insufficient description of randomization and without blinding [477]. Furthermore, studies on healthy volunteers have also demonstrated the improvement in cognitive performance but efficacy has been inconsistent [478], suggesting that similar to other traditional plant medicines for cognition, efficacy is greater for people who have not yet developed dementia [25, 436, 460].

Nicotine: Nicotine is a plant-derived alkaloids which has been reported as a nAChR agonists that can bind affinity for nAChRs, with the latter improving learning [25]. Epidemiological data has shown that smoking tobacco from Nicotiana species have no effect on AD risk or moderately increase AD risk. The result has revealed that nicotine is shown to inhibit the Aβ formation as well as the neurotoxic effects of glutamate, enhance the effects of nerve growth factor (NGF), modulate cholinergic function and improve cognitive functions [456]. However, this compound has not been developed pharmaceutically for the treatment perhaps because of toxicity concerns.

Curcumin: Epidemiological data has shown a lower prevalence of AD in some populations with a curcumin-rich diet [479] and improved cognitive function has been linked with consumption of a curry that frequently consists of turmeric (*Curcuma longa*), a source of curcumin [480]. Main active ingredient of *C. longa* is the curcumin and related curcuminoids (calebin A) which has been reported to protect neurons from Aβ toxicity through its antioxidant effects [481] and downregulate BACE1 thereby inhibit Aβ formation, prevent aggregation of Aβ into oligomers and fibrils and reduce amyloid plaques [482]. Although curcumin is a promising candidate for AD, there is only one clinical study of this compound has been completed to date [483]. Further and extensive clinical trials for assessment the effects of curcumin on both prevention and treatment of cognitive decline in dementia are still needed.

Resveratrol: Epidemiological studies have indicated that moderate consumption of wine reduces the risk of AD [484]. Although other interactive factors need to be considered when interpreting such data, documented activities of wine polyphenols appear to be supportive of a protective effect against AD. Resveratrol which is a polyphenols has been focused on numerous studies in relation to dementia. It has been greatly found in wine and grape juice at concentrations of 0.05–25 mg/L, but also occurs in a wide variety of other dietary sources such as peanuts, pistachios and blueberries [25]. Resveratrol has been reported to scavenge ROS to prevent oxidative stress and has neuroprotective effects, with the latter prevent cognitive impairments [485, 486]. It also activates sirt1, a histone deacetylase that is involved in the response to molecular damage and metabolic imbalances [487]. In addition, resveratrol treatment

can improve memory in rat model infused with Aβ in the cerebral ventricles [479] and reduce plaque formation in an AD transgenic mouse model [488]. To date, resveratrol has reached phase II trials as a neuroprotectant, although not specifically for AD [25].

5. <u>**Interesting plants in this study**</u>

Amaranthus plants are a promising food crop which belongs to Amaranthaceae family. Amaranthus is extensively cultivated throughout the world under a wide range of climatic conditions mainly due to its resistance to heat, drought, diseases and pests, and the high nutritional value of both seeds and leaves [489]. Amaranthus sp, generally known as spinach and locally known as ''pak khom'', is one of the most popular leafy vegetables consumed worldwide. Four types of Amaranthus can be distinguished; *A. viridis*, *A. lividus*, *A. spinosa* and *A. tricolor*, which are native vegetables in Thailand [490]. The leaves are rich in proteins and micronutrients such as carotenoids, vitamin C, and dietary fiber, minerals like calcium, iron, zinc, magnesium and phosphorus [491-493]. Amaranthus has also been reported to contain several antioxidant components such as polyphenols, flavonoids, beta-carotene phenolics, anthocyanins and ascorbic acid suggesting that it may prove be an efficient antioxidant [494, 495]. All parts of the plant are used as medicine to promote multiple health benefits. It has been extensively used in Aayurveda for treating menorrhagia, diarrhea, dysentery, haemorrhagic colitis, bowel hemorrhages, cough and bronchitis [496]. Several studies have shown that amaranth seed or oil may benefit those with hypertension and cardiovascular disease, regular consumption reduces blood pressure and cholesterol levels [489]. Apart from this, their leaves have been reported to possess wide range of pharmacological activities such as anti-tumor effect [497] and hepatoprotective activity [498]. In addition, recently research provides evidence that eating leafy vegetables such as spinach, kale, collards and mustard greens and other foods contained vitamin K, lutein, folate and beta-carotene can help slow cognitive decline and keep the brain healthy to preserve functioning [499]. Since declining cognitive ability strongly associate with AD dementia, consumption of these plants could offer a medical use given to these plants for protecting the brain from AD. Hence, it was thought worthwhile to carry out the potential beneficial attributes of two species of Amaranthus which are abundantly available Thailand; *Amaranthus lividus* and *Amaranthus tricolor*.

Amaranthus lividus **Linn.** (also known as *A. blitum*) Its common names are English wild amaranth, green amaranth or Thai amaranth, Known in Thai as Phak-khom-Thai. The plant grows with either red or green leaves. However, in Thailand, *A. lividus* is the green vegetable [490]. The nutrient contents of *A. lividus* are shown in Table 2. The plant has been reported as one of many vegetables rich in antioxidant components. Phenolic acids, β-carotene and ascorbic acid, flavonoids are found in *A. lividus* which might be some of the components able to contribute to their radical scavenging and metal chelating activities [493, 500]. In addition, it also contained α-tocopherol which is important biological antioxidants attributed to lipid peroxidation inhibitory for prevention oxidation of body lipids, including polyunsaturated fatty acids and lipid components of cells and organelle membranes [493, 500]. As A. *lividus* displays potent antioxidant properties, supporting the medical use given to this plant as a medicinal plant.

Amaranthus tricolor **Linn.** Its common names are Joseph's coat, English red spinach (Deighton), Chinese spinach or Tampala, Known in Thai as Phak-khom-Suan. The plant can be produced as a red leafy vegetable [490]. *A. tricolor* is rich in minerals such as calcium, iron, magnesium, phosphorus, potassium, zinc, copper and manganese and vitamins such as vitamin A, vitamin B6, vitamin C, riboflavin and foliate [491, 501]. This plant also possess the major unsaturated fatty acids; linoleic acid in seeds and stems and linolenic acid in leaves, while the major saturated fatty acid is palmitic acid found in seeds, stems and leaves [502]. Mature leaves of *A. tricolor* are red color which is reported to be caused by red-violet pigments, the betacyanins amaranthin and isoamaranthin [503] and anthocyanins, one of the phenolic compounds [504]. The nutrient contents of *A. tricolor* are shown in Table 2. *A. tricolor* has been used for the treatment of piles, bladder distress, blood disorders, tooth ache and dysentery and even as astringent, diuretic, haemorrhage and hepatoprotective agent [501, 505] and also externally use to treat inflammations [489]. Recent study has shown that *A. tricolor* possess antimicrobial activity which is endowed with a significant antioxidant activity which can counteract the oxidative damage induced by the malaria parasite [501]. It can be concluded that *A. tricolor* has a significant antioxidant activity due to the presence of active constituents, thereby have potential to use in the medicinal plants.

Table 2. Nutrient contents in the leaves of *A. lividus* and *A. tricolor* (on 100 g fresh weight of edible portion basis): adapted from Enoch G.A.D., et al., 2014 [489].

Species	Protein (g)	Vitamin A (mg)	Vitamin C (mg)	Ca (mg)	Fe (mg)	Zn (mg)
A. lividus	3.5	1.7	42	270	3.0	-
A. tricolor	3.9	1.8	62	358	2.4	0.8

Objective of the study

The general aim of the study was to find out two of Thai plants, *Amaranthus lividus* Linn. and *Amaranthus tricolor* Linn. on protective effects on AGEs-induced cellular damage by enhancing oxidative stress and inflammation that involved in Alzheimer's disease on cultured neuronal cells.

1. To determine the antioxidant capacity of the plant extracts.
2. To investigate the toxicity of AGEs on cultured neuronal cells and the effects on their receptor, RAGE, on neuroinflammatory mechanisms and secretases enzyme for Aβ production.
3. To examine the protective effects of the plants using crude extracts against AGEs induced-oxidative stress and neuroinflammation.
4. To evaluate the effects of both plant crude extracts on Aβ production cells through key enzymes for Aβ formation.

Research questions

1. Do BSA-AGEs adduct modified by glucose induces oxidative stress, inflammation and cell toxicity in cultured neuronal cells?
2. Do synthesized AGEs have any effects on RAGE, neuroinflammatory mechanisms, and key enzymes for Aβ production in cultured neuronal cells?
3. Do the chosen herbal extracts possess the antioxidant capacity and protect cultured neuronal cells against AGEs induced-neurotoxicity?
4. Do the chosen herbal extracts possess the protective effects on AGEs induced-oxidative stress and neuroinflammatory mechanisms?
5. Do the chosen herbal extracts possess the ability on prevention of Aβ production through key enzymes for Aβ production?

Hypotheses

1. A synthesized BSA-AGEs adduct *in vitro* can induce oxidative stress, inflammation causing progressive cell damage in cultured neuronal cells.
2. The synthesized BSA-AGEs adduct can activate RAGE, neuroinflammatory mechanisms and key enzymes for Aβ production in cultured neuronal cells in AGEs induced-oxidative stress condition.
3. The chosen herbal extracts possess antioxidant capacity and exert a protective activity toward cultured neuronal cells against AGEs-induced toxicity.
4. The chosen herbal extracts are capable of inhibition of AGEs induced-oxidative stress and neuroinflammation in cultured neuronal cells through inhibition of RAGE/NF-κB signal pathways.
5. The chosen herbal extracts are capable of inhibition of Aβ production in cultured neuronal cells through inhibition of secretases enzyme for Aβ production involving NF-κB-mediated secretases expression.

Conceptual framework
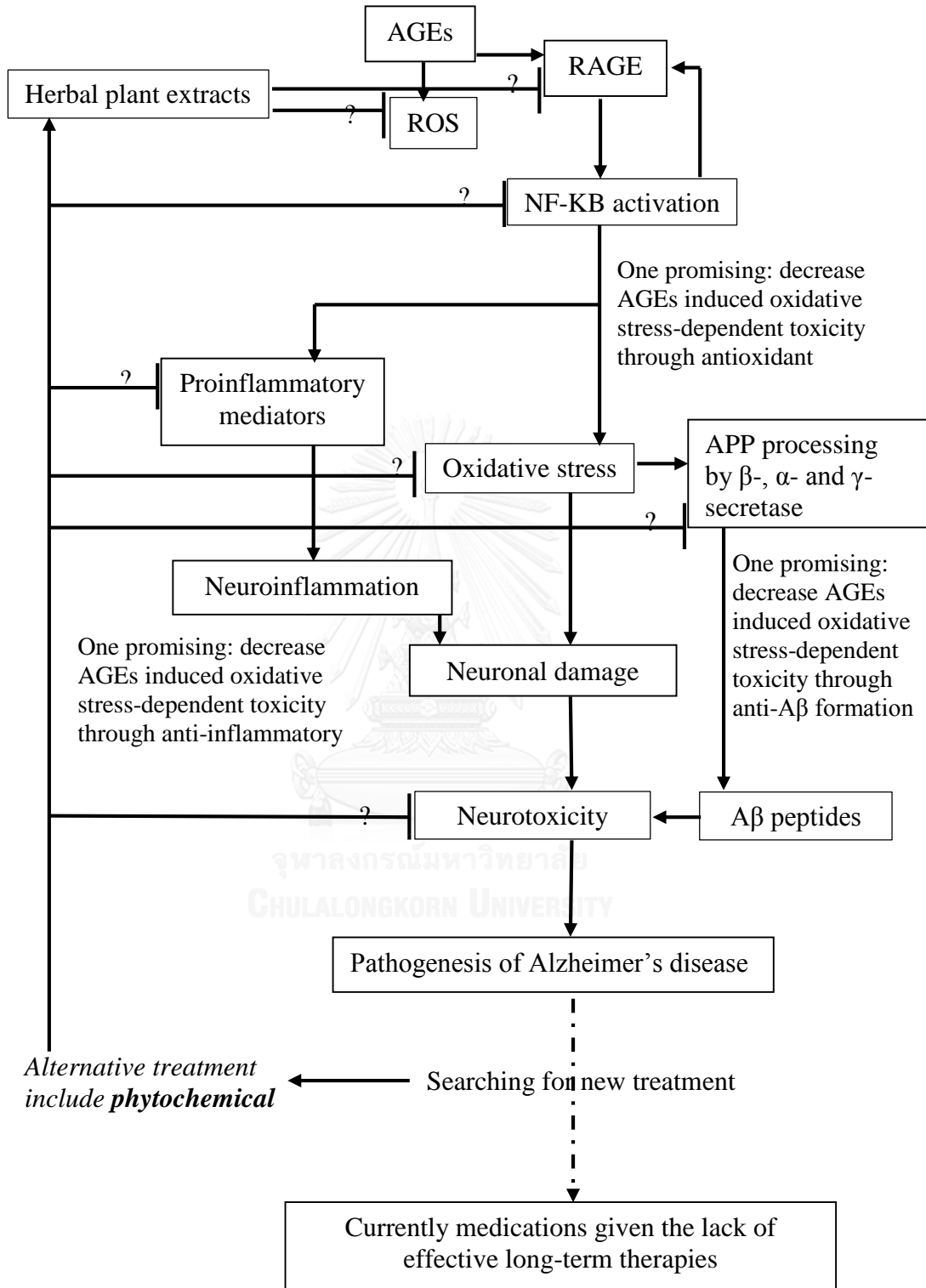
CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Material and solvents: All reagents were analytical grade. Water was purified using a Millipore Q water system and filter through 0.22 µM pore size, one carbon cartridge followed by two ion exchange cartridges (Millipore, USA).

Chemicals	Companies, Countries
2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid);ABTS	Sigma Aldrich, USA
Acetic acid	Sigma Aldrich, USA
Ascorbic acid	Merck, Germany
Agarose gel	Research Organics,USA
Bovine Serum Albumin (BSA)	VWR, USA
Cell lysis buffer	R&D Systems, USA
Chloroform	Sigma Aldrich, USA
Coomassie Brilliant Blue G	Sigma Aldrich, USA
D-glucose	Ajax Finechem, AUS
2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA)	Sigma Aldrich, USA
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Merck, Germany
Dimethyl Sulfoxide (DMSO)	Merck, Germany
Deoxyribonuclease I	Promega, USA
Dichloromethane	Merck, Germany
Diethyl pyrocarbonate (DEPC)	Sigma Aldrich, USA
DNA Ladder 100 bp	Fermentas, Lithuania
Ethylenediaminetetraacetic acid (EDTA)	Bio Basic, Canada

Ethanol	Merck, Germany
Ethidium Bromide	Sigma Aldrich, USA
Fetal Bovine Serum (FBS)	Hyclone, UT
Ham's F12 nutrient mixture	Hyclone, UT
Hank's balanced salt solution (HBSS)	Hyclone, UT
Hydrogen peroxide 30% W/V	Merck, Germany
Isopropanol	RCILabscan, Thailand
Minimum Essential Medium (MEM) with Earle's Balanced Salts (MEM/EBSS)	Hyclone, UT
Methanol	Merck, Germany
Oligo-dT 18 mer	Bioneer, South Korea
Orthophosphoric acid	Sigma Aldrich, USA
Penicillin-Streptomycin 100X solution	Hyclone, UT
Pertassium persulfate	Merck, Germany
Petroleum ether	Merck, Germany
Potassium chloride	Bio Basic, Canada
Potassium phosphate monobasic KH_2PO_4	Bio Basic, Canada
Phosphate Buffered Saline (PBS) 10X solution	Hyclone, UT
Phosphoric acid H_3PO_4	Sigma Aldrich, USA
Primer	Bioneer, South Korea
RNase inhibitor	Hyclone, UT
Sodium azide	Sigma Aldrich, USA
Trisbase	ResearchOrganics,USA
Sodium chloride	Merck, Germany
Sodium phosphate dibasic Na_2HPO_4	Bio Basic, Canada
Trypsin 0.25% (1X) solution with 0.1% EDTA	Hyclone, UT

| Trypan Blue Stain 0.4% | Invitrogen, USA |

2.1.2 Tool and Device:

Chemicals	**Companies, Countries**
-20°C Freezer	Sanyo Electric, Japan
-80°C ULT Deep Freezer	Liofreeze, USA
4°C Refrigerator	Sharp, Japan
6, 24, 96 well culture plate flat bottom with lid	Corning Inc., USA
96 well black culture plate flat bottom with lid	Corning Inc., USA
96 well plate medium binding	Corning Inc., USA
25, 75 cm^2 cell culture flask	Corning Inc., USA
15, 50 mL centrifuge tube	Corning Inc., USA
2 mL Cryovial tube	Corning Inc., USA
5, 10, 25 mL Disposable serological pipette	ProSourceScientific, Canada
0.2 mL PCR tube for Real-Time PCR	Bioneer, Korea
96 Real-Time Quantitative Thermal Block	Bioneer, Korea
Adhesive Optical Sealing Film	Bioneer, Korea
Analytical balances	MettlerToledo, Switzerland
Autoclave	Hirayama, Japan
Auto pipette	Gilson, France
Block heater	Wealtec Corp, USA
Centrifuge	Beckman Coulter, USA
CO_2incubator	Thermo Scientific, USA
Electrophoresis power supply	Bio-Radlaboratories, USA

Evaporator	Genevac, USA
Fluorescence spectrophotometer	PerkinElmer, Finland
Gel documentation (gel doc) systems	Syngene, UK
Gel electrophoresis apparatus	Bio-Radlaboratories, USA
Glassware	Pyrax, USA
Hemocytometer	Hausser Scientific, USA
Incubator	Memmert, Germany
Inverted microscope	Olympus Optical, Japan
Laminar Flow Cabinet	Haier, China
Laminar Flow Clean Bench	Esco, Singapore
Light microscope	Olympus Optical, Japan
Liquid Nitrogen Tank	Taylor Wharton, USA
Magnetic stirrer	DAIHANScientific, South Korea
Microcentrifuge	Beckman Coulter, USA
Microcentrifuge tube (1.5 mL)	Greiner Bio-One, Austria
Micro High Speed Refrigerated Centrifuge	Vision Scientific, South Korea
Multichannel pipette	Gilson, France
pH meter	MettlerToledo, Switzerland
Pipette controller	Jencons Scientific, UK
Pipette tips 10, 20 µl	Sorenson, USA
Pipette tips 100, 200 µl	Gilson, France
Pipette tips 1000 µl	Hycon, USA
Rotary evaporator	HeidolphInstruments, Germany

Soxhlet apparatus	Lab Heat, Germany
UV-Visible spectrophotometer	PerkinElmer, Finland
UV-Visible spectrophotometer for RNA	Beckman Coulter, USA
Vacuum Concentrator	ThermoElectron Corporation, USA
Vortex mixer	FINEPCR, South Korea
Water bath	Memmert, Germany

2.2 Herbal samples

Two species of herbs were used in this study (Figure 11 and 12). They were collected from a single source at Mahidol University, Salaya, Nakhon Pathom province, Thailand. The voucher specimen was botanically identified and given herbarium number by Department of Botany, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailand.

1. *Amaranthus lividus* Linn. Its common names are English wild amaranth, green amaranth or Thai amaranth, Known in Thai as Phak-khom-Thai which belongs to *Amaranthaceae* family, and given a herbarium number was 013696 (BCU).

Figure 11. *Amaranthus lividus* Linn.

2. *Amaranthus tricolor* Linn. Its common names are Joseph's coat, English red spinach (Deighton), Chinese spinach or Tampala, Known in Thai as Phak-khom-Suan which belongs to *Amaranthaceae* family, and given a herbarium number was 013695 (BCU).

Figure 12. *Amaranthus tricolor* Linn.

2.3 Cell culture model

Immortalized human neuroblastoma SH-SY5Y cell line (Figure 13), served as *in vitro* model for AGEs-induced cellular oxidative stress, was generous gift from Dr. Tewarit Sarachana, Faculty of Allied Health Sciences, Chulalongkorn University. The cell was maintained on MEM/F12 culture medium supplemented with 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2 in the CO_2 incubator. Collection of cells was carried out by de-adherence from the culture flask by trypsinization. The cells were counted using a Neubauer haemocytometer and seeded in a culture multiwell plate. The SH-SY5Y culture plate was then incubated in the CO_2 incubator, maintained at a temperature of 37°C in a humidified atmosphere at 5% CO_2 overnight with medium supplemented with 5% FBS. The adhered cells were grown to 70–80% confluence.
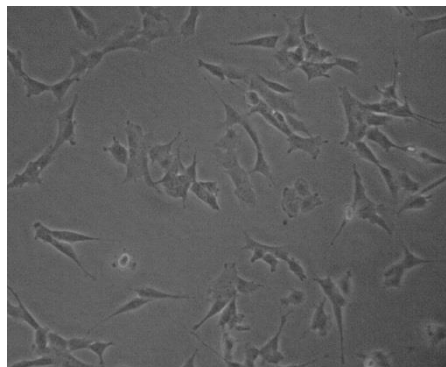
Figure 13. Morphology of immortalized human neuroblastoma cell line SH-SY5Y by phase contrast microscope 40X.

2.4 Experiment procedure

2.4.1 Herbal Extraction

The fresh leaves was cleaned with water, dried in a laboratory oven at 45°C for 5 days, and finally ground into a fine powder (Figure 14). The plant powder was successively extracted using a Soxhlet extractor (Figure 15) with organic solvents (1:10, w/v) using series of organic solvents with increasing polarities (petroleum ether, dichloromethane and methanol) until exhaustion. This method described by Franz von Soxhlet, it is the most commonly used example of a semi-continuous method applied to extraction from a solid material [506]. According to the Soxhlet's procedure, the desired compound from solid material was extracted by repeated washing (percolation) with an organic solvent under reflux in a special glassware. In this method the sample was dried, ground into small particles and placed in a porous cellulose thimble and an organic solvent was put into the flask, and then heated the solvent to reach its boiling point. The solvent evaporated and moved up into the condenser where it was converted into a liquid that trickles into the extraction chamber containing the sample. The extraction chamber is designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. The process was continued until exhaustion. At the end of the extraction process, the flask containing the solvent was removed. The plant extracts was concentrated using a vacuum rotary evaporator under reduced pressure and low temperature. The crude extracts were finally concentrated by a vacuum concentrator. The dried extracts were re-dissolved in DMSO and maintained as 100 mg/ml as stock solutions at -20°C and protected from light until further investigation.

Figure 14. *A. lividus* and *A. tricolor* in a laboratory oven at 45° and a fine plant powder.

Figure 15. A soxhlet extractor

2.4.2 Advanced glycation endproducts (AGEs) preparation

AGEs are formed endogenously by the non-enzymatic "browning"or Maillard biochemical reaction of a ketone or aldehyde group from reducing sugars such as glucose, with free amino groups from proteins, with further rearrangements, to form stable compounds, which crosslink to lysine residues of long-lived proteins [507].

2.4.2.1 AGEs preparation

AGEs-bovine serum albumin (AGEs-BSA) was prepared according to a previously described method with minor modification [243, 508, 509]. AGEs was prepared by incubating 10 mg/ml BSA with D-glucose (7 mmol/l) in PBS, pH 7.4, for 12 weeks at 37 °C. Control BSA was produced in a similar manner but without reducing sugars. All incubations were carried out in PBS and contained sodium azide to prevent bacterial contaminations. Unbound sugar was removed by dialysis through a semipermeable membrane against PBS. Then AGEs solution was filtered through sterile syringe filters (0.2 µm) for sterilization and confirmed to be endotoxin free, below the detection limit of the endotoxin assay, by using an endotoxin kit assay and then stored in the dark at -80°C until further investigation.
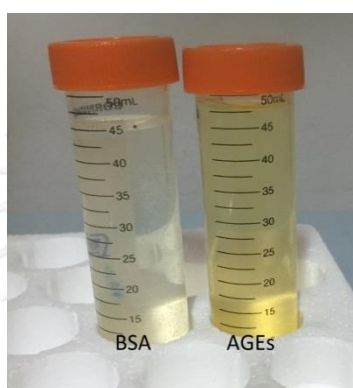
Figure 16. Long-term incubation of proteins with reducing sugars leads to AGEs formation with fluorescence and a brown color.

2.4.2.2 Dialysis of AGEs

After incubation, AGEs were dialyzed against PBS (pH 7.4) through a semipermeable membrane using a dialysis apparatus, Slide-A-Lyzer® Dialysis Cassette (Thermo Scientific, USA; Figure 17). This cassette facilitates simple and effective removal of small contaminants from proteins and other molecules that are larger than glucose molecule.

Procedure

1. Pipette15-30 ml of AGEs solution into the cassette.
2. Float cassette in PBS, pH 7.4 and stir gently with the stir bar. Dialyze for the amount of time sufficient to remove unwanted compounds. Using the dialysis buffer at 200-500 times the volume of the sample, a typical dialysis procedure is as follows: dialyze for 2 h at RT, then change the dialysis buffer and dialyze for another 2 h and finally change the dialysis buffer and dialyze overnight at 4°C.
3. Collect the sample from the cassette.
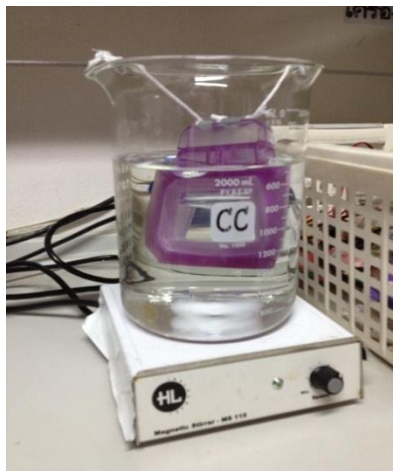
Figure 17. Dialysis of AGEs to remove unbound sugar by a Slide-A-Lyzer Dialysis Cassette in PBS.

2.4.2.3 Detection of AGEs fluorescence

At present, there is no universally accepted method to detect AGEs at a clinical level. Methods such as HPLC, ELISA, and immunohistochemistry have been employed, however no standard unit of measure exists to compare data. Since most AGEs have a characteristic fluorescence, with an excitation maximum approximately at 370, and emission around 445 nm, detection through fluorescence spectroscopy is a widely available method. For determination of the dependence of AGEs formation on incubation time, 200 μl of the incubation solution was recorded on the spectrofluorometer at excitation/emission wavelengths of 335/460 nm. The relative fluorescence of the AGEs was increased approximate 5-fold compared to the non-glycated BSA [509].

2.4.2.4 Detection of endotoxin levels of AGEs [510]

Endotoxin levels were tested from the stock solutions of AGEs. Endotoxin levels were measured by endotoxin test kit, E-TOXATE™ (Limulus Amebocyte Lysate) test kit (Sigma Aldrich, USA). The kit is intended for the detection and semi quantitation of endotoxins for research purposes. The E-TOXATE reagent is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*. When exposed to minute quantities of endotoxin (lipopolysaccharides from the walls of Gram-negative bacteria), the lysate increases in opacity as well as viscosity and may gel, depending on the concentration of endotoxin. AGEs were found to be below the detection limit of the endotoxin assay.

Procedure

1. Prepare nine standard solutions of the endotoxin standard stock solution, containing 0.015, 0.03, 0.06, 0.125, 0.25, 0.50, 4, 40 and 400 EU/ml, prepare dilutions of endotoxin standard stock using E-TOXATE water as shown in Table 3.

Table 3. The procedure step to dilute endotoxin standard stock solution

Tube No.	Endotoxin	E-TOXATE water (ml)	Final concentration (EU/ml)
1	0.2 ml Endotoxin Std. Stock Soln.	1.8	400
2	0.2 ml from Tube No. 1	1.8	40
3	0.2 ml from Tube No. 2	1.8	4
4	0.3 ml from Tube No. 3	2.1	0.5
5	1 ml from Tube No. 4	1.0	0.25
6	1 ml from Tube No. 5	1.0	0.125
7	1 ml from Tube No. 6	1.0	0.06
8	1 ml from Tube No. 7	1.0	0.03
9	1 ml from Tube No. 8	1.0	0.015

2. Add sample, water, and endotoxin standard dilutions directly to the bottom of tubes as shown in Table 4.
3. Add E-TOXATE reagent working solution to each tube by inserting pipette to just above the contents and allowing lysate to flow down the side of tube, thereby avoiding contact and possible cross contamination.
4. Mix tube contents gently. Cover mouths of tubes with parafilm and incubate for 1 h undisturbed at water bath 37°C to avoid disruption gel structure and cause an irreversible liquefaction.
5. Gently remove tubes and slowly invert 180° while observing for evidence of gelation. A positive test is the formation of a hard gel that permits complete inversion of the tube without disruption of the gel. All other results including soft gels, turbidity, increasing in viscosity, or clear liquid are considered negative.

Table 4. The procedure step to measure endotoxin levels by endotoxin test kit, E-TOXATE™

	Tube	Sample	E-TOXATE water	Endotoxin Std. Dilution	E-TOXATE reagent working solution
A	test for endotoxin in sample	0.1 ml	-	-	0.1 ml
B	test for inhibitor in sample	0.1 ml	-	0.01 ml of 4 EU/ml	0.1 ml
C	negative control	-	0.1 ml	-	0.1 ml
D	standard	-	-	0.1 ml of 0.5 EU/ml	0.1 ml
E	standard	-	-	0.1 ml of 0.25 EU/ml	0.1 ml
F	standard	-	-	0.1 ml of 0.125 EU/ml	0.1 ml
G	standard	-	-	0.1 ml of 0.06 EU/ml	0.1 ml
H	standard	-	-	0.1 ml of 0.03 EU/ml	0.1 ml
I	standard	-	-	0.1 ml of 0.015 EU/ml	0.1 ml

2.4.2.5 Total protein determination by Bradford assay [511]

The Bradford protein assay was used to measure the concentration of total protein in the AGEs sample. The principle of this assay is that the binding of protein molecules to Coomassie Brilliant Blue G-250 under acidic conditions results in a color change from brown to blue. The absorbance maximum for an acidic solution of Coomassie dye shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range [512]. This assay is very fast and fairly accurate and samples that are out of range can be retested within minutes. This assay use BSA as standard.

Procedure
1. Prepare five standard solutions (1 ml each) containing 0, 0.0625, 0.125, 0.25, 0.50 and 1 mg/ml.
2. Dilute unknown protein samples to obtain 0.0625-1 mg protein/ml.

3. Add 10 µl each of standard solution or unknown protein sample to an appropriately labeled test tube. Protein solutions are normally assayed in duplicate or triplicate.
4. Add 200 µl of dye reagent concentrate to each tube and vortex.
5. Pipet of each standard and sample solution into a fresh microtiter plate and incubate at RT shaker for 10-30 min.
6. Measure absorbance at 595 nm.
7. Prepare a standard curve of absorbance versus micrograms protein and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor.

2.4.3 Determination of antioxidant activity of herbal extracts

2.4.3.1 The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging assay

The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable 2, 2- diphenyl 2-picrylhyorazyl (DPPH) free radical according to the method described by Brand-Williams et al [513] with slight modifications. DPPH is very popular for the study of natural antioxidants. This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. DPPH is a stable free radical (DPPH•) which accepting hydrogen from a corresponding donor. DPPH• accepts hydrogen from an antioxidant. DPPH• shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. Therefore, the antioxidant effect is proportional to the disappearance of DPPH• in test samples.

Procedure

1. Prepare 2.5 mM DPPH solution with absolute methanol. It should be protected from light and the DPPH solution was prepared daily.
2. Adjust the absorbance to 0.650 ± 0.020 at 517 nm using absolute methanol.
3. Prepare ascorbic acid as a reference standard between 0, 6.25, 12.5, 25, 50 and 100 µg/ml as shown in Table 5 and dilute the herbal extracts from 100 mg/ml stock solutions to 0.001, 0.01, 0.1 and 1 mg/ml as shown in Table 6.

Table 5. The procedure step to dilute ascorbic acid stock solution

Tube	Ascorbic acid	Absolute methanol (µl)	Final Concentration
1	Ascorbic acid stock solution	-	1000 µg/ml
2	100 µl from Tube No. 1	900	100 µg/ml
3	500 µl from Tube No. 2	500	50 µg/ml
4	500 µl from Tube No. 3	500	25 µg/ml
5	500 µl from Tube No. 4	500	12.5 µg/ml
6	500 µl from Tube No. 5	500	6.25 µg/ml

Table 6. The procedure step to dilute herbal extracts stock solution

Tube	Herbal extracts	Absolute methanol (µl)	Final Concentration
1	Herbal extracts stock solution	-	100 mg/ml
2	5 µl from Tube No. 1	495	1 mg/ml
3	10 µl from Tube No. 2	90	0.1 mg/ml
4	10 µl from Tube No. 3	90	0.01 mg/ml
5	10 µl from Tube No. 4	90	0.001 µg/ml

4. 20 µl of the herbal extracts were mixed with 180 µl of DPPH solution and incubated for 30 min in the dark at RT.

5. Negative control was prepared by 20 µl of absolute methanol and 180 µl of DPPH solution whereas blank sample was prepared by 180 µl of absolute methanol and 20 µl of the herbal extracts.

6. Measure absorbance at 517 nm. The procedure step is shown in Table 7.

7. Prepare a standard curve of absorbance versus concentration of ascorbic acid and determine antioxidant activity from the curve.

8. The results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of dry plant material. The % scavenging activity was calculated using the following formula:

$$\% \text{ DPPH Scavenged} = \frac{[\text{Abs(control)} - (\text{Abs(sample)} - \text{Abs(blank of sample)})]}{\text{Abs(control)}} \times 100$$

Table 7. The procedure step of DPPH assay

Reagents	Control (µl)	Standard (µl)	Sample blank (µl)	Sample (µl)
Ascorbic acid	-	20	-	-
Herbal extracts	-	-	20	20
Absolute methanol	20	-	180	-
DPPH solution	180	180	-	180
Mix and incubate at RT for 30 min in the dark and then measure at 517 nm				

2.4.3.2 The ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) radical scavenging assay

The ABTS (2, 2´-azinobis(3-ethylbenzthiazoline]-6-sulfonic acid)) assay was used to determine the antioxidant activity by estimating peroxide formation of both hydrophilic and hydrophobic compounds [514]. The ABTS performed following the procedure described by Delgado-Andrade et al [515]. This assay is based on the ability of the antioxidants to scavenge the loge-life radical cation ABTS●+.The formation of the colored ABTS radical is suppressed by antioxidants by electron donation radical scavenging and inhibit. The quantity of antioxidant in the test sample is inversely proportional to the ABTS radical development.

Procedure
1. The ABTS●+ was freshly prepared by mixing 8 ml of 7 mM ABTS with 12 ml of 2.45 mM potassium persulfate and stored in the dark at RT for 16-18 h.
2. The ABTS●+ solution was diluted with absolute ethanol to an absorbance at 734 nm of 0.70 ± 0.02.
3. Prepare ascorbic acid as a reference standard between 0, 6.25, 12.5, 25, 50 and 100 µg/ml as shown in Table 8 and dilute the herbal extracts from 100 mg/ml stock solutions to 0.001, 0.01, 0.1 and 1 mg/ml as shown in Table 9.

Table 8. The procedure step to dilute ascorbic acid stock solution

Tube	Ascorbic acid	Absolute methanol (µl)	Final Concentration
1	Ascorbic acid stock solution	-	1000 µg/ml
2	100 µl from Tube No. 1	900	100 µg/ml
3	500 µl from Tube No. 2	500	50 µg/ml
4	500 µl from Tube No. 3	500	25 µg/ml
5	500 µl from Tube No. 4	500	12.5 µg/ml

Tube	Ascorbic acid	Absolute methanol (µl)	Final Concentration
6	500 µl from Tube No. 5	500	6.25 µg/ml

Table 9. The procedure step to dilute herbal extracts stock solution

Tube	Herbal extracts	Absolute methanol (µl)	Final Concentration
1	Herbal extracts stock solution	-	100 mg/ml
2	5 µl from Tube No. 1	495	1 mg/ml
3	10 µl from Tube No. 2	90	0.1 mg/ml
4	10 µl from Tube No. 3	90	0.01 mg/ml
5	10 µl from Tube No. 4	90	0.001 µg/ml

4. 20 µl of the herbal extracts were mixed with 180 µl of ABTS●+ solution and incubated for 45 min in the dark at RT.
5. Negative control was prepared by 20 µl of absolute ethanol and 180 µl of ABTS●+ solution whereas blank sample was prepared by 180 µl of absolute ethanol and 20 µl of the herbal extracts.
6. Measure absorbance at 734 nm. The procedure step is shown in Table 10.
7. Prepare a standard curve of absorbance versus concentration of ascorbic acid and determine antioxidant activity from the curve.
8. The results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of dry plant material. The % scavenging activity was calculated using the following formula:

$$\% \text{ ABTS Scavenged} = \frac{[\text{Abs(control)} - (\text{Abs(sample)} - \text{Abs(blank of sample))}]}{\text{Abs(control)}} \times 100$$

Table 10. The procedure step of ABTS assay

Reagents	Control (µl)	Standard (µl)	Sample blank (µl)	Sample (µl)
Ascorbic acid	-	20	-	-
Herbal extracts	-	-	20	20
Absolute methanol	20	-	180	-
ABTS●+ solution	180	180	-	180
Mix and incubate at RT for 45 min in the dark and then measure at 734 nm				

2.4.4 The effect of herbal extract and AGEs on cell culture system

2.4.4.1 Routine maintenance of SH-SY5Y cell line protocol

SH-SY5Y cells were maintained in MEM/F12 culture medium, supplemented with 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 95% humidified air and 5% CO_2 at 37°C.

2.4.4.1.1 Subculture of SH-SY5Y cells

These cells are grown as a mixture of floating and adherent cells. The cells are grown as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites) and aggregate, form clumps and float. The cell cultures include both adherent and floating cells, both types of which are viable. Few studies address the biological significance of the adherent versus floating phenotypes, but most reported studies utilize adherent populations and discard the floating cells during media changes [516].

These adherent cells were grown to near confluence and passaged as follows:

1. Change the medium twice a week. Healthy passage of SH-SY5Y cells were indicated by determined cell morphology and their doubling time. SH-SY5Y cells were subcultured every 5-6 days using standard trypsinization procedures to maintain the cell line by removed the medium with the floating cells and recovered the cells by centrifugation. The cells were rinsed the adherent cells with fresh 0.25% trypsin and incubated the culture at 37°C in CO_2 incubator until the cells detached. Then added fresh medium, aspirate, combined with the floating cells recovered above and dispensed into new flasks.

2. All the experiments were carried out while cells were grown to 70–80% confluence. Seeding SH-SY5Y cells on to fresh feeder onto a fresh culture multiwell plate in the medium supplemented with 5% FBS at a density shown in Table 11. The culture plate was then incubated in the CO_2 incubator, maintained at a temperature of 37°C in a humidified atmosphere at 5% CO_2 overnight before initiation of experiments to make the cells grow to 70–80% confluence.

Table 11. The density of SH-SY5Y cells in culture plates

Culture plate	Density (cells/cm^2)
6 well culture plate	1,100,000
24 well culture plate	50,000
96 well culture plate	10,000

2.4.4.2 Exposure SH-SY5Y to herbal extracts/AGEs solution

-Herbal extracts: the plant extracts were dissolved in DMSO in a concentration of 100 mg/ml as stocks. The stocks were diluted with cell culture medium with 5% FBS to get range the concentration between 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 500 and 1000 µg/ml. Final concentration of DMSO not above 0.1%.

-AGEs solution: the concentration of total protein in AGEs solution and BSA control were calculated from Bradford method. Both solutions were diluted with cell culture medium with 5% FBS to get range the concentration between 0.5, 1, 2, 3, 4, 5, 6 and 8 mg/ml.

-Both herbal extracts/AGEs or BSA solutions were diluted into fresh complete growth medium with 5% FBS and pre-treated the cells for 24 and 48 h. At the end of the treatments the cells were used for the experiments describes below. All treatments were carried out at least 3 independent replications.

2.4.4.3 The cytotoxic effect of herbal extracts/AGEs solution on cell culture system

2.4.4.3.1 Cytotoxic effect of herbal extracts/AGEs solution on cellular viability using MTS assay

The MTS is a colorimetric method that used for sensitive quantification of viable cells in proliferation. The method is based on the reduction of a 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. The formazan dye produced by viable cells can be quantified by measuring the absorbance at 490-500 nm [517]. The CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega, USA) contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. Because the MTS formazan product is soluble in culture medium, this assay requires fewer steps than procedures that use tetrazolium compounds such as MTT. The formazan product of MTT reduction is a crystalline precipitate that requires an additional step in the procedure to dissolve the crystals before recording absorbance readings at 570nm. The quantity of MTS formazan product as measured by absorbance at 490nm is directly proportional to the number of living cells in culture.

Procedure
1. Culture the cells in 96 well plates and after treatment with herbal extracts or AGEs solution, 100 µl of cultural medium in each well was subjected to a fresh microtiter plate.
2. Pipet 20 µl of MTS reagent into each well of the 96 microtiter plate.
3. Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO_2 atmosphere.
4. Record the absorbance at 490 nm using a spectrophotometer.
5. Results were expressed as the percentage of cell viability compared with the corresponding control value. The % cell viability was calculated according to the following formula:

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

2.4.4.3.2 Cytotoxic effect of AGEs solution on cellular viability using trypan blue dye exclusion assay

The dye exclusion test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. Trypan blue is a ~960 Daltons molecule that is cell membrane impermeable and therefore only enters cells with compromised membranes. Upon entry into the cell, trypan blue binds to intracellular proteins thereby rendering the cells a bluish color. The trypan blue exclusion assay allows for a direct identification and enumeration of live (unstained) and dead (blue) cells in a given population [518]. The trypan blue dye exclusion assay is recommended to use for cultured cell lines with viabilities greater than 70 %.
Procedure
1. Culture the cells in 24 well plates and after treatment with herbal extracts or AGEs solution, the cells were washed with PBS.
2. Detach the cells from culture plate using standard trypsinization procedures.
3. Make a 1:1 mixture of the cell suspension and the 0.4% trypan blue solution and gently mix and let stand for 3 min at RT.
4. Load 10 µl of the cell suspension to the edge of a hemocytometer counting chamber between the cover slip and the V-shaped groove in the chamber, allow the cell suspension to be drawn into the chamber by capillary action and let sit for 1–2 min and then count under a microscope.
5. Results were expressed as the percentage of cell survival compared with the corresponding control value. The % cell survival was calculated according to the following formula:

$$\% \text{ Cell survival} = \frac{\text{Live unstained cells}}{(\text{Live unstained cells} + \text{Dead stained cells})} \times 100$$

2.4.4.3.3 Cytotoxic effect of herbal extracts/AGEs solution on cellular toxicity using LDH assay

Cytotoxicity was evaluated by the quantification of plasma membrane damage using LDH assay. Lactate dehydrogenase (LDH) is a cytosolic enzyme that is an indicator of cellular toxicity. The LDH-cytotoxicity assay provides a fast and simple method for quantitating cytotoxicity based on the measurement of activity of LDH released from damaged cells. Unlike many other cytoplasmic enzymes which exist in many cells either in low amount or unstable such as alkaline and acid phosphatase, LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity can be determined by a coupled enzymatic reaction [519]. The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA) is a colorimetric cytotoxicity assay kit. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay, which results from LDH oxidizes lactate to pyruvate which then convert a tetrazolium salt (iodonitrotetrazolium violet; INT) into a red formazan product. The amount of color formed by formazan produced in culture supernatant is proportional to the number of lysed cells.

Procedure
1. Culture the cells in 96 well plates.
2. Treat the cells with the herbal extracts or AGEs solution, as well as a set of control including untreated cells to serve as a vehicle control, a no-cell control to serve as the negative control to determine background and a maximum LDH release control to serve as positive control by lyse the cells with a lysis solution to determine the maximum LDH release.
3. Incubate the cells at 37°C for the desired test exposure period.
4. Transfer 50 μl of cultural medium in each well as well as a set of control and lysed cells to a fresh microtiter plate.
5. Add 50 μl of CytoTox 96® reagent to the cell culture medium present in each well.
6. Cover the 96 well plate with foil to protect it from light and incubate for 30 min at RT
7. Add 25 μl of Stop Solution to each well of the 96-well plate and carefully pop any large bubbles using a syringe needle.
8. Record the absorbance at 490 nm within 1 h using a spectrophotometer.
9. Results were expressed as the percentage of cell toxicity compared with the corresponding control value. The % cytotoxicity was calculated according to the following formula:

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

2.4.4.4 The effects of AGEs/herbal extracts on oxidative stress on cell culture system

2.4.4.4.1 Effect of AGEs on cellular oxidative stress using DCFH-DA assay

Accumulation of ROS coupled with an increase in oxidative stress has been implicated in the pathogenesis of several disease states. Free radicals and other reactive species are constantly generated in vivo and cause oxidative damage to biomolecules, a process held in check by the existence of multiple antioxidant and repair systems as well as the replacement of damaged nucleic acids, proteins and lipids [7]. Measuring the effect of antioxidant therapies and ROS activity intracellularly is crucial to suppressing or treating oxidative stress inducers. DCFH-DA assay is a cell-based assay providing a convenient and sensitive means to monitor oxidative activity in living cells. The assay employs the cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA). DCFH-DA has been widely used as a marker for oxidative stress, and has been suggested to be a good indicator of the overall oxidative status of the cell [520]. This hydrophobic non-fluorescent molecule, DCFH-DA, penetrates rapidly into the cell and is hydrolysed by intracellular esterases to give the DCFH molecule which can be oxidised to its fluorescent 2-electron product 2′,7′dichlorofluorescein (DCF) by ROS. The fluorescence intensity is proportional to the ROS levels within the cell cytosol.

Procedure
1. Culture cells in either a black 96 well cell culture plate and after treatment with herbal extracts or AGEs solution, the cultural medium were removed from all wells and discarded.
2. Wash cells gently with HBSS 2-3 times and remove the last wash and discard.
3. Dilute the DCFH-DA stock solution to 100 μM in the cell culture medium without FBS.
4. Add 100 μL of 100 μM DCFH-DA to cells and cover the black 96 well plate with foil to protect it from light and incubate for 30-60 min at 37°C in CO_2 incubator.
5. Remove solution. Repeat step three using multiple washes with HBSS to remove excess dye. Remove the last wash and discard.
6. Add pre-warm PBS and monitor the fluorescence intensity by a fluorescence plate reader at 485 nm excitation and 530 nm emission.

7. The results were normalized using protein concentration. Results were expressed as the percentage of fluorescence of cells compared with the corresponding control groups.

2.4.4.4.2 Effect of AGEs on byproducts of lipid peroxidation during oxidative stress using MDA assay

Malondialdehyde (MDA) is one of the most prevalent byproducts of lipid peroxidation during oxidative stress. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as MDA which can be measured as thiobarbituric acid reactive substances (TBARS) [521]. The Parameter TBARS assay kit (R&D Systems, USA) offers a simple, reproducible, and consistent system for the detection of lipid peroxidation generated during oxidative stress. MDA forms a 1:2 adduct with thiobarbituric acid (TBA).Typically, free MDA is quite low, requiring release of MDA by acid treatment of proteins and breakdown of peroxides by heat and acid to product that absorbs light at 530-540 nm. Measuring TBARS levels corresponds to the level of oxidative stress in a sample.

Procedure
1. Culture cells in a 6 well cell culture plate and after treatment with herbal extracts or AGEs solution, the cells were washed with PBS.
2. Detach the cells from culture plate using standard trypsinization procedures.
3. Wash the cells two times in cold PBS.
4. Resuspend cells at 1×10^6 cells/ml in diluted cell lysis buffer.
5. Incubate with gentle agitation for 30 min at 4 °C in the refrigerator and freeze/thaw cells once at -80 °C.
6. Collect the cell lysates for acid treatment to remove interfering proteins and other substances and catalyze the TBARS reaction, 300 μl the cell lysates were added to 300 μl TBARS acid reagent and mix well.
7. Incubate for 15 minutes at RT, then centrifuge at $\geq$ 12,000 x g for 4 min.
8. Carefully remove and retain the supernatant.
9. Prepare eight TBARS standard containing 0, 0.26, 0.52, 1.04, 2.09, 4.18, 8.35 and 16.7 μM, prepare dilutions of TBARS standard stock as shown in Table 12.

Table 12. The procedure step to dilute TBARS standard stock solution

Tube No.	TBARS	Deionized water (μl)	Final concentration (μM)
1	167 μM TBARS stock solution	-	167
2	100 μl from Tube No. 1	900	16.7
3	500 μl from Tube No. 2	500	8.35
4	500 μl from Tube No. 3	500	4.18
5	500 μl from Tube No. 4	500	2.09
6	500 μl from Tube No. 5	500	1.04
7	500 μl from Tube No. 6	500	0.52
8	500 μl from Tube No. 7	500	0.26
9	-	500	0

10. Add 150 μl each of standard solution or sample to a microplate.
11. Add 75 μl of TBA reagent to each well and cover with the adhesive strip provided and incubate the microplate for 2-3 h at 45-50°C.
12. Determine the optical density using a spectrophotometer at 532 nm.
13. Create a standard curve of absorbance versus concentrations and determine amounts from the curve. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

2.4.4.5 The effects of AGEs/herbal extracts on genes expression that involve in oxidative stress, RAGE/NF-kB signaling pathway, secretases in APP processing and proinflammatory cytokines using qRT-PCR method

Effects of AGEs/herbal extracts on cellular oxidative stress, RAGE/NF-kB, secretases that involve in Aβ production and proinflammatory cytokines were evaluated by a quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis to determine gene expression levels between untreated SH-SY5Y group and treated SH-SY5Y group. The process started with reverse transcribing RNA of interest into its DNA complement through the use of reverse transcriptase. Subsequently, the newly synthesized specific cDNA was amplified using real-time polymerase chain reaction or quantitative PCR (qPCR) with design primers.

Procedure

A. Isolation of RNA

1. Culture cells in a 6 well cell culture plate and after treatment with herbal extracts or AGEs solution, the cultural medium were removed from all wells and discarded.

2. Wash cells gently with twice with PBS and remove the last wash and discard.

3. Lyse the cells with 1 ml TRI reagent by passing the lysate through a pipette several times.

4. Add 200 µl chloroform, then and vigorous mix and centrifuge at 12,000 g for 10 min at 4°C.

5. Transfer the colorless upper aqueous phase to a fresh tube (RNA is on the upper layer, lower layer are protein, DNA and debris). Precipitate the RNA from the aqueous phase by mixing with 500 µl isopropanol.

6. Incubate samples at RT for 10 minutes and centrifuge at 12,000 g for 5 min at 4°C.

7. Remove the supernatant, the RNA precipitation, a gel-like pellet on the side and bottom of the tube, was purified 2-3 times with 1 ml of 75% ethanol.

8. Mix the sample by vortexing and centrifuge at no more than 7,500g for 5 minutes at 4°C.

9. Dry the RNA pellet and the dried pellets were re-dissolved in RNase-free water passing the solution a few times through a pipette tip, and incubating for 10 min at 65°C.

10. The purity of total RNA was determined as a ratio of absorbance at 260 and 280 nm and the yield was calculated using absorbance at 260 nm.

B. Elimination of DNA contaminate

1. Each sample was incubated with DNaseI in a mixture of DNaseI incubation buffer as shown in Table 13 for 15-30 min at 37°C.

2. Terminate DNaseI reaction by adding 25mM EDTA and incubate for 10 min at 65°C.

Table 13. DNase step

Reagents	Concentration	Volume per 1 reaction (µl)
RNA	1 µg/ml	to 10 µl
DNaseI	0.1 unit/ml	1
DNaseI buffer	10X	1
DEPC treated water	-	to 10 µl
Incubate at 37°C for 15 min		
EDTA	25 mM	1
Incubate for 10 min at 65°C		

C. Reverse transcription reaction

1. Set up the following components in as shown in Table 14, add the enzyme last.

Table 14. Reverse transcription components

Reagents	Concentration	Volume per 1 reaction (µl)
RT buffer	5X	4
$MgCl_2$	25 mM	1.2
dNTPs	10 mM	1
Oligo dT	100 µM	1
Reverse transcriptase (RT)	200 unit/µl	1
RiboLock RNase Inhibitor	40 unit/l	0.5
DEPC treated water	-	1.3
RNA sample	1 µg/ml	10

2. Mix gently, spin briefly.
3. Incubate in the thermal cycler at:
   25°C for 5 min
   42°C for 65 min
   70°C for 10 min
4. Store reaction at −20°C until proceed to the PCR.

D. Real-time RT-PCR using SYBR Green

The procedure began with reverse transcription of total RNA. The cDNA was then used as template for real-time PCR with gene specific primers. The specific primers for each gene expression are shown in the Table 15.

Table 15. Specific nucleotide primer used in qPCR

Gene		Sequence	Annealing temp (°C)	Product size (bp)
HMOX-1	Forward primer	5' GACCCATGACACCAAGGACC 3'	57	399
	Reverse primer	5' GGATGTGCTTTTCGTTGGGG 3'		
RAGE	Forward primer	5' GTGGGGACATGTGTGTCAGAGGGAA 3'	68	383
	Reverse primer	5' TGAGGAGAGGGCTGGGCAGGGACT 3'		
RelA	Forward primer	5' TGGCCCCTATGTGGAGATCA 3'	56	371
	Reverse primer	5' AGGGGTTGTTGTTGGTCTGG 3'		
NF-κB1	Forward primer	5' AGCCCCCAATGCATCCAACTT 3'	60	402
	Reverse primer	5' CAACCGCCGAAACTATCCGAAAAA 3'		
NF-κB2	Forward primer	5' CAGTGAGAAGGGCCGAAAGAC 3'	60	421

Gene		Sequence	Annealing temp (°C)	Product size (bp)
NF-κB2	Reverse primer	5' CAGGGGCAGGGAGAAGGAG 3'		
BACE1	Forward primer	5' GAGTCACAGCTTTCCCAGGT 3'	55.5	345
	Reverse primer	5' TAATCCCCCTCCCCCAGAAG 3'		
ADAM10	Forward primer	5' ACCACAGACTTCTCCGGAATC 3'	55.5	238
	Reverse primer	5' CTGAAGGTGCTCCAACCCAA 3'		
PS1	Forward primer	5' ATGATGGCGGGTTCAGTGAG 3'	56	207
	Reverse primer	5' TGGCTGTTGCTGAGGCTTTA 3'		
TNF-α	Forward primer	5' TCTCGAACCCCGAGTGACAA 3'	55	181
	Reverse primer	5' TGAAGAGGACCTGGGAGTAG 3'		
IL-1	Forward primer	5' ACCAAACCTCTTCGAGGCAC 3'	56	300
	Reverse primer	5' CATGGCCACAACAACTGACG 3'		
IL-6	Forward primer	5' GAAGAGAGCCCTCAGGCTGGACTG 3'	64	627
	Reverse primer	5' TGAACTCCTTCTCCACAAGCGC 3'		
GAPDH	Forward primer	5' GAAAGCCTGCCGGTGACTAA 3'	60	370
	Reverse primer	5' TCGCCCCACTTGATTTTGGA 3'		

1. Set up the experiment and the following PCR program on Exicycler™ 96 Real-Time Quantitative Thermal Block.
2. Amplify cDNA by an AccuPower® 2X GreenStar qPCR master mix (Bioneer, South Korea), a master mix containing SYBR Green I dye, Hotstart Top DNA polymerase.
3. Set up the following components in as shown in Table 16 on to a PCR tube.

Table 16. Real-time PCR components

Reagents	Concentration	Volume per 1 reaction (µl)
Greenstar master mix	2X	12.5
PCR F-Primer	10 pM	0.5
PCR R-Primer	10 pM	0.5
cDNA template	1 µg/ml	2
DEPC treated water	-	9.5

4. Seal an optical adhesive film for real-time PCR on tube.

5. Completely mix by vigorous vortexing for resuspension of the master mix.

6. Load them and start real-time PCR setting.

7. After reaction was completed, performed data analysis and removed the tubes from the machine. The PCR specificity was examined by 2% agarose gel using 5-10 µl from each reaction.

8. The gene expression of GAPDH was used for normalization.

9. Values were calculated in a fold change in mRNA levels using the ΔΔct method ($2^{-\Delta\Delta ct}$) of interested genes over those of GAPDH compared with untreated group.

2.5   Statistical analysis

All experiments were performed independently at least three times and in triplicates or quadruplicates as indicated. Data are expressed as the mean $\pm$ SE of the mean (SEM). Statistically significant differences between two groups was determined using Student's *t*-test and statistically significant differences between more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by the Scheffe's post hoc test using SPSS version 20.0 for Windows. The p-value was two-tailed, and $P < 0.05$ was considered to be statistically significant when comparing the data sets.

CHAPTER III

RESULTS

3.1 Examination of herbal extracts

3.1.1 Extraction yield

The extraction yield is a measure of the solvent efficiency to extract specific components from the plant material. In this case, the Soxhlet extraction was carried out to extract the desired compounds from *A. lividus* and *A. tricolor* under different organic solvents. We took advantage of this successive extraction method to use small quantity of plant materials and organic solvents to reduce organic wastes. The percentage yield of *A. lividus* and *A. tricolor* extracts that were successively extracted with petroleum ether, dichloromethane and methanol was calculated according to the method of Zhang S., et al. [522] using the following formula: (weight of the dried extract x 100) / (weight of the original sample). The percentage yields of different herbal extracts were range from 0.835% to 5.629% (Table 17). The highest percentage yields were obtained from methanol fractions.

Table 17. Extraction yield (%) of *A. lividus* and *A. tricolor* extracted successively in petroleum ether, dichloromethane and methanol.

Types of plant	% Yield of herbal extracts (w/w)		
	Petroleum ether	Dichloromethane	Methanol
A. lividus	0.995	2.123	5.544
A. tricolor	0.835	2.177	5.629

3.1.2 Assessment of antioxidant activities of herbal extracts in a cell-free system

Although the antioxidant activity was usually reported in all plants, it was still important to determine the antioxidant properties of our herbal extracts in order to indicate the potential of the herbal extracts as the medicinal food with potential application to diminish or prevent the oxidative stress associated with the risk of brain aging and neurodegenerative diseases. Antioxidants have been found to exhibit their activities by various mechanisms and at different stages if oxidations such as antioxidant activities. Two different experimental approaches were employed to determine of antioxidant activities; DPPH and ABTS assay.

3.1.2.1 Antioxidant activity of herbal extracts using DPPH assay

DPPH assay is based on hydrogen donor property of antioxidants and is widely used in natural antioxidant because of its simplicity and sensitivity. Result of antioxidants activities of *A. lividus* and *A. tricolor* on the DPPH assay was listed in

Table 18. The DPPH scavenging activities of the extracts showed methanol extract of *A. lividus* had the greatest amount of radical-scavenging activity (28.87 $\pm$ 2.38 % SC, 15.18 $\pm$ 1.59 mg VCEAC/g extract), followed by methanol extract of *A. tricolor* (26.65 $\pm$ 3.09 % SC, 13.86 $\pm$ 0.28 mg VCEAC/g extract). The lowest antioxidant activity was found in petroleum ether extract of *A. tricolor* (4.21 $\pm$ 1.51 % SC, 1.73 $\pm$ 0.86 mg VCEAC/g extract). In general, the extracts obtained from solvents with highest polarities (methanol) showed the strongest antioxidant activities.

Table 18. Antioxidant activities of *A. lividus* and *A. tricolor* extracts derived from different solvents by DPPH assay.

Types of plant	% DPPH Scavenged	Antioxidant activity (mg VCEAC/g dry plant material)
A. lividus (petroleum ether)	7.74 $\pm$ 1.88	3.43 $\pm$ 1.13
A. lividus (dichloromethane)	14.35 $\pm$ 3.35	6.81 $\pm$ 1.93
A. lividus (methanol)	28.87 $\pm$ 2.38	15.18 $\pm$ 1.59
A. tricolor (petroleum ether)	4.21 $\pm$ 1.51	1.73 $\pm$ 0.86
A. tricolor (dichloromethane)	14.70 $\pm$ 1.39	7.66 $\pm$ 0.68
A. tricolor (methanol)	26.65 $\pm$ 3.09	13.86 $\pm$ 0.28

3.1.2.2 Antioxidant activity of herbal extracts using ABTS assay

ABTS assay is based on the reduction of the ABTS•+ radical cation by single electron transfer property of antioxidants and has been widely used to evaluate antioxidant activities due to its applicability in both aqueous and liquid phase [514]. Result of antioxidants activities of *A. lividus* and *A. tricolor* on the ABTS assay was listed in Table 19. The methanol extract of the *A. tricolor* contained the most radical-scavenging activity in the ABTS assay (52.89 $\pm$ 2.71 % SC, 25.03 $\pm$ 1.58 mg VCEAC/g extract), followed by dichloromethane and methanol fraction of the *A. lividus* (42.89 $\pm$ 2.03 % SC, 20.90 $\pm$ 0.70 and 42.19 $\pm$ 1.46 % SC, 20.89 $\pm$ 0.69 mg VCEAC/g extract, respectively). The lowest antioxidant activity was found in the *A. tricolor* from petroleum ether extract (5.39 $\pm$ 2.29 % SC, 2.07 $\pm$ 0.95 mg VCEAC/g extract). Of note, the extracts obtained from solvents with higher polarities (dichloromethane and methanol) exhibited the greater antioxidant activities than those of solvents with lower polarities (petroleum ether).

3.1.3 Assessment the toxicity of herbal extracts using SH-SY5Y cell culture as the model

3.1.3.1 Determined the cell damage effect of herbal extracts on SH-SY5Y cells using MTS assay

In order to examine the effects of herbal extracts on viability of SH-SY5Y cells, a MTS assay was performed. The MTS assay is carried out to determine the optimum concentration and incubation time of each herbal extracts to be used in the subsequent procedures. SH-SY5Y cells were incubated with various concentrations of *A. lividus* and *A. tricolor* extracts for 24 and 48 h. Figure 19 and 20 presented % viability of cells following treatment with different herbal extracts ranging from 1.5625 to 1000 µg/ml. and 0.1% DMSO that corresponding the final content in the incubation medium with the highest concentration of extract, 1000 µg/ml. The result demonstrated that both *A. lividus* and *A. tricolor* had cytotoxic effect in SH-SY5Y cells in dose and time dependent manner. Loss of cell viability upon herbal treatment became much more pronounced with increasing concentration of herbal extracts and time of incubation. The result showed at 24 herbal extract treatments, most of the extracts were revealed not toxic to the cells, with the exception as detailed below. Petroleum ether, dichloromethane, and methanol fraction of *A. lividus* and *A. tricolor* at the 500 and 1000 µg/ml and methanol fraction of *A. lividus* at 100 µg/ml significantly reduced the numbers of living cells. At 48 h the result indicated all of the extracts showed less cell viability than 24 h herbal treatment. There was a significant reduction in cell viability after treatment with most of the extracts and appeared in a high concentration of 100 to 1000 µg/ml.

A

*A. lividus* **petroleum ether fraction (µg/ml)**
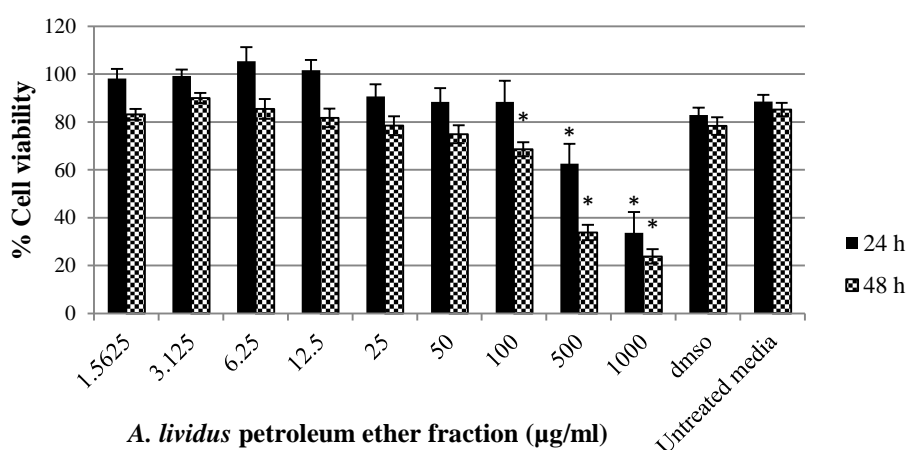
B
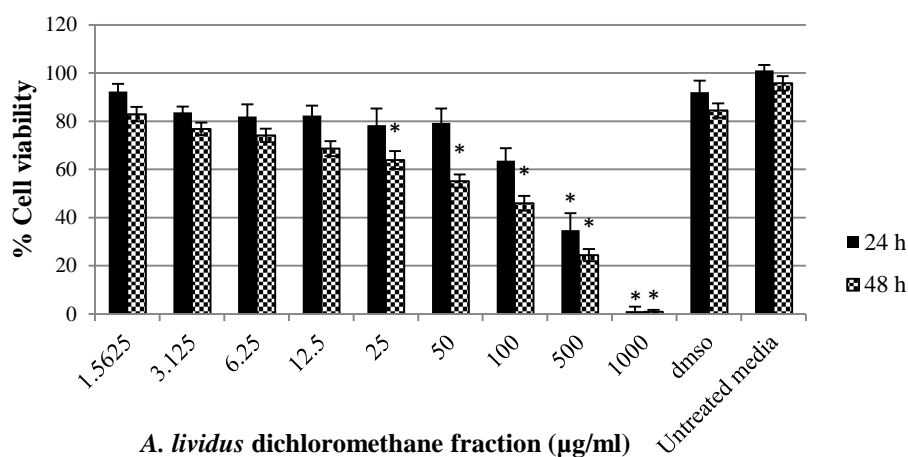
C
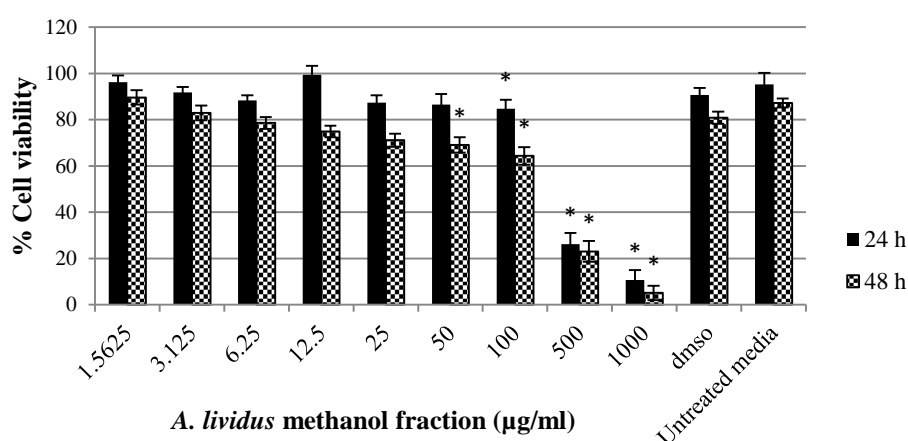
Figure 19. Effect of *A. lividus* extracts on the viability of SH-SY5Y cells using MTS assay. The cell viability was measured at 24 and 48 h. The cells were treated with various concentrations of the extract (A) petroleum ether; (B) dichloromethane and (C) methanol. The results were reported as means with their standard error of the mean (SEM), depicted by vertical bars. *P < 0.05 for significant change as compared to control (no treatment).
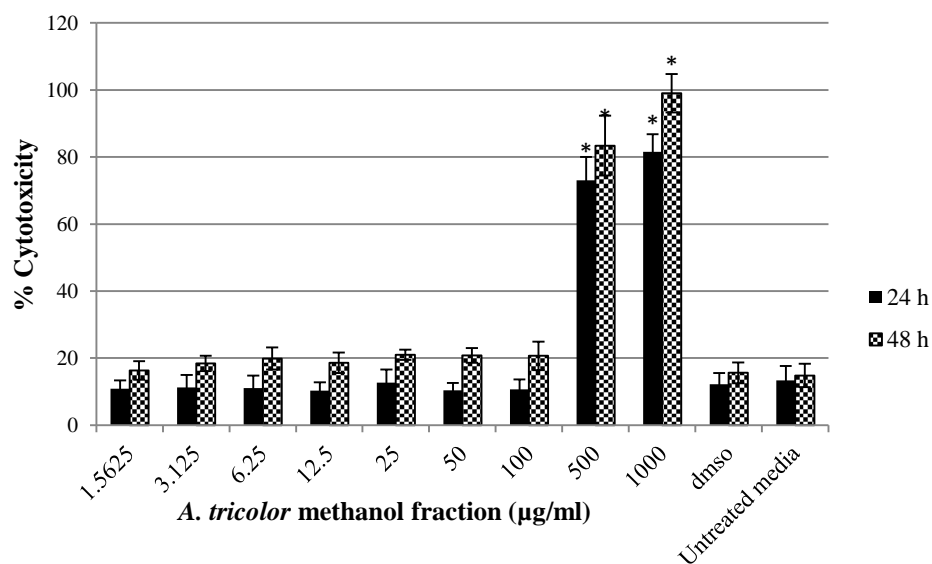
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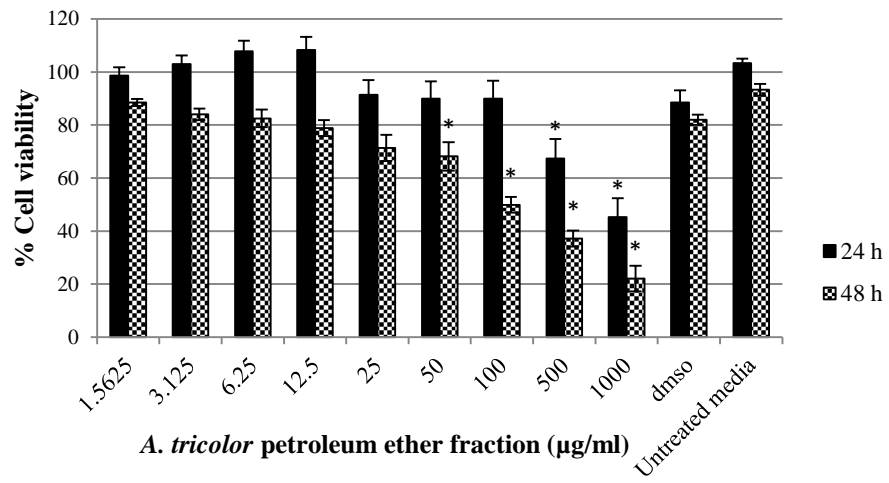
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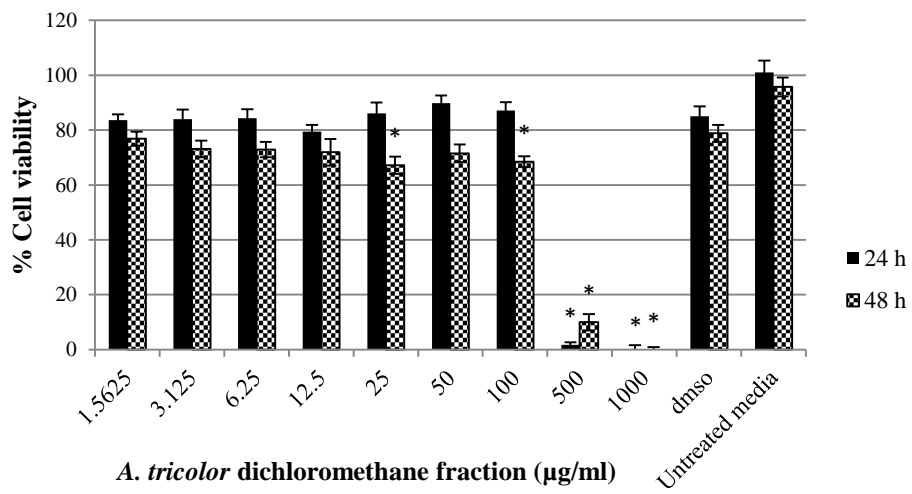
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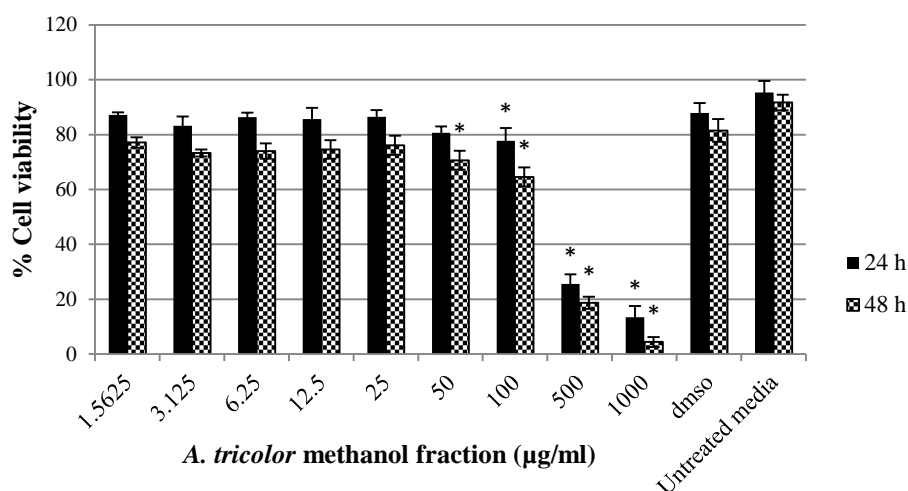
Figure 20. Effect of *A. tricolor* extracts on the viability of SH-SY5Y cells using MTS assay. The cell viability was measured at 24 and 48 h. The cells were treated with various concentrations of the extract (A) petroleum ether; (B) dichloromethane and (C) methanol. The results were reported as means with their standard error of the mean (SEM), depicted by vertical bars. *P < 0.05 for significant change as compared to control (no treatment).

3.1.3.2 Determined the cell toxicity effect of herbal extracts on SH-SY5Y cells using LDH assay

These cytotoxic effects of herbal extracts from MTS results were confirmed by measurement of cellular toxicity by the LDH assay. A dose and time course for measurement of LDH that released upon cell damaged was shown in Figure 21 and 22. The results demonstrated that *A. lividus* and *A. tricolor* increased cell toxicity in a concentration and time dependent fashion. At 24 h of herbal treatment, most of the extracts were revealed not toxic to the cells, with the exception as detailed below. All solvents of *A. lividus* and *A. tricolor* at 500 to 1000 µg/ml and 100 µg/ml dichloromethane extract of *A. lividus* revealed significantly cytotoxicity. These LDH data showed that most of the extracts were relative more toxic when incubated with SH-SY5Y cells for 48 h.

As shown in Table 20, there were good correlation between the values of both LDH and MTS assay ($R^2 > 0.9$) in 24 and 48 herbal extracts treatment. Therefore both assays could be used to screen the cell toxicity effect of herbal extracts on SH-SY5Y. Regarding the SH-SY5Y cells, the viability test using MTS assay was over 80% as shown in Figure 19 and 20 and cytotoxicity test using LDH assay was under 20% as shown in Figure 21 and 22, for the further experiment we chose to investigate more depth the protective effect of petroleum ether, dichloromethane, and methanol extracts

of *A. lividus* and *A. tricolor* at a concentration range of 1.5625 to 100 μg/ml, with the exception of the dichloromethane extract of the *A. lividus* at a concentration range of 1.5625 to 50 μg/ml for 24 h of treatment.

A
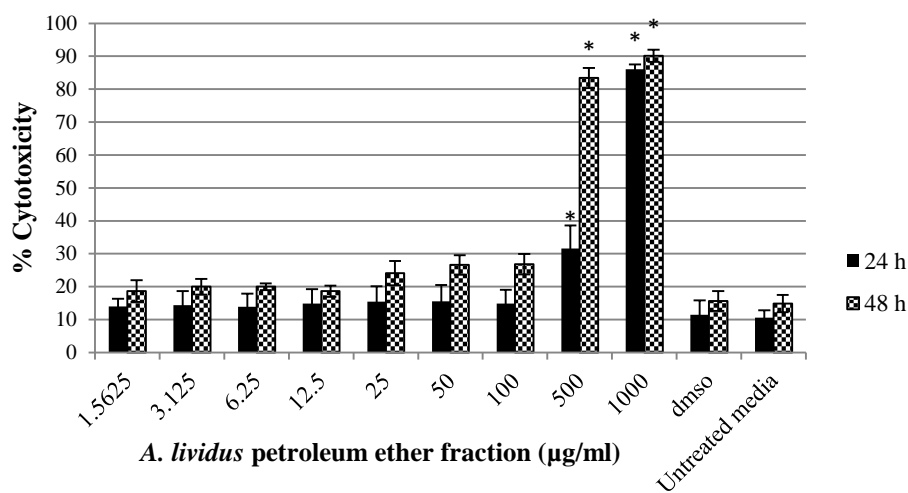
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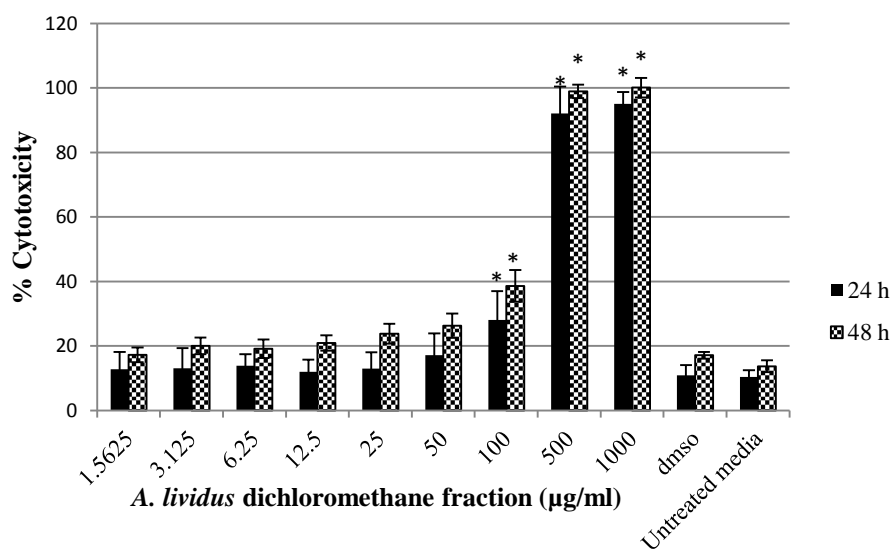
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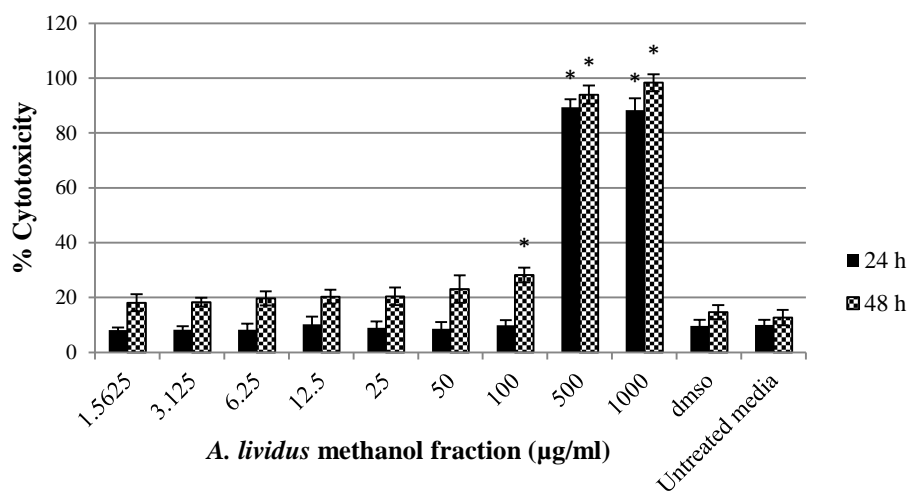
Figure 21. Effect of *A. lividus* extracts on the cell toxicity of SH-SY5Y cells using LDH assay. The cytotoxicity was measured at 24 and 48 h. The cells were treated with various concentrations of the extract (A) petroleum ether; (B) dichloromethane and (C) methanol. The results were reported as means with their standard error of the mean (SEM), depicted by vertical bars. *P < 0.05 for significant change as compared to control (no treatment).

A
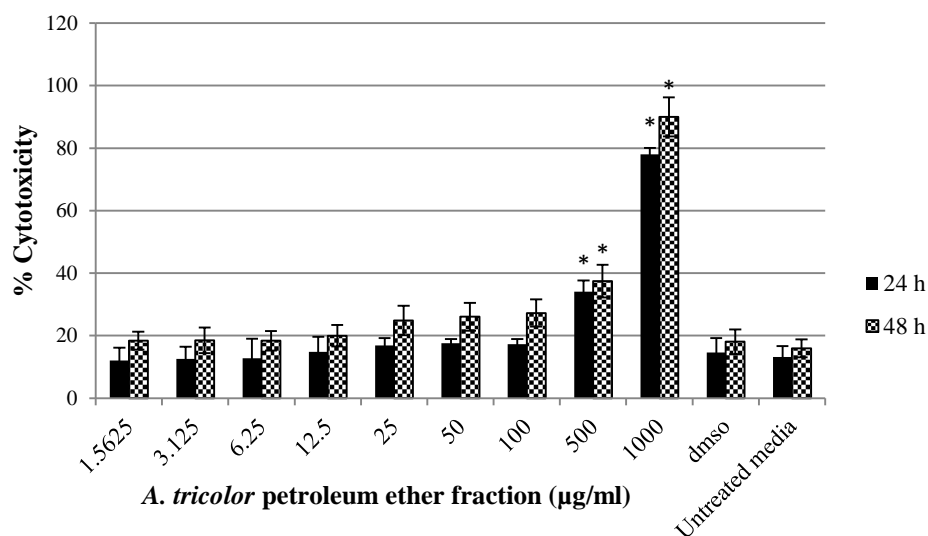
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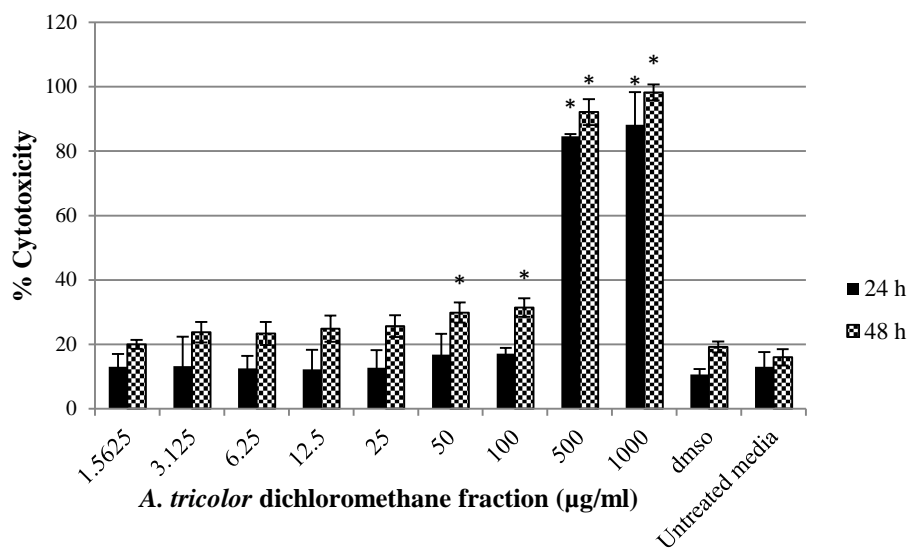
C

Figure 22. Effect of *A. tricolor* extracts on the cell toxicity of SH-SY5Y cells using LDH assay. The cytotoxicity was measured at 24 and 48 h. The cells were treated with various concentrations of the extract (A) petroleum ether; (B) dichloromethane and (C) methanol. The results were reported as means with their standard error of the mean (SEM), depicted by vertical bars. *P < 0.05 for significant change as compared to control (no treatment).

Table 20. Statistical analysis using Pearson's correlation coefficient (R^2) to determine correlation between LDH assay and MTS assay for determination the cell toxicity effect of herbal extracts on SH-SY5Y.

A) *A. lividus* petroleum ether fraction

24 h	LDH	MTS	48 h	LDH	MTS
LDH Pearson's correlation coefficient (R^2)	1	0.904**	LDH Pearson's correlation coefficient (R^2)	1	0.973**
MTS Pearson's correlation coefficient (R^2)	0.904**	1	MTS Pearson's correlation coefficient (R^2)	0.973**	1

** Correlation was significant at P value < 0.01 (2-tailed)

B) *A. lividus* dichloromethane fraction

24 h	LDH	MTS	48 h	LDH	MTS
LDH Pearson's correlation coefficient (R^2)	1	0.946**	LDH Pearson's correlation coefficient (R^2)	1	0.963**
MTS Pearson's correlation coefficient (R^2)	0.946**	1	MTS Pearson's correlation coefficient (R^2)	0.963**	1

** Correlation was significant at P value < 0.01 (2-tailed)

C) *A. lividus* methanol fraction

24 h	LDH	MTS	48 h	LDH	MTS
LDH Pearson's correlation coefficient (R^2)	1	0.980**	LDH Pearson's correlation coefficient (R^2)	1	0.981**
MTS Pearson's correlation coefficient (R^2)	0.980**	1	MTS Pearson's correlation coefficient (R^2)	0.981**	1

** Correlation was significant at P value < 0.01 (2-tailed)

D) *A. tricolor* petroleum ether fraction

24 h	LDH	MTS	48 h	LDH	MTS
LDH Pearson's correlation coefficient (R^2)	1	0.918**	LDH Pearson's correlation coefficient (R^2)	1	0.942**
MTS Pearson's correlation coefficient (R^2)	0.918**	1	MTS Pearson's correlation coefficient (R^2)	0.942**	1

** Correlation was significant at P value < 0.01 (2-tailed)

E) *A. tricolor* dichloromethane fraction

24 h	LDH	MTS	48 h	LDH	MTS
LDH Pearson's correlation coefficient (R^2)	1	0.984**	LDH Pearson's correlation coefficient (R^2)	1	0.950**
MTS Pearson's correlation coefficient (R^2)	0.984**	1	MTS Pearson's correlation coefficient (R^2)	0.950**	1

** Correlation was significant at P value < 0.01 (2-tailed)

F) *A. tricolor* methanol fraction

24 h	LDH	MTS	48 h	LDH	MTS
LDH Pearson's correlation coefficient (R^2)	1	0.981**	LDH Pearson's correlation coefficient (R^2)	1	0.964**
MTS Pearson's correlation coefficient (R^2)	0.981**	1	MTS Pearson's correlation coefficient (R^2)	0.964**	1

** Correlation was significant at P value < 0.01 (2-tailed)

3.2 Examination of advanced glycation endproducts (AGEs)

3.2.1 Determination of the AGEs formation

AGEs formation is not very specific, as it is not directed by enzymes, and is quite slow. Solving the mysteries of AGEs is likely to be a long process, as conducting research on the molecules can be difficult. We really concerned about the *in vitro* preparation of AGEs. Since the results are dependent on the stimulation of the cells with AGEs prepared *in vitro*, it is very important that we prepared the AGEs properly. To find the condition to synthesis AGEs *in vitro*, we considered many factors that might influence such as incubation duration, concentration of D-glucose and concentration of BSA. Using fluorescence content, measured with a fluorescence spectrometer at 335 nm emission after 460 nm excitation, as an indicator of AGEs content, we found that incubating 7 mmol/l D-glucose with 10 mg/ml BSA at 37°C in the dark for 12 weeks was the best condition because it presented an approximate 5-fold increase in fluorescence for AGEs compared with that of controls as shown in Figure 23.

A) 2 mmol/l D-glucose and 1 mg/ml BSA
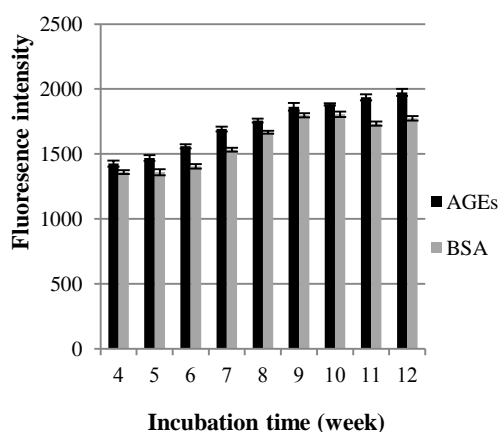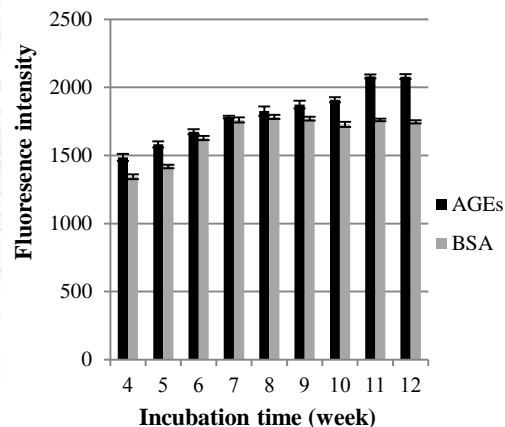
B) 5 mmol/l D-glucose and 1 mg/ml BSA

C) 7 mmol/l D-glucose and 1 mg/ml BSA
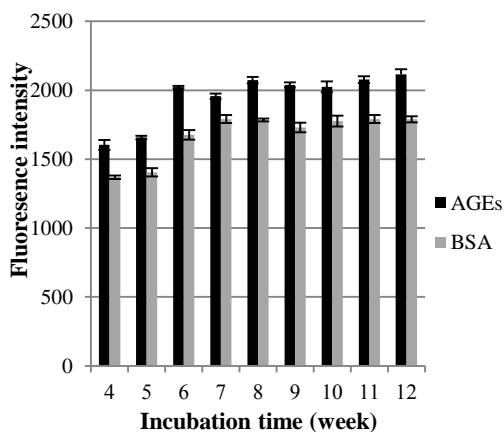
D) 2 mmol/l D-glucose and 5 mg/ml BSA
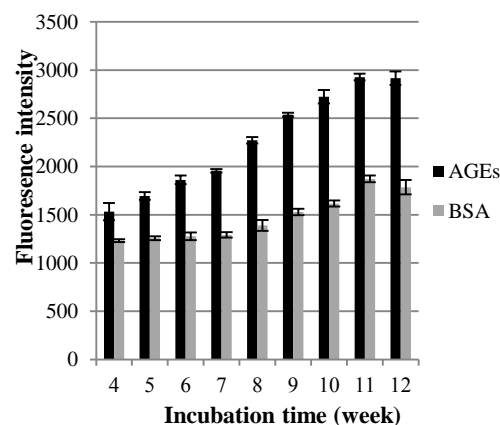
E) 5 mmol/l D-glucose and 5 mg/ml BSA
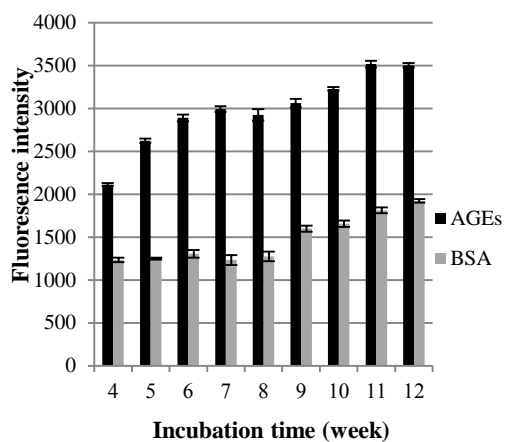
F) 7 mmol/l D-glucose and 5 mg/ml BSA
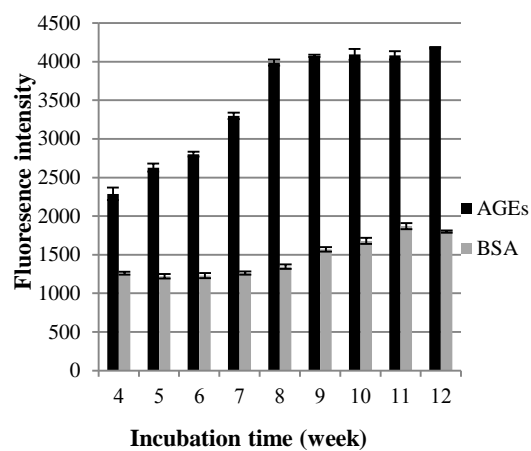
G) 2 mmol/l D-glucose and 10 mg/ml BSA
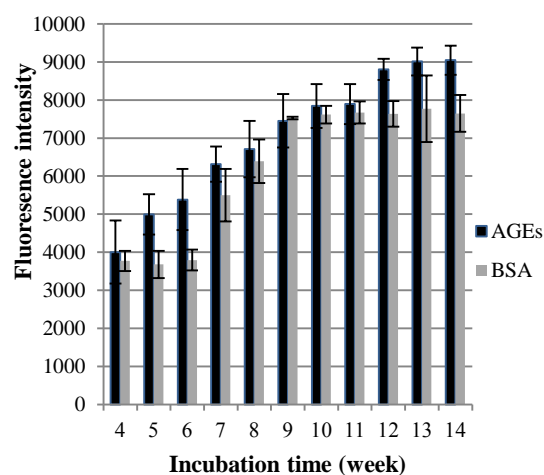
H) 5 mmol/l D-glucose and 10 mg/ml BSA
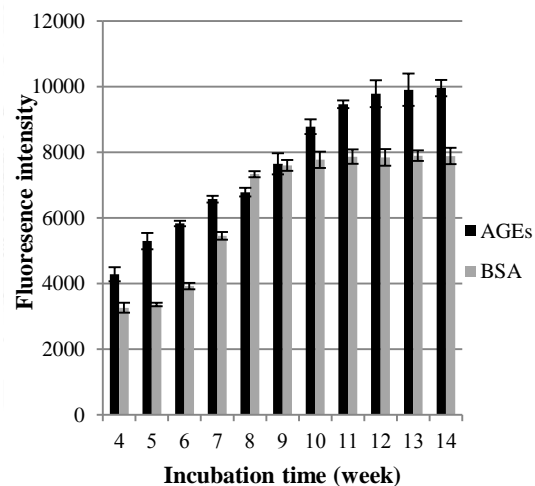
I)   7 mmol/l D-glucose and 10 mg/ml BSA
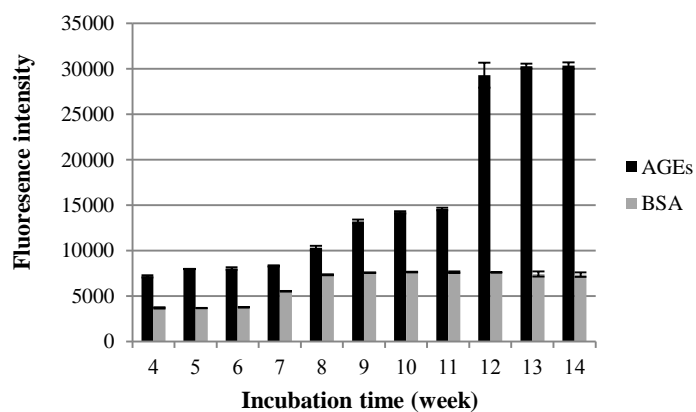
Figure 23. Fluorescent intensity of AGEs using different concentration of D-glucose and BSA for 4-14 weeks. The fluorescence intensity was measured at Ex 335 nm and Em 460 nm. (A) 2 mmol/l D-glucose and 1 mg/ml BSA; (B) 5 mmol/l D-glucose and 1 mg/ml BSA; (C) 7 mmol/l D-glucose and 1 mg/ml BSA; (D) 2 mmol/l D-glucose and 5 mg/ml BSA; (E) 5 mmol/l D-glucose and 5 mg/ml BSA; (F) 7 mmol/l D-glucose and 5 mg/ml BSA; (G) 2 mmol/l D-glucose and 10 mg/ml BSA; (H) 5 mmol/l D-glucose and 10 mg/ml BSA; (F) 7 mmol/l D-glucose and 10 mg/ml BSA. Values were reported as means with their standard error of the mean (SEM), depicted by vertical bars.

Correlation analysis was used to evaluate the correlation between the different concentration of D-glucose and BSA and incubation time for AGEs synthesis (Table 21). All concentration of D-glucose showed high correlation with incubation time for AGEs synthesis. The results also showed the strong correlation even though their concentrations differed. Furthermore, high correlation between 5 and 10 mg/ml BSA and incubation time were observed, but low correlation with 1 mg/ml BSA and incubation time. These results could suggest that D-glucose, BSA and incubation time were important for determination the fluorescence of AGEs.

Table 21. Statistical analysis using Pearson's correlation coefficient (R^2) to determine correlation between incubation time and different concentration of D-glucose and BSA.

Incubation time and different concentration of D-glucose and BSA	Pearson's correlation coefficient (R^2)
2 mmol/l D-glucose and 1 mg/ml BSA	0.984
5 mmol/l D-glucose and 1 mg/ml BSA	0.986
7 mmol/l D-glucose and 1 mg/ml BSA	0.824
1 mg/ml BSA	0.799
2 mmol/l D-glucose and 5 mg/ml BSA	0.989
5 mmol/l D-glucose and 5 mg/ml BSA	0.941
7 mmol/l D-glucose and 5 mg/ml BSA	0.933
5 mg/ml BSA	0.943
2 mmol/l D-glucose and 10 mg/ml BSA	0.983
5 mmol/l D-glucose and 10 mg/ml BSA	0.982
7 mmol/l D-glucose and 10 mg/ml BSA	0.909
10 mg/ml BSA	0.904

3.2.2 Assessment the toxicity of AGEs using SH-SY5Y cell culture as the model

3.2.2.1 Determined the cell toxicity effect of AGEs on SH-SY5Y cells using LDH assay

The above data showed that *A. lividus* and *A. tricolor* extracts have antioxidant activities, which may be protect against AGEs induced oxidative damage. We therefore attempted to assess the potential for these extracts to prevent oxidative stress induced by AGEs. In the first set of experiment, we used the LDH assay to examine the effects of AGEs on cytotoxicity of SH-SY5Y cells. A LDH assay is carried out to determine the optimum concentration and incubation time of AGEs to be used in the subsequent procedures. SH-SY5Y cells were incubated with various concentrations of AGEs and BSA control for 24 and 48 h. Figure 24 presented % cytotoxicity of cells following treatment with different AGEs or BSA control ranging from 0.5 to 8 mg/ml and 100% LDH released served as a positive control by lysed all cells with lysis solution. The results demonstrated that AGEs increased cell toxicity in a concentration and time dependent manner. At 24 h AGEs treatment, a high concentration of AGEs ($\geq$ 5 mg/ml) revealed toxic to the cells indicated by % cytotoxicity was over 20% as shown in Figure 24A. Whereas BSA control treatment showed lower toxicity than AGEs but became much more pronounced with increasing dose. IC_{50} value of AGEs were more than 8 mg/ml for 24 and more than 4 mg/ml for 48 h. It was indicated that exposure of SH-SY5Y cells to AGEs or BSA for 48 h showed more cytotoxic effect than 24 h treatment. At 48 h, all concentrations of AGEs were % cytotoxicity more than 20% as shown in Figure 24B.
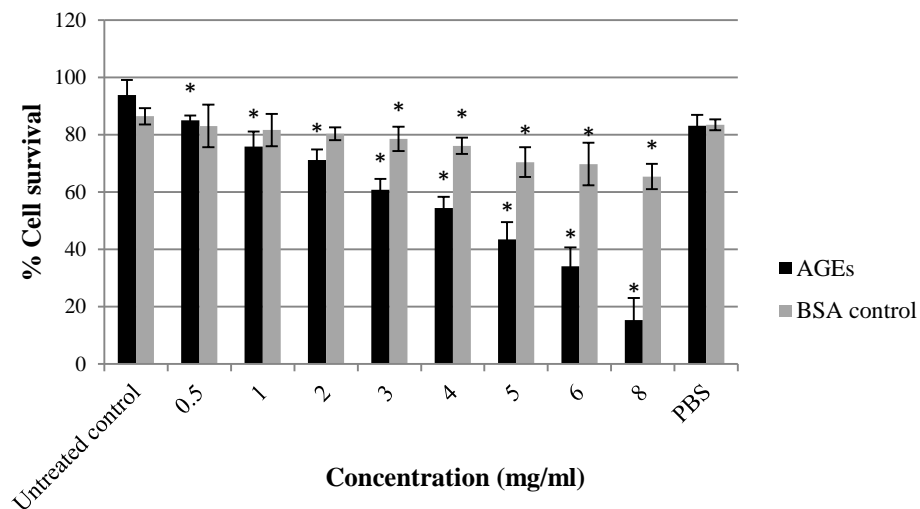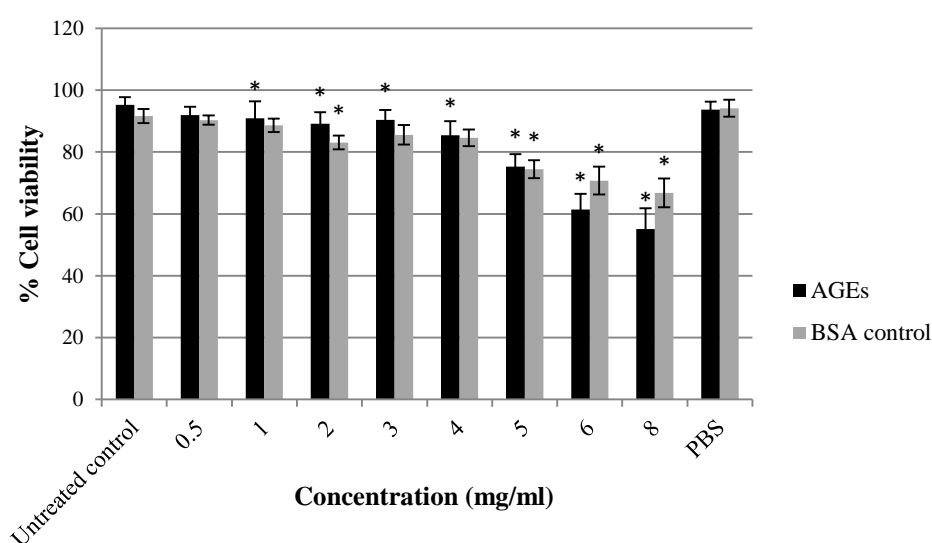
A
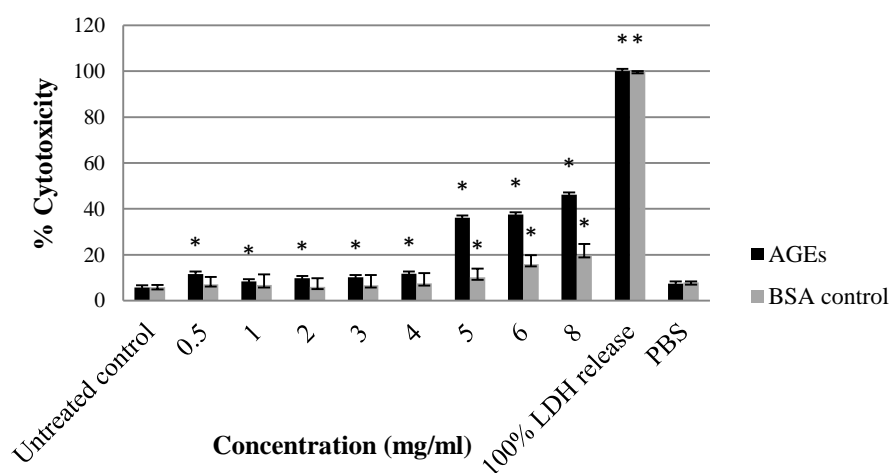
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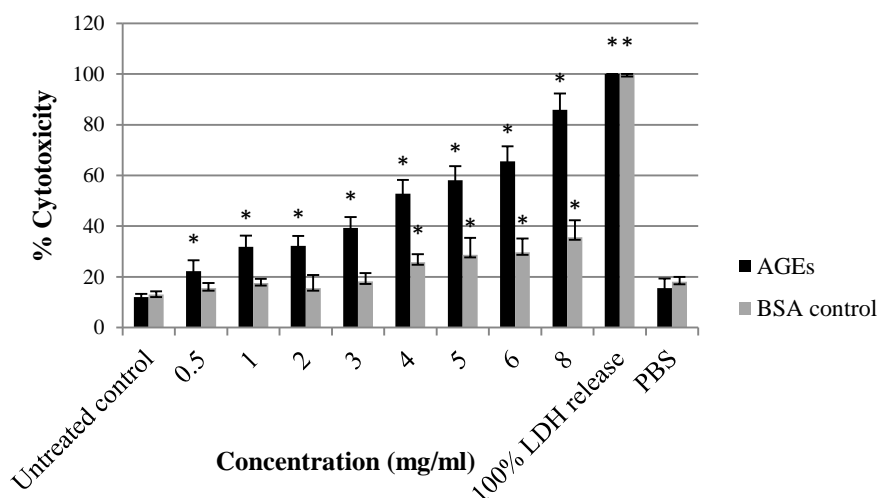
Figure 24. AGEs have an effect on cell toxicity of SH-SY5Y cells. SH-SY5Y cells were incubated with different concentrations of AGEs and BSA-control (0.5 to 8 mg/ml) for 24 to 48 h. (A) 24 h and (B) 48 h. The release of LDH of damaging SH-SY5Y cells was assessed using the LDH assay. The results were reported as means with their standard error of the mean (SEM), depicted by vertical bars.*P < 0.05 for significant change as compared to control (no treatment).

3.2.2.2 Determined the cell viability effect of AGEs on SH-SY5Y cells using trypan blue dye exclusion assay

The cytotoxic effects of AGEs results from LDH assay was confirmed by trypan blue dye exclusion assay. A dose and time course for decreasing in cell survival was shown in Figure 25 for 24 h and 48 h. The trypan blue staining demonstrated that AGEs treatment substantial inducing cell death in a concentration and time dependent manner. At 24 h, the trypan blue data showed that AGEs treatment was slightly toxic to the cells at low concentration range 0.5 to 4 mg/ml as indicated by % cell survival was over 80% and became more toxic when the cells were incubated with high concentration of AGEs (≥ 5 mg/ml) as shown in Figure 25A. Whereas BSA treatment indicated a slightly loss in cell viability but also became more toxic only at high concentration range 5 to 8 mg/ml. In addition, we further assessed the toxicity of AGEs for a period of incubation up to 48 h. Unsurprising, AGEs treatment at all concentrations were found to be the potential agents for induction of cell death while BSA treatment also revealed slightly cytotoxic at low concentration but became more toxic only at high concentration range 3 to 8 mg/ml (Figure 25B).

A
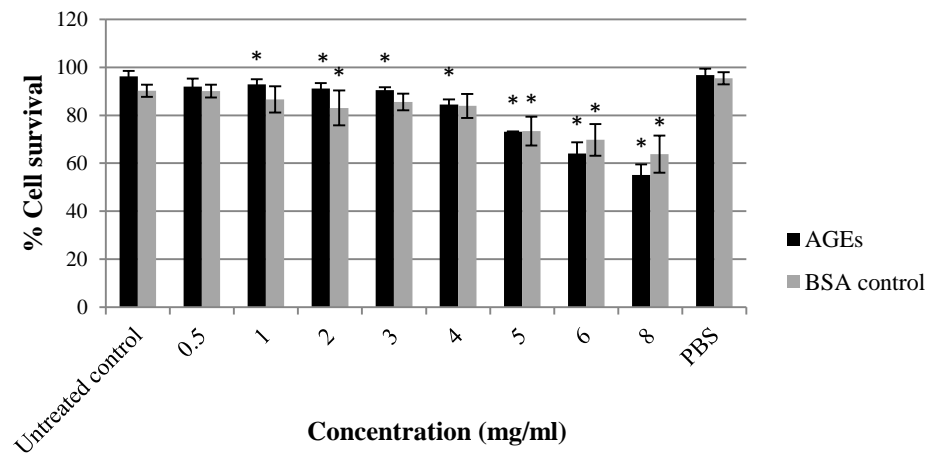
B

Figure 25. AGEs have an effect on cell survival of SH-SY5Y cells. SH-SY5Y cells were incubated with different concentrations of AGEs and BSA-control (0.5 to 8 mg/ml) for 24-48 h. (A) 24 h and (B) 48 h. The cell survival of living SH-SY5Y cells was assessed using the trypan blue exclusion assay. The results were reported as means with their standard error of the mean (SEM), depicted by vertical bars.*P < 0.05 for significant change as compared to control (no treatment).

3.2.2.3 Determined the cell damage effect of AGEs on SH-SY5Y cells using MTS assay

Next, the extent of cellular damage of AGEs was further quantified a reduction in metabolic activity using the MTS assay. SH-SY5Y cells were treated for 24 and 48 h with AGEs or BSA control at concentration ranging between 0.5 to 8 mg/ml. Figure 26 presented the % cell viability following treatment with various concentrations of AGEs and BSA for 24 h and 48 h. The result demonstrated that AGEs had cytotoxic effect on SH-SY5Y cells in a dose dependent manner while BSA indicated a slightly loss in cell viability at low concentration and became more toxic only at high concentration range 5 to 8 mg/ml by 24 h incubation. At 48 h, both AGEs and BSA became much more toxic in a concentration dependent manner. These data revealed that AGEs was more toxic than BSA in SH-SY5Y cells. A 50% cell death occurred at 4 mg/ml while BSA showed lower toxicity, $IC_{50} \geq 8$ mg/ml for 48 h. It was indicated that cytotoxic effect of AGEs and BSA was different.

As shown in Table 22, there were good correlation among the values of LDH assay, MTS assay and trypan blue exclusion assay ($R^2 > 0.8$) in 24 and 48 of AGEs treatment. Therefore these assays could be used to screen the cell toxicity effect of AGEs on SH-SY5Y. Regarding the SH-SY5Y cells, the viability test using MTS assay and trypan blue exclusion assay were over 80% as shown in Figure 25 and 26 and cytotoxicity test using LDH assay was under 20% as shown in Figure 24, for the further experiment we chose to investigate the effect of AGEs at a concentration range of 0.5 to 4 mg/ml for 24 h toward cellular oxidative stress induced by AGEs.

A

B
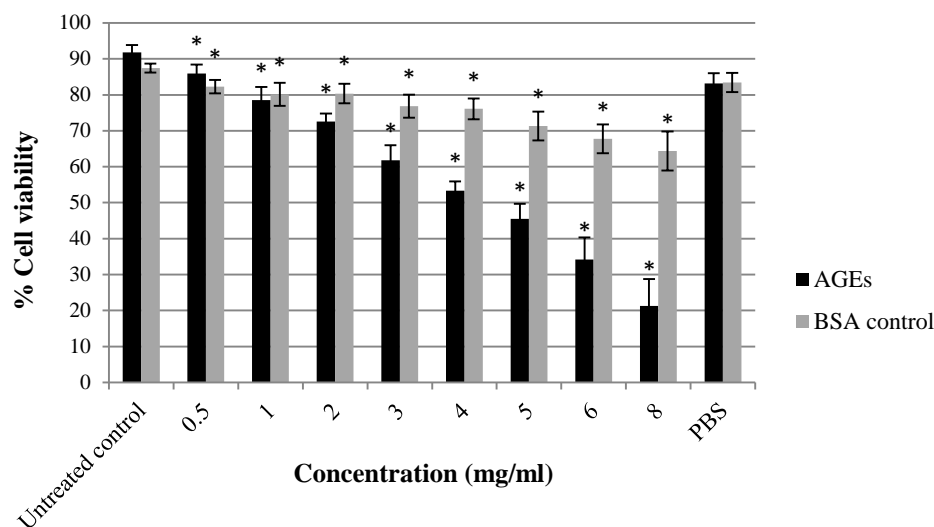
Figure 26. Effect of AGEs on the viability of SH-SY5Y cells using MTS assay. SH-SY5Y cells were incubated with different concentrations of AGEs and BSA-control (0.5 to 8 mg/ml) for 24-48 h. (A) 24 h and (B) 48 h. The results were reported as means with their standard error of the mean (SEM), depicted by vertical bars. *P < 0.05 for significant change as compared to control (no treatment)

Table 22. Statistical analysis using Pearson's correlation coefficient (R^2) to determine correlation with the finding of LDH assay, MTS assay and trypan blue exclusion assay for determination the cell toxicity effect of AGES on SH-SY5Y cells

A) 24 h

Pearson's correlation coefficient (R^2)	LDH assay for AGEs	LDH assay for BSA	MTS assay for AGEs	MTS assay for BSA	Trypan blue staining for AGEs	Trypan blue staining for BSA
LDH assay for AGEs	1	0.922**	0.966**	0.951**	0.976**	0.947**
LDH assay for BSA	0.922**	1	0.967**	0.869**	0.948**	0.872**
MTS assay for AGEs	0.966**	0.967**	1	0.961**	0.994**	0.960**
MTS assay for BSA	0.951**	0.869**	0.961**	1	0.970**	0.995**
Trypan blue staining for AGEs	0.976**	0.948*	0.994**	0.970**	1	0.971**
Trypan blue staining for BSA	0.947**	0.872**	0.960*	0.995**	0.971**	1

** Correlation was significant at P value < 0.01 (2-tailed)

B) 48 h

Pearson's correlation coefficient (R^2)	LDH assay for AGEs	LDH assay for BSA	MTS assay for AGEs	MTS assay for BSA	Trypan blue staining for AGEs	Trypan blue staining for BSA
LDH assay for AGEs	1	0.960**	0.990**	0.979**	0.993**	0.979**
LDH assay for BSA	0.960**	1	0.946**	0.970**	0.947**	0.963**
MTS assay for AGEs	0.990**	0.946**	1	0.976**	0.998**	0.981**
MTS assay for BSA	0.979**	0.970**	0.976**	1	0.979**	0.992**
Trypan blue staining for AGEs	0.993**	0.947*	0.998**	0.979**	1	0.982**
Trypan blue staining for BSA	0.979**	0.963**	0.981*	0.992**	0.982**	1

** Correlation was significant at P value < 0.01 (2-tailed)

3.3 Assessment the effects of AGEs/herbal extracts on oxidative stress on cell culture system

3.3.1 Effect of AGEs on cellular oxidative stress using DCFH-DA assay

There are several reports suggest that AGEs could produce ROS and subsequently exert cytotoxic effects [8]. We first sight to assess the cytotoxic effect of AGEs in SH-SY5Y cells. Alterations in cell viability and occurrence of cell toxicity were evaluated in cultures for 24 to 48 h with increasing concentrations of AGEs. AGEs and BSA control were insignificant cytotoxic effect at a concentration range of 0.5 to 4 mg/ml for 24 h (Figure 24, 25 and 26). In the following experiments we used the intracellular fluorescent ROS dye DCFH-DA, to examine the level of ROS in the SH-SY5Y cells after treated with un-effective doses of AGEs. The cells were treated with different concentrations of AGEs for 24 h. Hydrogen peroxide (H_2O_2) used as a positive control in the assay because it can diffuse across the plasma membrane and induced oxidative stress. The changes of intracellular ROS in SH-SY5Y cells were shown in Figure 27. Treatment of the cells with 10 μM H_2O_2 could lead to oxidative stress that ultimately resulted in a rapid increased of DCF fluorescence. The cells exposed to AGEs exhibited increased DCF fluorescence in a dose dependent manner while BSA treatment indicated a slight effect on intracellular ROS in SH-SY5Y cells suggesting only AGEs at level 0.5 to 4 mg/ml induced oxidative stress.
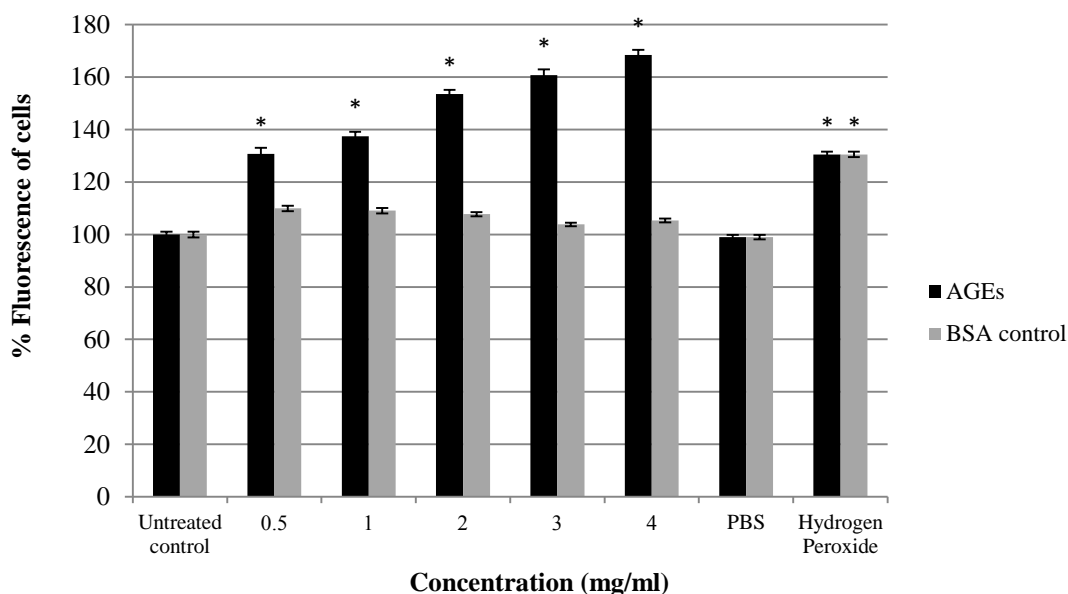
Figure 27. Effect of AGEs on oxidative stress in SH-SY5Y cells. SH-SY5Y cells were incubated with different concentrations of AGEs and BSA-control (0.5 to 4 mg/ml) for 24 h. The oxidative stress levels were measured at 24 h of incubation using DCFH-DA assay. The results were expressed as the percentage fluorescence of untreated control cells and represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.

3.3.2 Effect of AGEs on byproducts of lipid peroxidation during oxidative stress using MDA assay

In order to confirm that the increased intracellular ROS seen in Figure 27 were resulted from incubation the cells with AGEs, measurement of thiobarbituric acid reactive substances (TBARS) using MDA assay was used to examine the byproducts of lipid peroxidation during oxidative stress in the SH-SY5Y cells. As the oxidative degradation of lipids by ROS, results in the formation of highly reactive and unstable lipid peroxides. Decomposition of lipid peroxides results in the formation of TBARS. The CNS is particularly susceptible to oxidative stress, mainly because the brain is rich in polyunsaturated fatty acids therefore accumulates ROS and eventually oxidative stress can cause neuroinflammation and neurotoxicity [523]. We used MDA assay to determine whether exposure of the SH-SY5Y cells to AGEs would lead to the appearance of TBARS in the cells. A parallel set of the cultures were treated with BSA control. Hydrogen peroxide used as a positive control in the assay. As shown in Figure 28, treatment of the cells with 10 µM H_2O_2 could lead to generate 1.88 µM TBARS. The TBARS level was significantly increased in a dose dependent manner when SH-SY5Y cells were incubated with 1 to 4 mg/ml AGEs but this effect was insignificant at low concentration (0.5 mg/ml) of AGEs. Whereas BSA treatment resulted in ineffectiveness with any concentrations suggesting only AGEs at level 1 to 4 mg/ml could induce TBARS products of the damage produced by oxidative stress induced by AGEs.
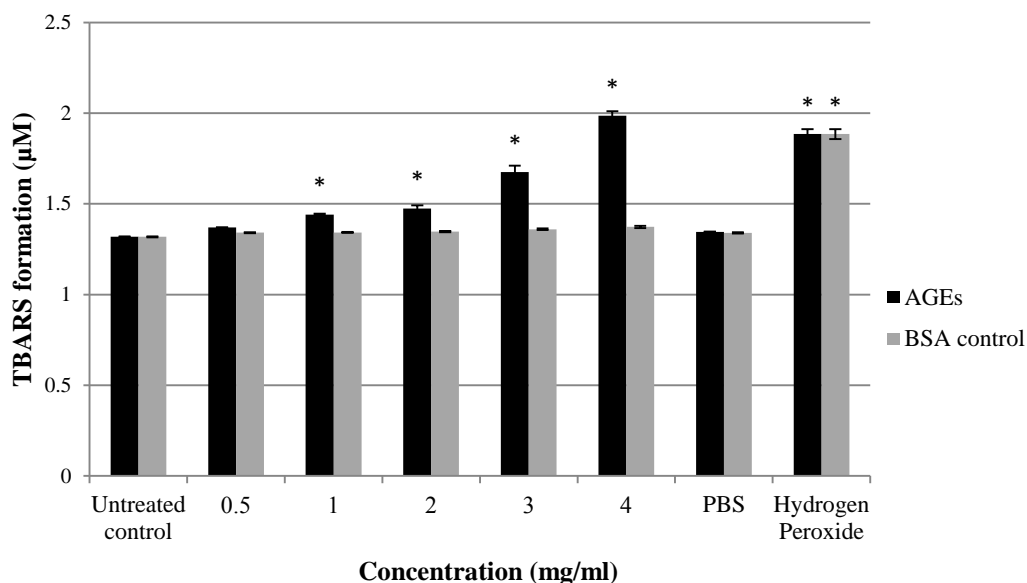
Figure 28. Effect of AGEs on TBARS formation during oxidative stress in SH-SY5Y cells. SH-SY5Y cells were incubated with different concentrations of AGEs and BSA-control (0.5 to 4 mg/ml) for 24 h. The generation of TBARS was assessed using the MDA assay. The results were expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *$P < 0.05$ when compared with untreated cells.

3.3.3 Effects of the herbal extracts on oxidative stress induced by AGEs on SH-SY5Y cells

In order to evaluate the effect of herbal extracts on oxidative stress, levels of intracellular ROS were examined using DCF fluorescence method along with MDA level against oxidative stress in SH-SY5Y cells. Based on cytotoxic data, antioxidant and toxicity test, SH-SY5Y cells were pretreated with 1.5625 to 100 µg/ml *A. lividus* and *A. tricolor* extracts except for 1.5625 to 50 µg/ml *A. lividus* dichloromethane extract. In these experiments, we applied a protocol using a dosage of AGEs of 0.5 to 4 mg/ml which were shown to induce oxidative stress by 24 h post-incubation in SH-SY5Y cells (Figure 27). SY5Y cells were pretreated with the herbal extracts for 24 h and then further incubated with AGEs for 24 h. Hydrogen peroxide used as a positive control in the assay. In this study, *A. lividus* and *A. tricolor* extracts were dissolved in DMSO. The final content of DMSO in the cultural medium with the highest concentration of the extract, 100 µg/ml, was 0.01%. As shown in Figure 29 and 31, while both herbs in all solvents could inhibit oxidative stress in SH-SY5Y cells, DMSO showed no significant reducing of oxidative stress. We then tested the protective effects of pretreatment of the cells with *A. lividus* petroleum ether fraction (Figure 29A), dichloromethane fraction (Figure 29B) and methanol fraction (Figure 29C). The results revealed that *A. lividus* extracts in all solvents were effective at reducing DCF fluorescence in a dose dependent manner in response to all concentrations of AGEs (0.5 to 4 mg/ml) and H_2O_2 when compared to the untreated cells. The changes of ROS in SH-SY5Y cells exposed to each concentration of AGEs were shown; 0.5 mg/ml (Figure 30A), 1 mg/ml (Figure 30B), 2 mg/ml (Figure 30C), 3 mg/ml (Figure 30D) and 4 mg/ml

(Figure 30E). A 50% inhibition of oxidative stress occurred at 100 µg/ml of *A. lividus* methanol extract for 0.5 mg/ml AGEs (Figure 30A), 25 to 50 µg/ml of *A. lividus* dichloromethane extract and 100 µg/ml of methanol extract for 1 mg/ml AGEs (Figure 30B), 25 to 100 µg/ml of *A. lividus* petroleum ether and dichloromethane extracts and 50 to 100 µg/ml of methanol extract for 2 mg/ml AGEs (Figure 30C), 50 to 100 µg/ml of *A. lividus* petroleum ether, 25 to 50 µg/ml dichloromethane extracts and 50 to 100 µg/ml of methanol extract for 3 mg/ml AGEs (Figure 30D) and 100 µg/ml of *A. lividus* petroleum ether, 50 µg/ml dichloromethane extracts and 50 to 100 µg/ml of methanol extract for 4 mg/ml AGEs (Figure 30E). For *A. tricolor,* as shown in Figure 31, pretreatment of the cells with petroleum ether fraction (Figure 31A), dichloromethane fraction (Figure 31B) and methanol fraction (Figure 31C) exhibited significantly reduced intracellular ROS in a concentration dependent manner in the cells under oxidative stress condition induced by 0.5 to 4 mg/ml AGEs and H_2O_2 when compared to the untreated cells. A 50% inhibition of oxidative stress occurred at 100 µg/ml of *A. tricolor* methanol extract for 0.5 mg/ml AGEs (Figure 32A), 100 µg/ml of *A. lividus* dichloromethane and methanol extracts for 1 mg/ml AGEs (Figure 32B), 100 µg/ml of *A. tricolor* petroleum ether, 50 to 100 µg/ml dichloromethane and methanol extracts for 2 mg/ml AGEs (Figure 32C), 50 to 100 µg/ml of *A. tricolor* petroleum ether, dichloromethane and methanol extracts for 3 mg/ml AGEs (Figure 32D) and 100 µg/ml of *A. tricolor* petroleum ether, 25 to 100 µg/ml dichloromethane extracts and 50 to 100 µg/ml of methanol extract for 4 mg/ml AGEs (Figure 32E). However, both *A. lividus* and *A. tricolor* methanol extract showed maximum protection against ROS generation in SH-SY5Y cells at concentration of 100 µg/ml under oxidative stress condition over 24 h of observation. Moreover, under this concentration of *A. lividus* methanol extract and all solvents of *A. tricolor* treatment with oxidative stress condition, ROS generation was found lowered than untreated control. These results could indicate that *A. tricolor* was relatively effective than *A. lividus*.

A

B

A. lividus dichloromethane extract (μg/ml)
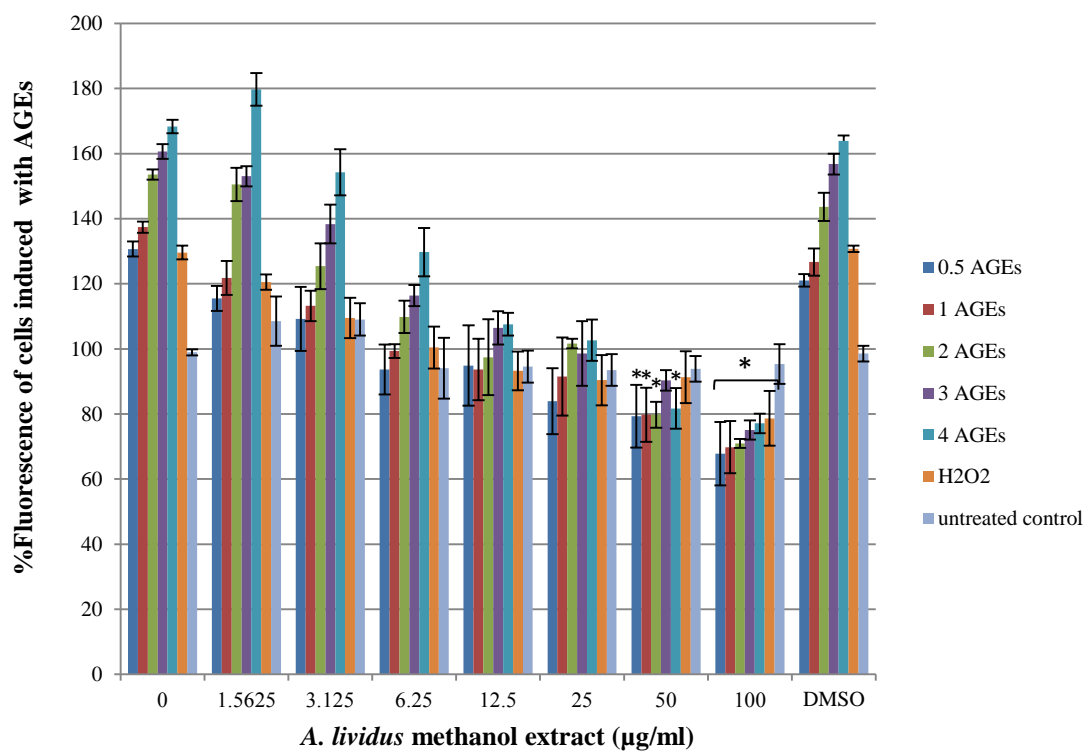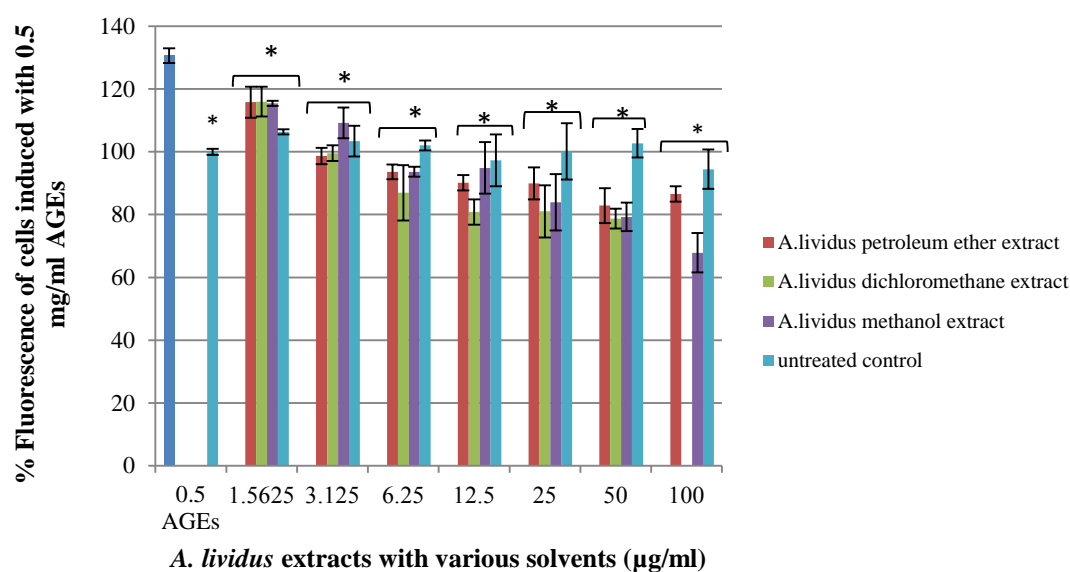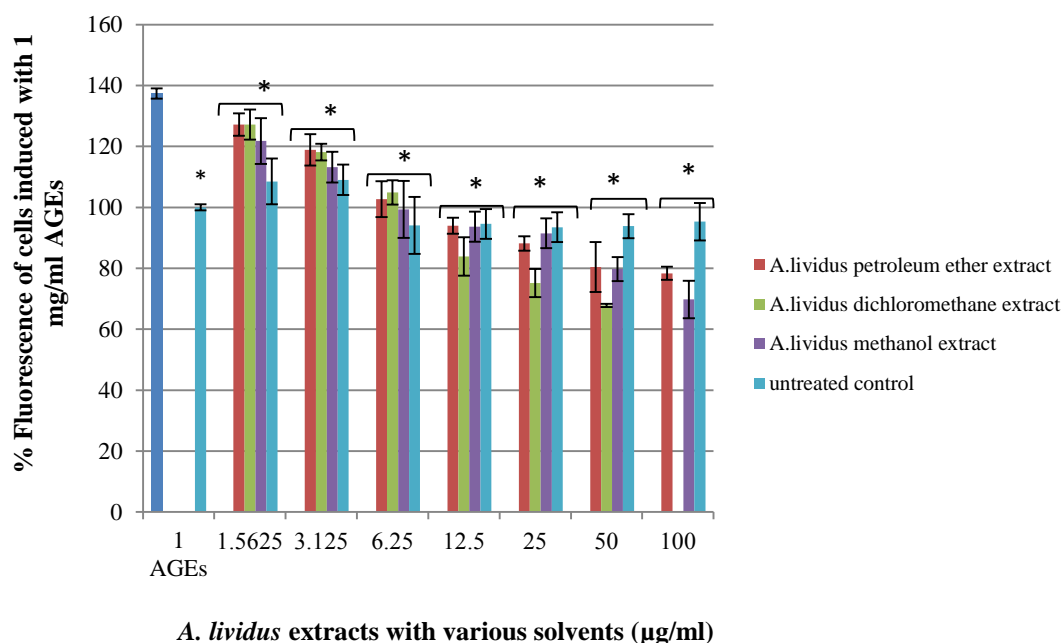
C

A. lividus methanol extract (μg/ml)
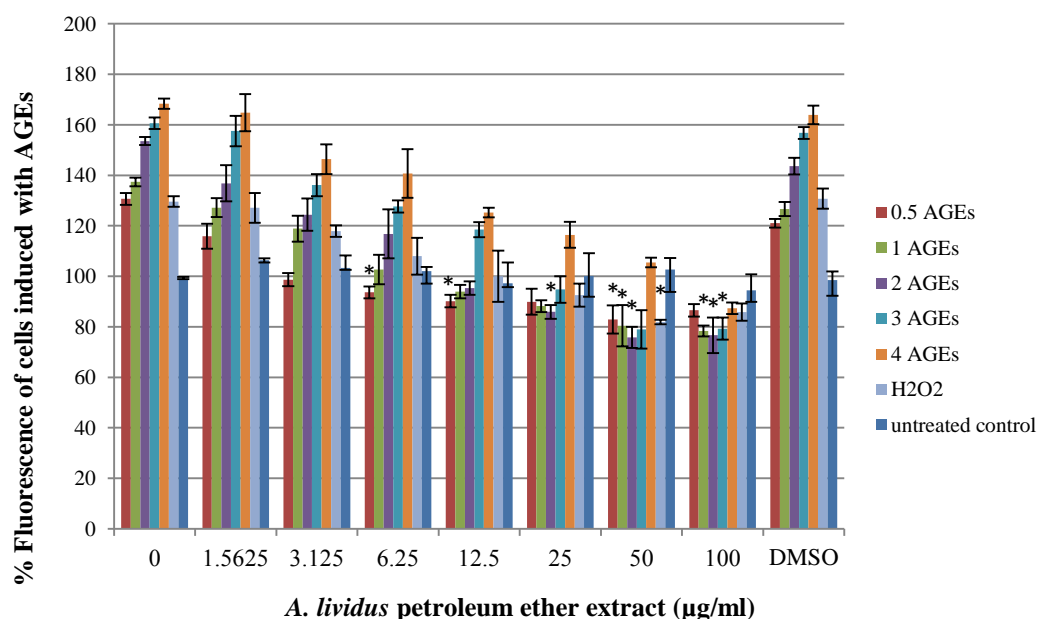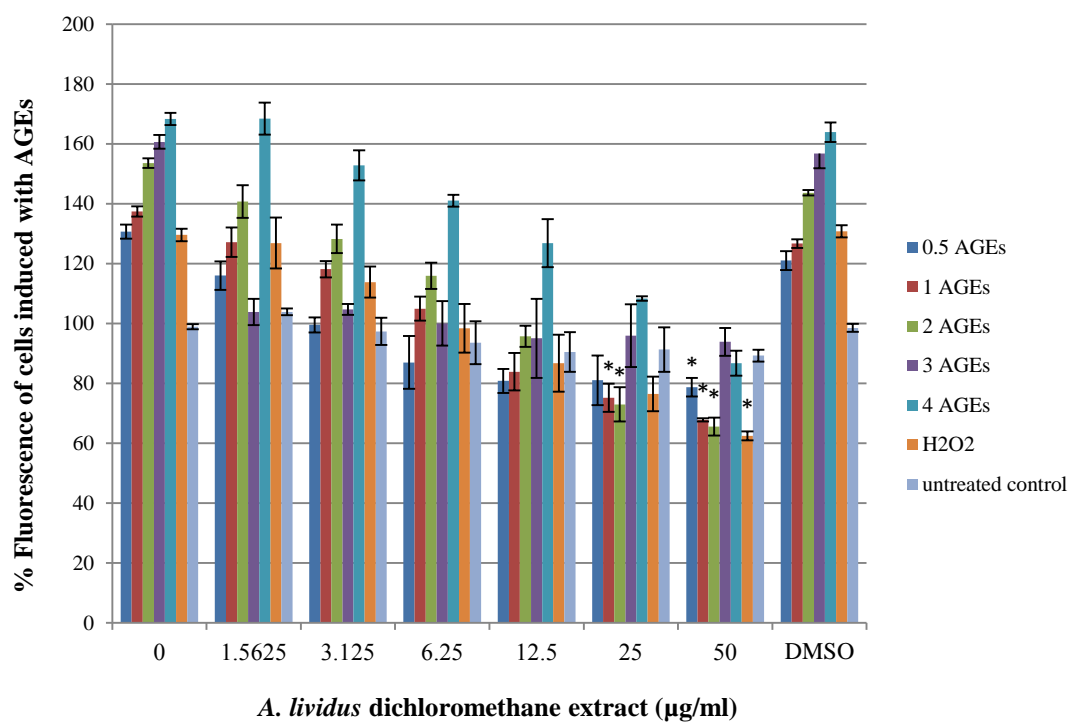
Figure 29. Ameliorative effect of different *A. lividus* extracts on oxidative stress in SH-SY5Y cells. SH-SY5Y cells were preincubated with different concentrations of three different *A. lividus*; (A) petroleum ether; (B) dichloromethane and (C) methanol extracts. The oxidative stress levels were measured using DCFH-DA assay. The results were expressed as the percentage fluorescence of untreated control cells and represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated control.

A

B

C
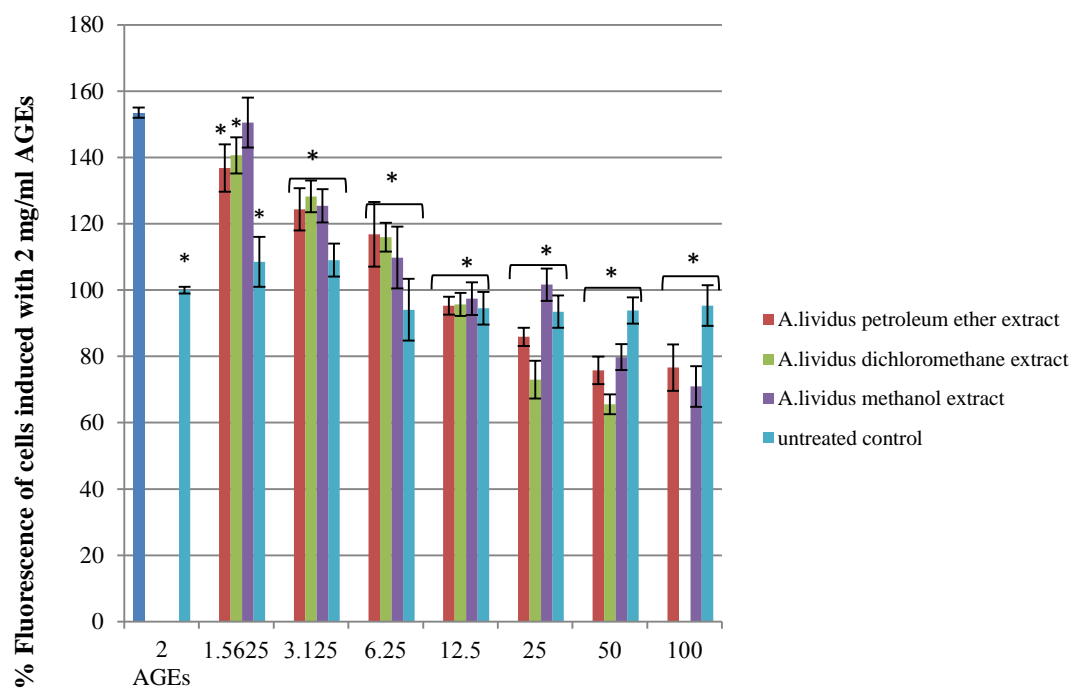
A. lividus extracts with various solvents (μg/ml)

D
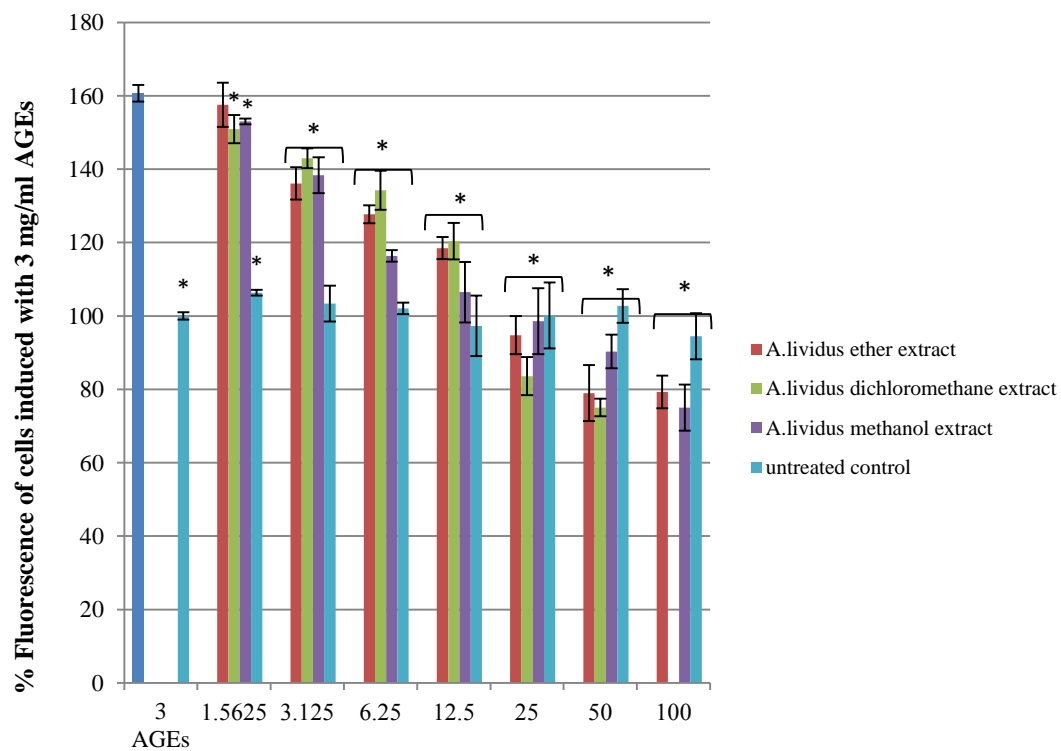
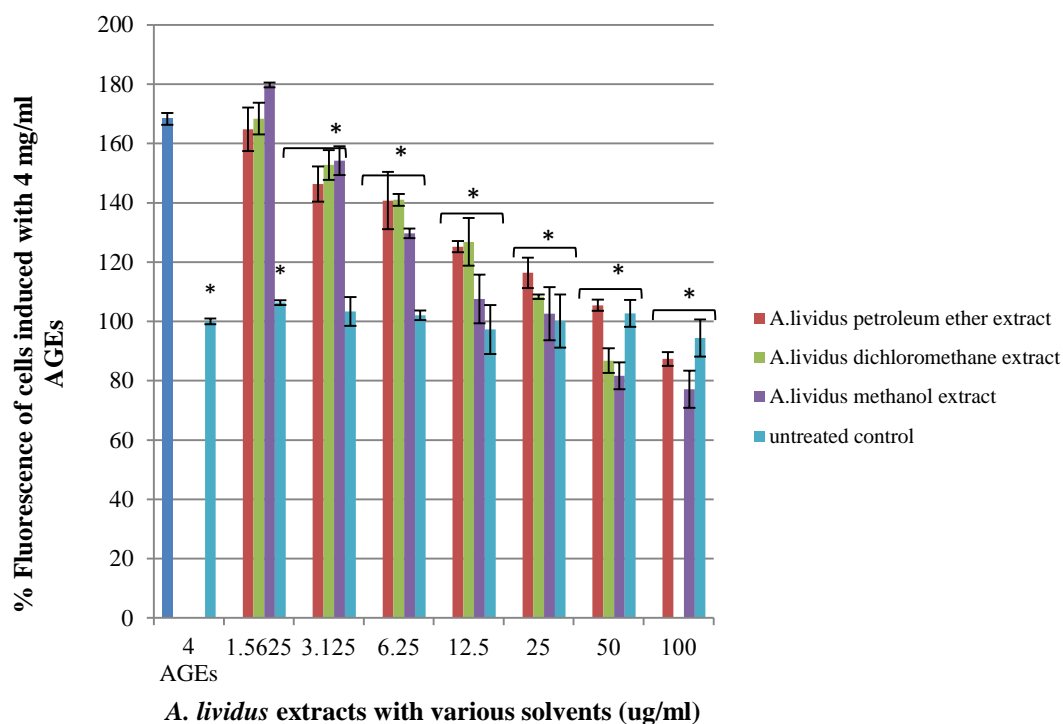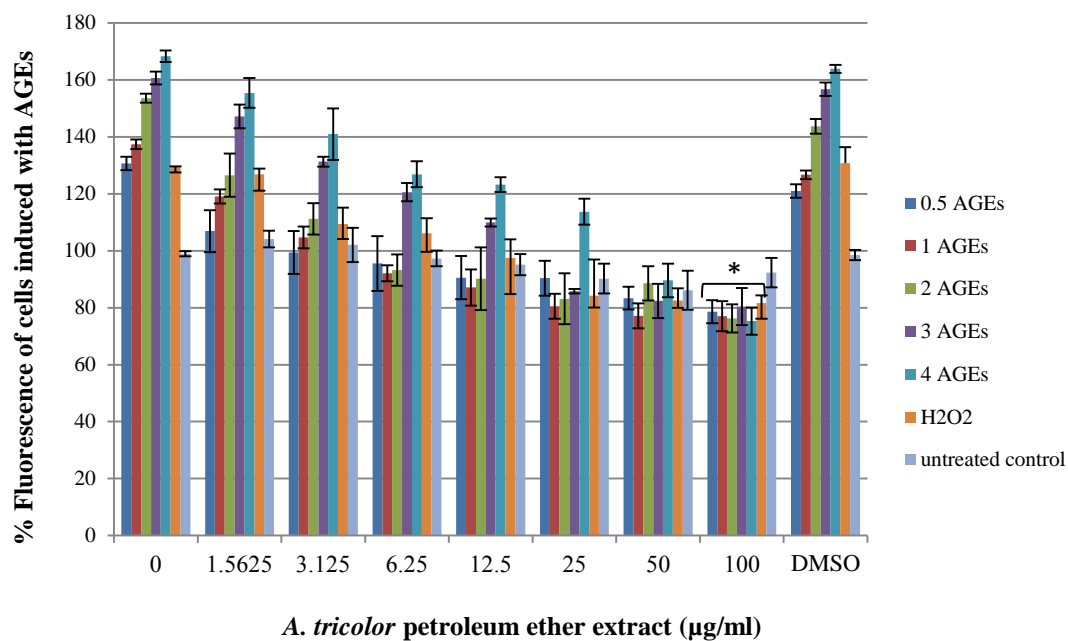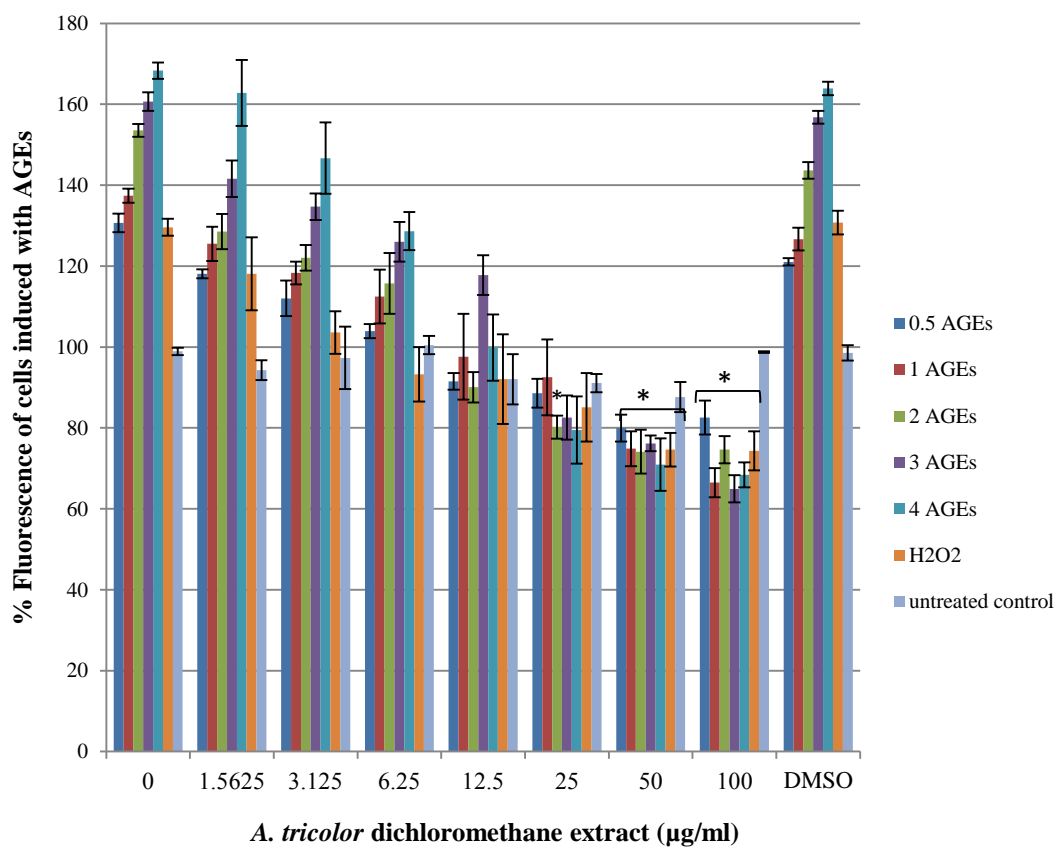**A. lividus extracts with various solvents (μg/ml)**

E

Figure 30. Ameliorative effect of different *A. lividus* extracts on oxidative stress induced by AGEs in SH-SY5Y cells. SH-SY5Y cells were preincubated with different concentrations of three different *A. lividus* for 24 h and further incubated with different concentrations of AGEs (A) 0.5 mg/ml; (B) 1 mg/ml; (C) 2 mg/ml; (D) 3 mg/ml and (E) 4 mg/ml. The oxidative stress levels were measured using DCFH-DA assay. The results were expressed as the percentage fluorescence of untreated control cells and represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by $*P < 0.05$ when compared with AGEs treated cells.

A

A. tricolor **petroleum ether extract (µg/ml)**

B

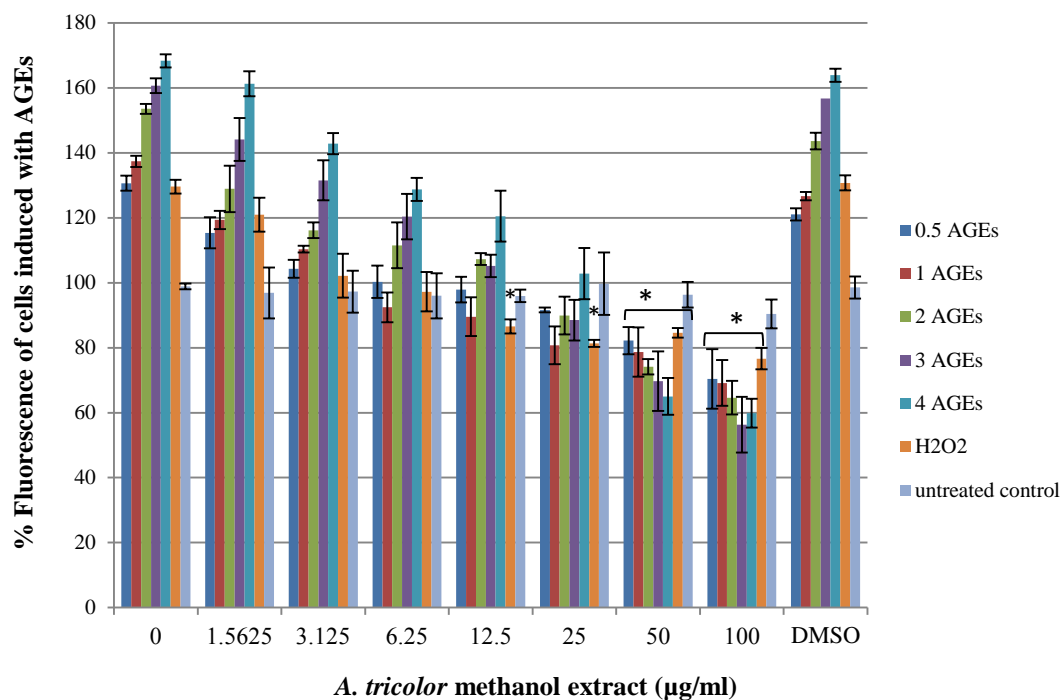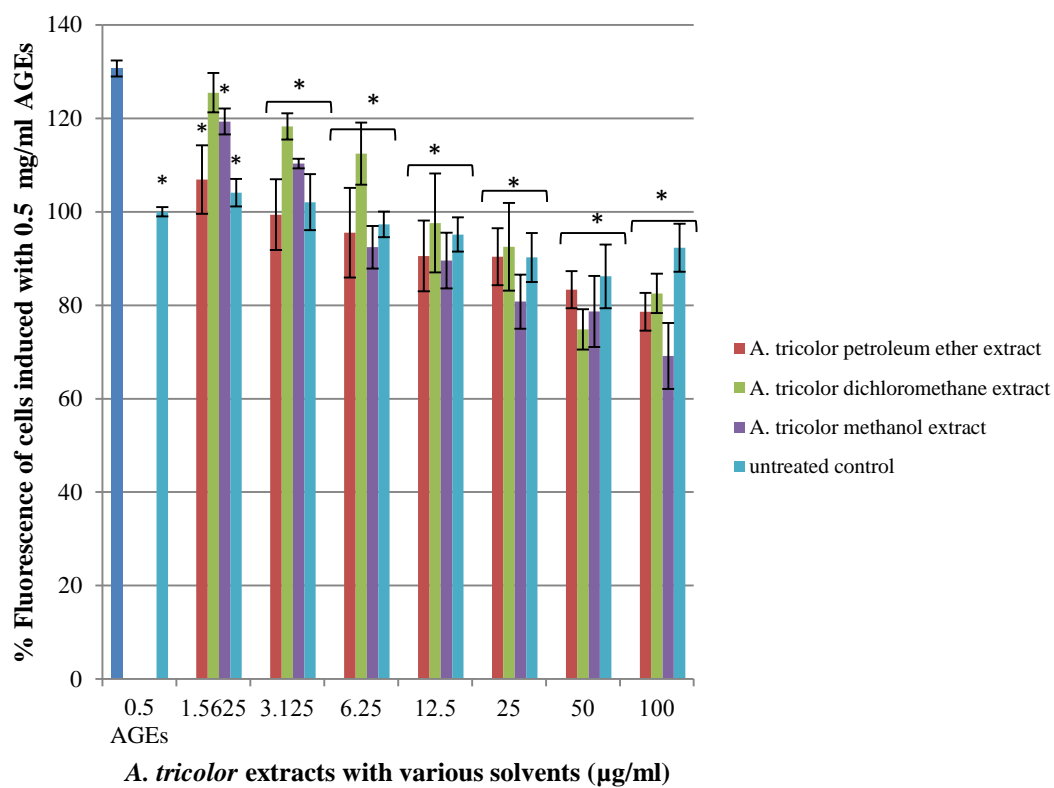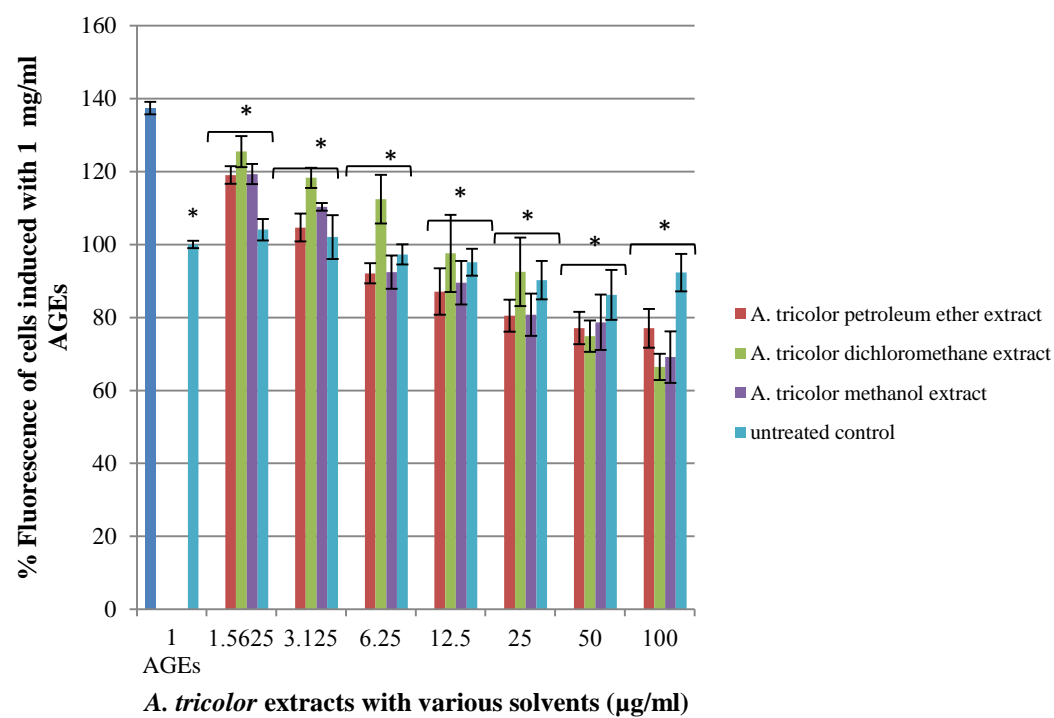*A. tricolor* **dichloromethane extract (µg/ml)**

C

Figure 31. Ameliorative effect of different *A. tricolor* extracts on oxidative stress in SH-SY5Y cells. SH-SY5Y cells were preincubated with different concentrations of three different *A. lividus*; (A) petroleum ether; (B) dichloromethane and (C) methanol extracts. The oxidative stress levels were measured using DCFH-DA assay. The results were expressed as the percentage fluorescence of untreated control cells and represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated control.
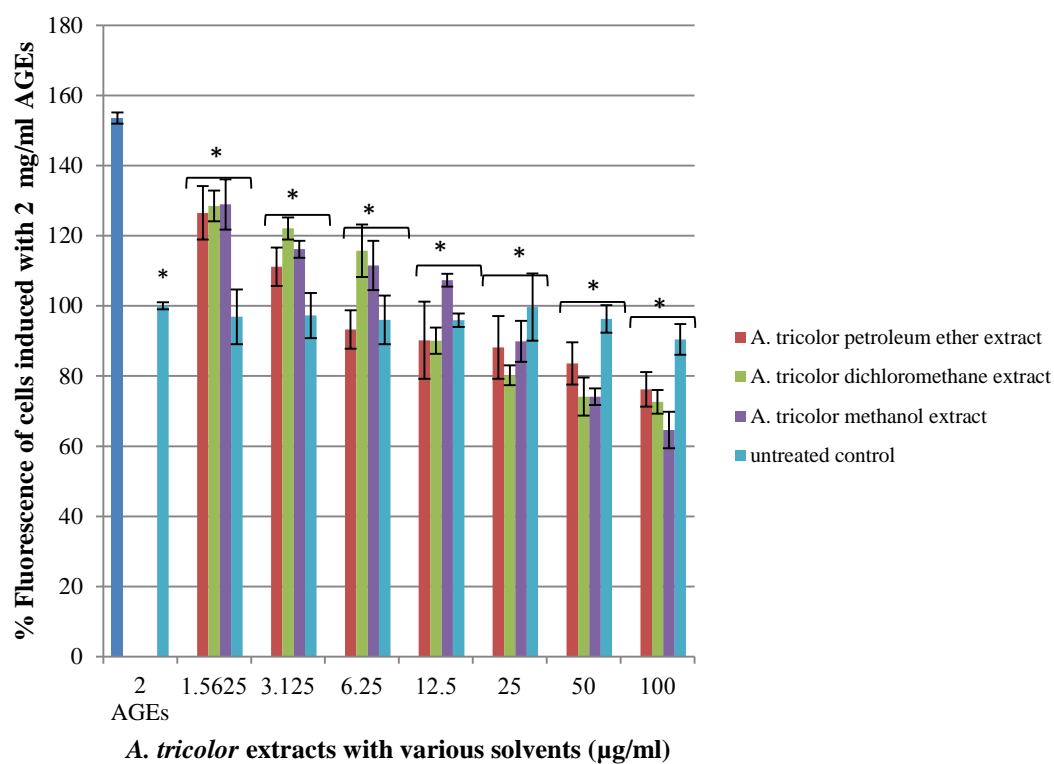
A

B

113
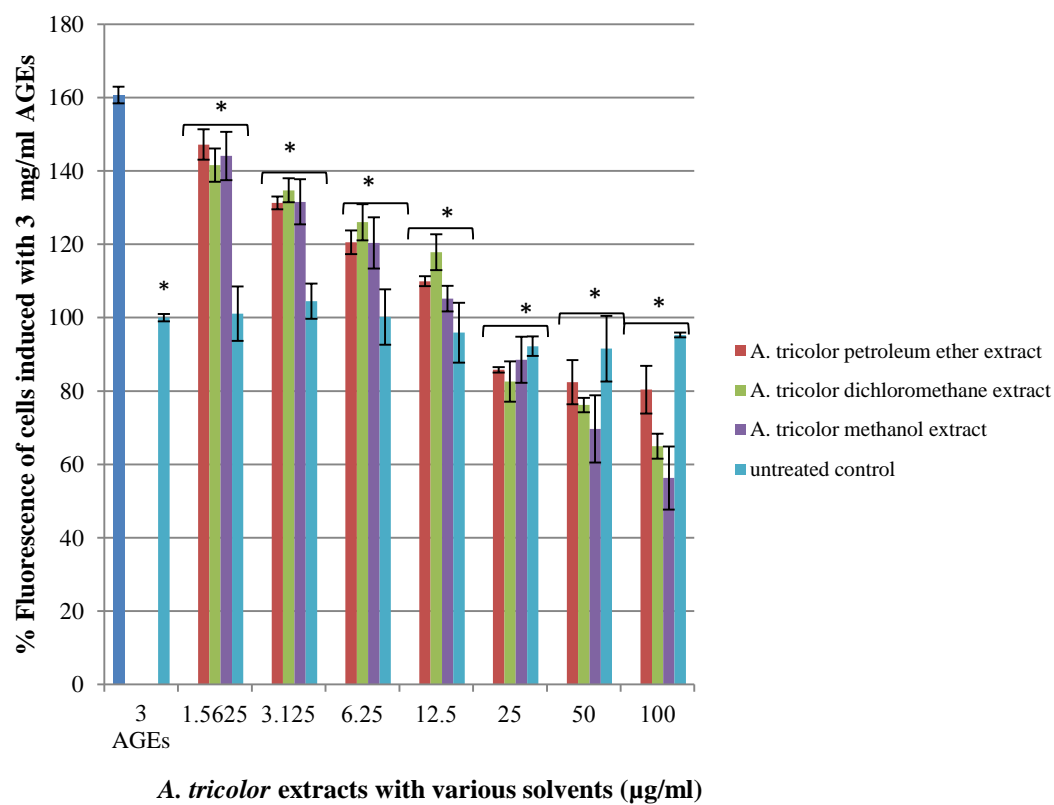
E
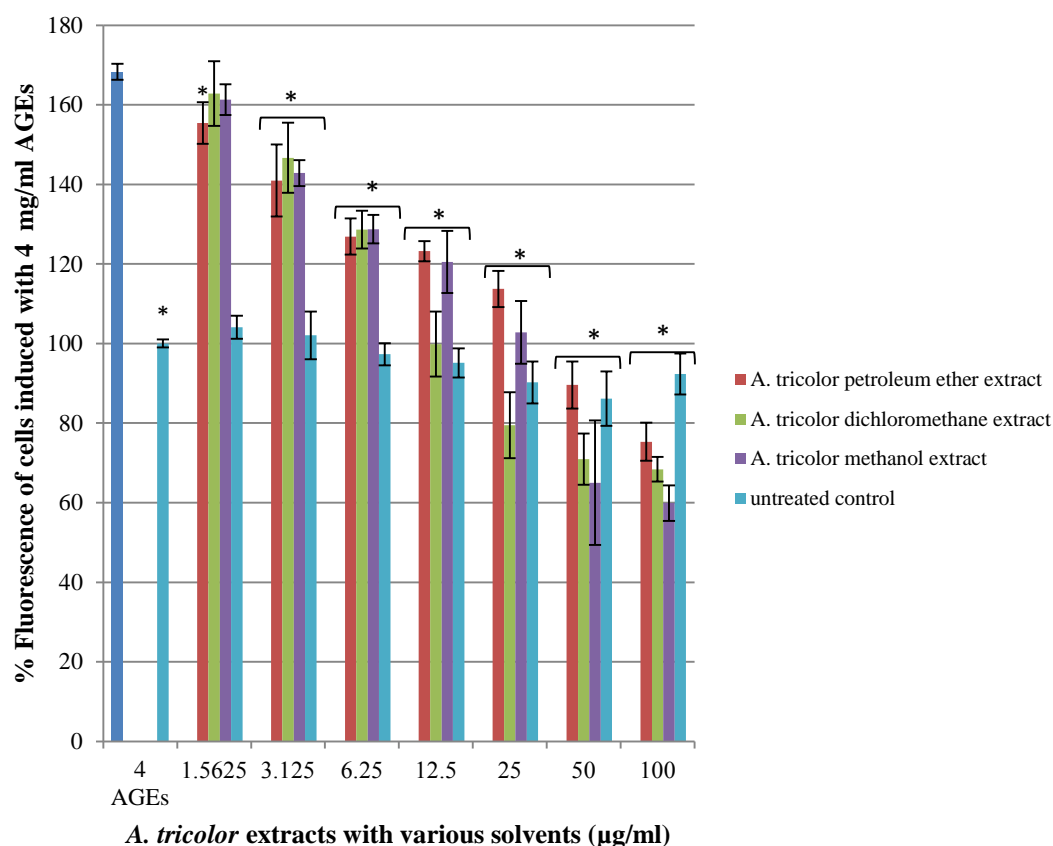
Figure 32. Ameliorative effect of different *A. tricolor* extracts on oxidative stress induced by AGEs in SH-SY5Y cells. SH-SY5Y cells were preincubated with different concentrations of three different *A. tricolor* for 24 h and further incubated with different concentrations of AGEs; (A) 0.5 mg/ml; (B) 1 mg/ml; (C) 2 mg/ml; (D) 3 mg/ml and (E) 4 mg/ml. The oxidative stress levels were measured using DCFH-DA assay. The results were expressed as the percentage fluorescence of untreated control cells and represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by $*P < 0.05$ when compared with AGEs treated cells.

3.3.4 Effects of the herbal extracts on oxidative stress byproducts induced by AGEs on SH-SY5Y cells using MDA assay

Another approach that used to explore the protective effect of *A. lividus* and *A. tricolor* extracts against AGEs induced oxidative stress was MDA assay. We used MDA assay to measure the generation of TBARS during oxidative stress induced by AGEs in SH-SY5Y cells and to find out whether the *A. lividus* and *A. tricolor* extracts were capable of reducing oxidative stress in the cells. As shown in Figure 28, exposure of SH-SY5Y cells to 1 to 4 mg/ml AGEs led to significantly increase TBARS formation by 24 h post-incubation. The highest rising level of TBARS occurred when the cells treated with 4 mg/ml of AGEs resulted in an approximate 1.98 μM TBARS formation. Hydrogen peroxide, a positive control, could induce TBARS level to 1.88 μM. The present study used herbal extracts that dissolved in DMSO. The final content of DMSO

in the cultural medium with the highest concentration of the extract, 100 µg/ml, was 0.01%. As shown in Figure 33 and 34, pretreated the cells with DMSO was revealed not effect for TBARS formation whereas both herbs in all solvents could inhibit TBARS generation in SH-SY5Y cells exposed to AGEs and H_2O_2. The results of protective effects of pretreatment of the cells with *A. lividus* petroleum ether fraction (Figure 33A), dichloromethane fraction (Figure 33B) and methanol fraction (Figure 33C) and *A. tricolor* as shown in Figure 34, pretreatment of the cells with petroleum ether fraction (Figure 34A), dichloromethane fraction (Figure 34B) and methanol fraction (Figure 34C) were shown. The TBARS level was decreased in a dose dependent manner in pretreatment the cells with herbal extracts and then exposed to AGEs but this effect was slightly changed when treatment SH-SY5Y cells alone with herbal extracts. The results revealed that the pretreated of the cells with *A. lividus* concentration of 6.25 to 100 µg/ml petroleum ether extract, 3.125 to 50 mg/ml dichloromethane extract, and 1.5625 to 100 µg/ml of *A. lividus* methanol extract and all concentrations of *A. tricolor* extracted with petroleum ether, dichloromethane, and methanol, exhibited significantly decreasing in TBARS level when compared to the cells treated with 4 mg/ml. It was indicated that both *A. lividus* and *A. tricolor* have protective effect against AGEs induced oxidative stress.

A

***A. lividus* petroleum ether extract (µg/ml)**
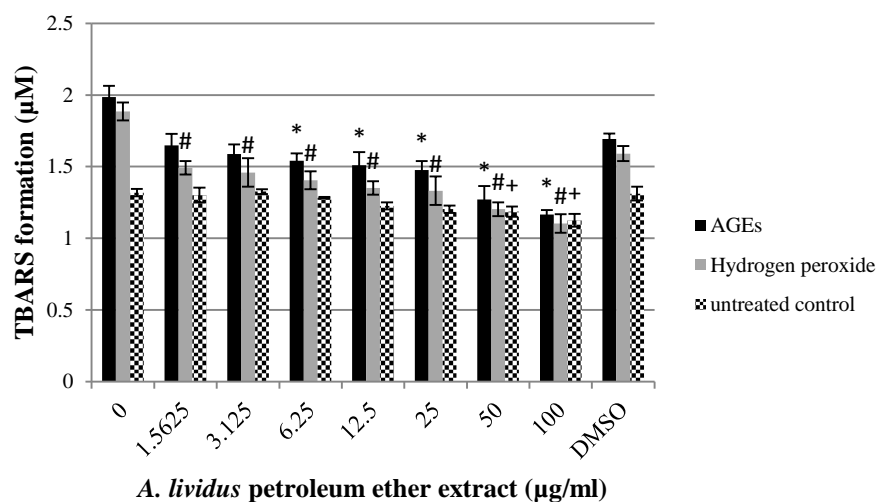
B
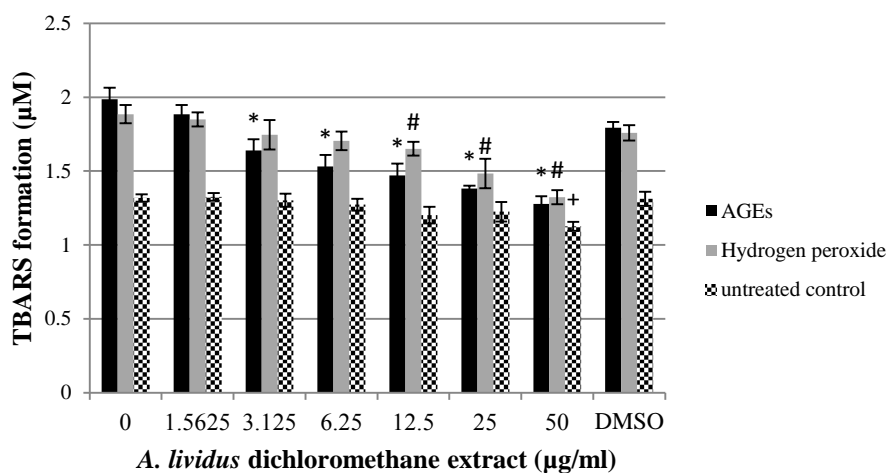
C

Figure 33. Protective effect of *A. lividus* extracts on AGEs-induced oxidative stress in SH-SY5Y cells. The generation of TBARS was assessed using the MDA assay. SH-SY5Y cells were preincubated with different concentrations of three different *A. lividus*; (A) petroleum ether; (B) dichloromethane and (C) methanol extracts. SH-SY5Y cells were further incubated with 4 mg/ml AGEs. The results were represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with AGEs treated cells, #P < 0.05 when compared with H_2O_2 treated cell and +P < 0.05 when compared with non-herbal extract treated cells.

A

A. tricolor **petroleum ether extract (µg/ml)**

B

A. tricolor **dichloromethane extract (µg/ml)**

C

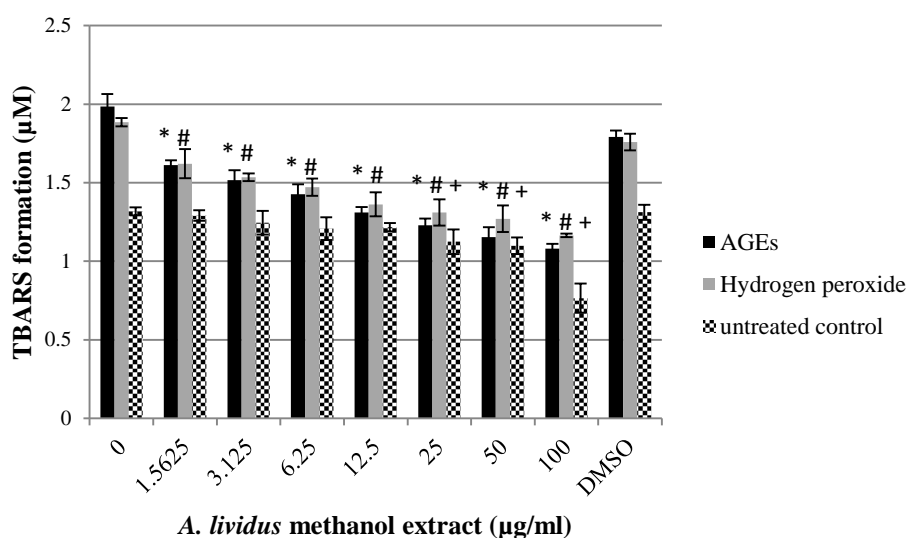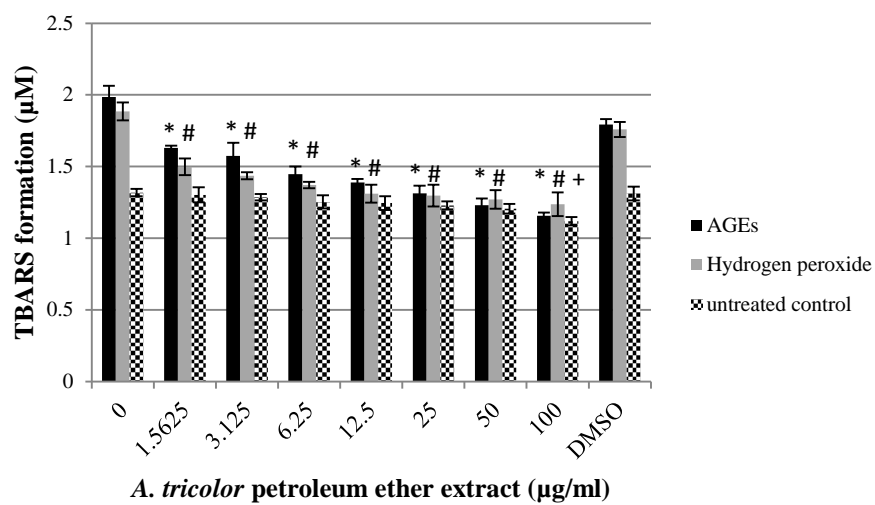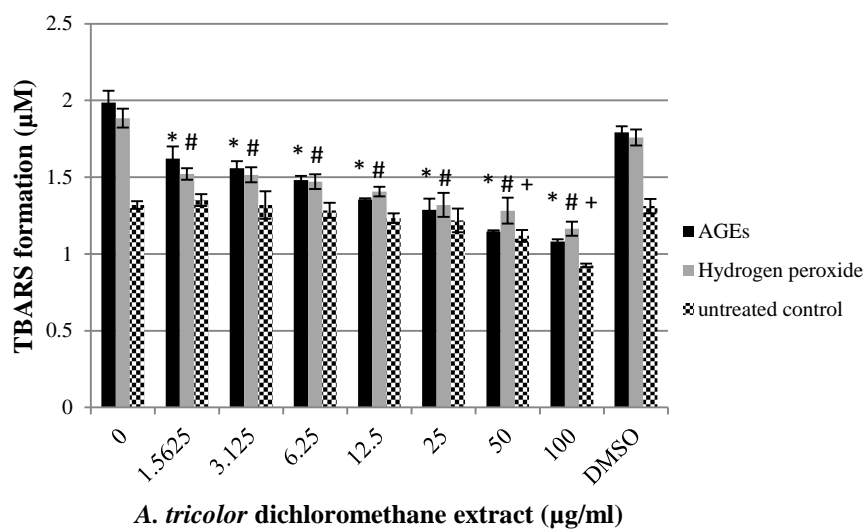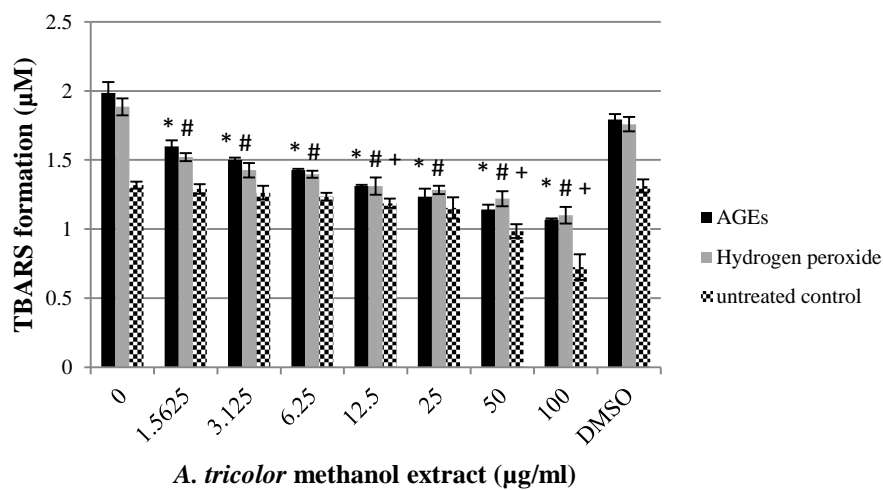*A. tricolor* **methanol extract (µg/ml)**

Figure 34. Protective effect of *A. tricolor* extracts on AGEs-induced oxidative stress in SH-SY5Y cells. The generation of TBARS was assessed using the MDA assay. SH-SY5Y cells were preincubated with different concentrations of three different *A. lividus*; (A) petroleum ether; (B) dichloromethane and (C) methanol extracts. SH-SY5Y cells were further incubated with 4 mg/ml AGEs. The results were represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with AGEs treated cells, #P < 0.05 when compared with H_2O_2 treated cell and +P < 0.05 when compared with non-herbal extract treated cells.

3.4 Assessment the protective effects of herbal extracts against AGEs induced neurotoxicity in SH-SY5Y cells

In order to measure the potential for *A. lividus* and *A. tricolor* extracts to prevent toxicity induced by AGEs. The protective effect of herbal extracts on AGEs induced cell death was estimated by pretreatment of SH-SY5Ycells with *A. lividus* and *A. tricolor* extracts and then exposed to the toxic concentration of AGEs. As shown on Figure 24, 25 and 26, alteration of cell morphology and occurrence of cell death, ability of cell proliferation and the release of LDH from damaged cells were evaluated in the cultures that were exposed to AGEs with increasing concentrations. In these experiments, we adopted a protocol using a dosage of AGEs of 4 mg/ml which were shown to cause approximately 50 % increasing of LDH release (Figure 24), approximately 55 % reduction of cell survival staining (Figure 25) and approximately 55 % reduction of ability of cell proliferation (Figure 26) by 48 h post-AGEs incubation in SH-SY5Y cells. Based on antioxidant activity and cytotoxicity test, we chose *A. lividus* extracts with petroleum ether and methanol and *A. tricolor* extracts with petroleum ether, dichloromethane, and methanol at concentration high doses up to 100 µg/ml and *A. lividus* extract with dichloromethane up to 50 µg/ml. As shown in Figure 35, 36 and 37, pretreated the cells with DMSO were not effect on prevention of cell death induced by AGEs examined using LDH assay, trypan blue exclusion assay and MTS assay, respectively. Under this condition, the result showed that *A. lividus* and *A. tricolor* extracts were capable in a dose dependent manner to attenuate the neuron cell toxicity caused by AGEs treatment. In LDH assay, treatment with AGEs resulted in a 50% increase in LDH above control levels (Figure 35). Whereas pretreatment with of 3.125 to 100 µg/ml petroleum ether, 1.56 to 50 µg/ml dichloromethane extract and 1.56 to 100 µg/ml of *A. lividus* methanol extracts (Figure 35A) and all solvents of *A. tricolor* extracts (Figure 35B) resulted in a significant decrease in LDH release compared to AGEs alone. We further assessed the protection against AGEs toxicity provided by *A. lividus* and *A. tricolor* in SH-SY5Y cells by trypan blue exclusion assay. The presence of dead cells in the cells treated with 4 mg/ml of AGEs was approximate 45% and remaining 55% in live cells as shown in Figure 36. When the cells were pretreated with 3.125 to 50 µg/ml dichloromethane fraction, 6.25 to 100 µg/ml of petroleum ether and methanol extracts of *A. lividus* were sufficient to significant increase cell survival (Figure 36A), while all solvents of *A. tricolor* at concentration ranging from 3.125 to 100 µg/ml showed a significant increase in cell viability (Figure 36B). The protective effect of herbal extracts determined by MTS assay also gave similar results, although

the exception as detailed below. The MTS assay showed that the cells treated 48 h with AGEs (4 mg/ml) resulted in an approximate 55% reduction in cell viability (Figure 37). When the cells were pretreated with 3.125 to 100 µg/ml petroleum ether, 3.125 to 50 µg/ml dichloromethane fractions and 6.25 to 100 µg/ml methanol extracts of *A. lividus*, there were a significant increase in cell viability (Figure 37A). The pretreatment of 6.25 to 100 µg/ml petroleum ether fraction of *A. tricolor* and dichloromethane and methanol extracts of *A. tricolor* concentration ranging from 3.125 to 100 µg/ml were shown a significant protection the cells from AGEs-induced cell toxicity (Figure 37B). According to the *in vitro* biological tests of protection against AGEs by herbal extracts revealed that *A. lividus* and *A. tricolor* methanol extract exhibited the best of all the extracts in providing protection from AGEs-induced cell death in SH-SY5Y cells. In addition, the best concentration of these extracts that provided the maximum cellular viability under AGEs-induced cytoxicity was observed at 100 µg/ml. Under this condition, the cell viability was returned to control level.

A

A. lividus extract (µg/ml)

B

A. tricolor extract (µg/ml)
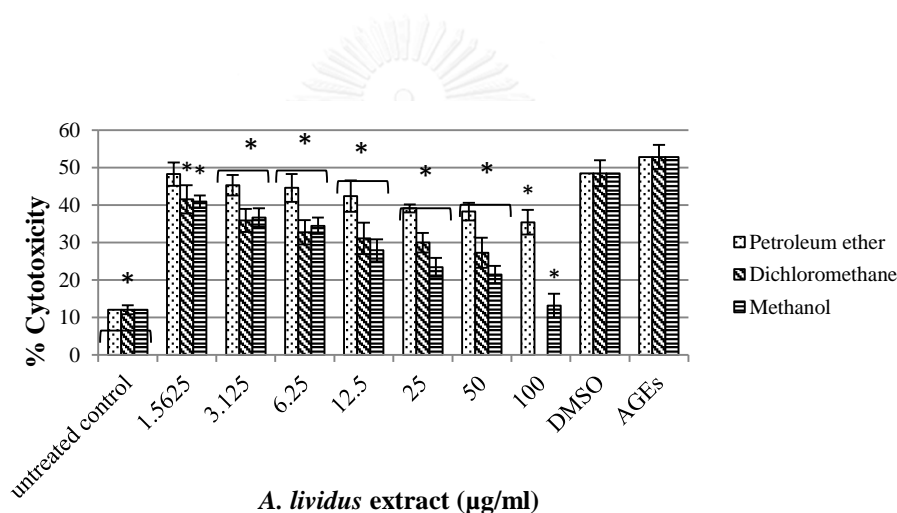
Figure 35. Protective effect of herbal extracts on AGEs-cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were preincubated with different concentrations of three different solvents of (A) *A. lividus*; (B) *A. tricolor*. SH-SY5Y cells were further incubated with 4 mg/ml AGEs for 48 h. The release of LDH of damaging SH-SY5Y cells was assessed using the LDH assay. The results were represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with AGEs treated cells.

A

B

Figure 36. Protective effect of herbal extracts on AGEs-cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were preincubated with different concentrations of three different solvents of (A) *A. lividus*; (B) *A. tricolor*. SH-SY5Y cells were further incubated with 4 mg/ml AGEs for 48 h. Cell survival of living SH-SY5Y cells was assessed using trypan blue exclusion assay. The results were represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with AGEs treated cells.
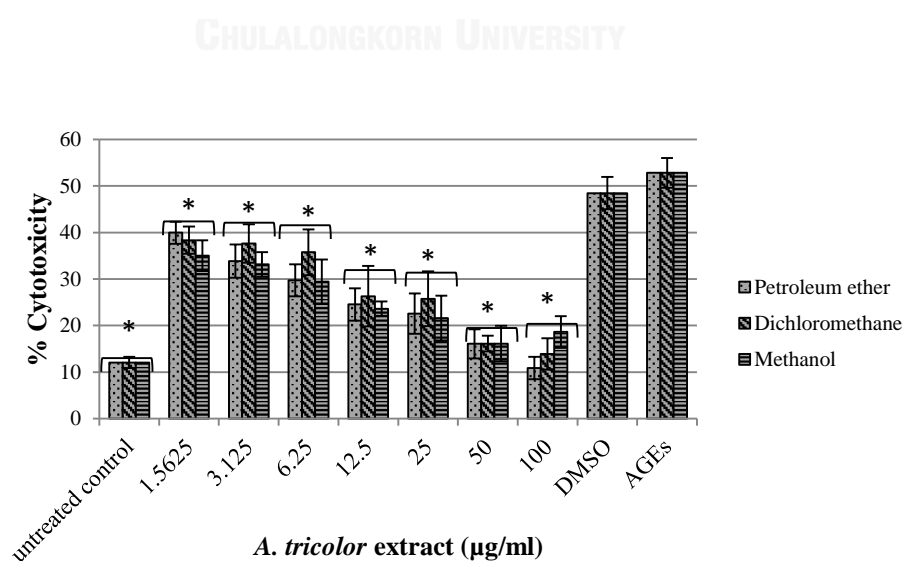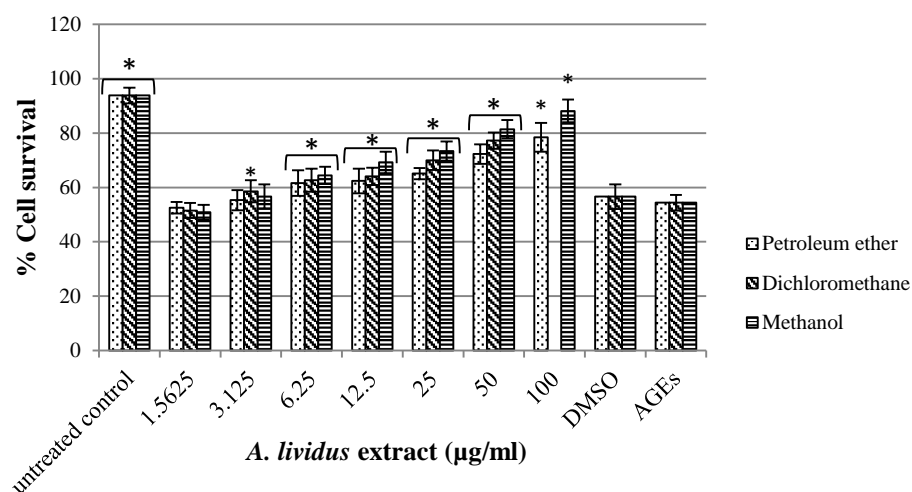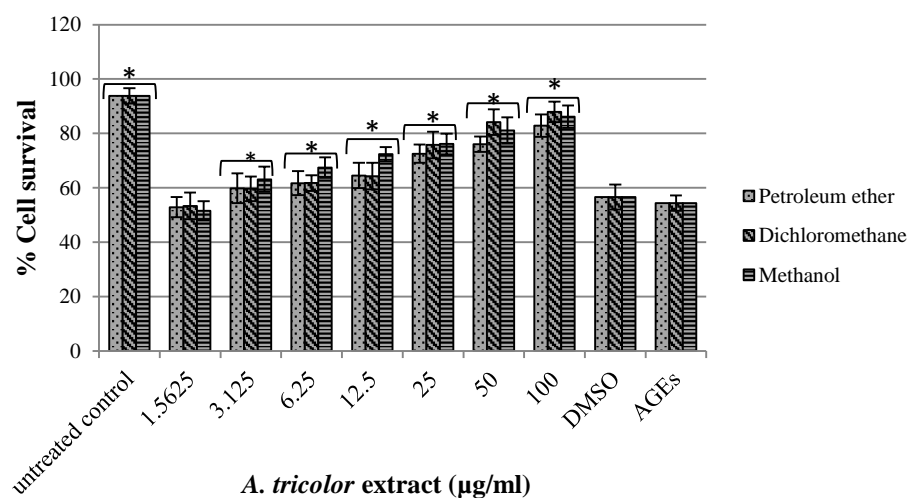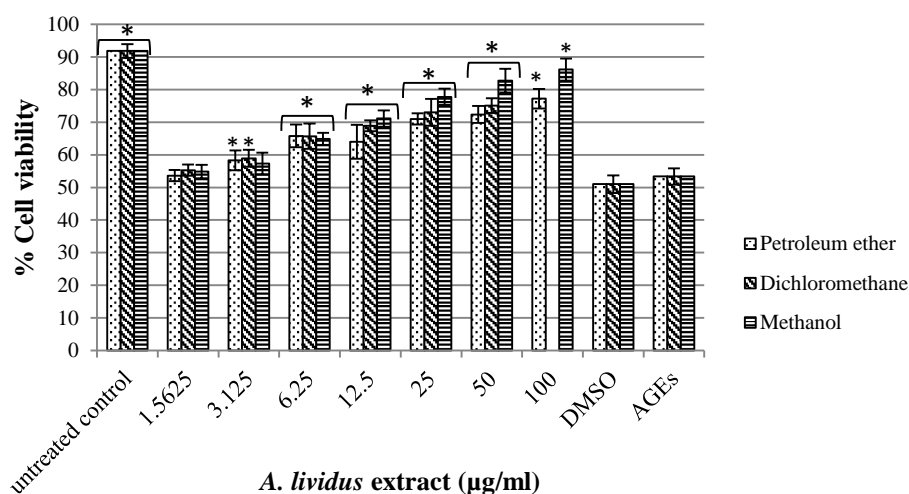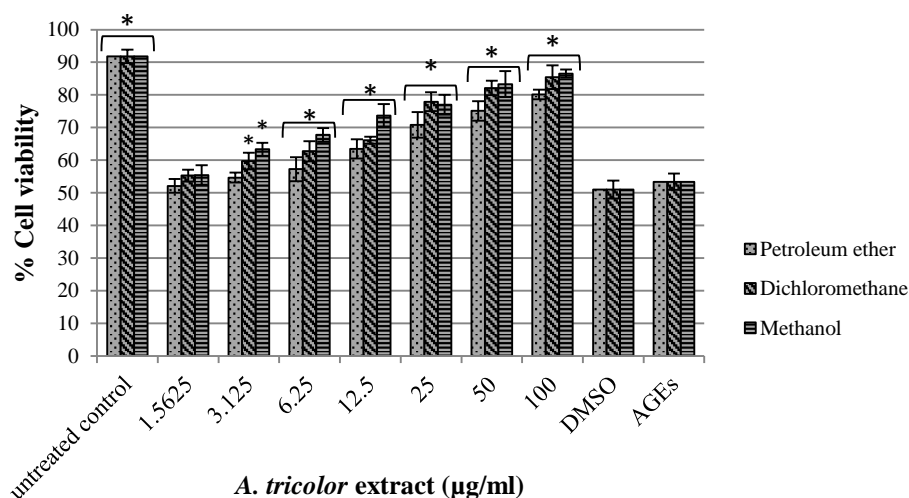
A

B

Figure 37. Protective effect of herbal extracts on AGEs-cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were preincubated with different concentrations of three different solvents of (A) *A. lividus*; (B) *A. tricolor*. SH-SY5Y cells were further incubated with 4 mg/ml AGEs for 48 h. Cellular viability of living SH-SY5Y cells was assessed using MTS assay. The results were represented as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with AGEs treated cells.

3.5 Gene expression in SH-SY5Y in response to AGEs and herbal extracts exposure

We first sought to assess the effects of AGEs in SH-SY5Y cells. The above data revealed that AGEs could induce oxidative damage. We therefore attempted to determine the potential of AGEs on the relative expression of oxidative stress, AGEs receptor (RAGE), secretases enzyme for Aβ formation, inflammation and redox-sensitive transcription factors using qPCR in SH-SY5Y cells. Non-toxic concentrations of 0.5, 1, 2, 3 and 4 mg/ml AGEs or BSA control were incubated with the cells and genes expression were analyzed after 24 h. Exposure of SH-SY5Y cells to AGEs results in alteration genes expression over time as presented in Table 23 and Figure 38. The relative fold change of each gene was listed. AGEs and BSA showed different gene expression profiles. The reports suggested that mRNA levels of all interested genes were higher upon incubation with AGEs. Transcript of HMOX-1, RAGE, BACE1, PS1, RelA, NF-kB1, NF-κB2, TNF-α, IL-1 and IL-6 but not ADAM10 were upregulated only in AGEs-induced cells. The gene expression level of HMOX-1, an indicator of cellular oxidative stress, was found to be increased in oxidative stress condition in a dose-dependent manner upon incubation with AGEs (Figure 38A). The roles of AGEs and its receptor, RAGE and its signaling pathway have resulted in increased scientific focus. The our present study demonstrated that exposure of SH-SY5Y cells with AGEs in the concentration of 0.5 to 4 mg/ml showed a significant increase of RAGE gene expression (Figure 38B) as well as a significant increase in NF-κB signaling genes including RelA (Figure 38C), NF-κB1 (Figure 38D) and NF-κB2 (Figure 38E) expression levels in a dose dependent manner. The expressions of BACE1, ADAM10 and PS1, secretases involved in APP processing, were also analyzed in SH-SY5Y cells treatment with AGEs. Our data also demonstrated a dose–dependent relation between mRNA levels of BACE1 and PS1 shown in Figure 38F and 38G, respectively and AGEs concentrations. On the other hand, ADAM10 expression was unchanged when incubated with various concentrations of AGEs except for incubation with 4 mg/ml AGEs (Figure 38H). In addition, there has recently been reported that AGEs can induce oxidative stress which can induce cell damage and promote neuroinflammation. Exposure of SH-SY5Y cells to AGEs for 24 h significantly induced TNF-α (Figure 38I), IL-6 (Figure 38J) and IL-1 (Figure 38K) genes expression in a dose dependent manner.

To determine whether the herbal extract can alter the cell physiology and function was correlated to mRNA levels in SH-SY5Y cells, the effects of herbal extracts on the relative expression of HMOX-1, RAGE, BACE1, PS1, ADAM10, RelA, NF-kB1, NF-κB2, TNF-α, IL-1 and IL-6 were also examined using qPCR. Based on

the protective effects of herbal extracts against AGEs induced oxidative stress and neurotoxicity data, for these experiments we chose to investigate the protective potential of 100 μg/ml *A. lividus* petroleum ether, *A. lividus* methanol, *A. tricolor* petroleum ether, *A. tricolor* dichloromethane, *A. tricolor* methanol and 50 μg/ml *A. lividus* dichloromethane. In these experiments we adopted a protocol using the lowest (0.5 mg/ml) and highest (4 mg/ml) concentrations of AGEs that shown to cause significant increasing of the interested genes as shown above. The herbal extracts were pretreated with SH-SY5Y cells then cells were further incubated with 0.5 or 4 mg/ml AGEs and mRNA were analyzed. Moreover, the final content of DMSO in the cultural medium with the highest concentration of the extract, 0.01%, was also tested in SH-SY5Y cells. As shown in Figure 39 to 49, pretreated the cells with DMSO were revealed insignificant modulate the interest genes. We found that HMOX-1 gene expression in SH-SY5Y cells which were preincubated with *A. lividus* with petroleum ether, dichloromethane and methanol fraction, and *A. tricolor* with petroleum ether and methanol extracts were significantly decreased when compared with the oxidative stress condition induced by 0.5 mg/ml AGEs as shown in Figure 39. Figure 40 presented that pretreatment with all extracts of *A. lividus* and *A. tricolor* were significantly decreased RAGE mRNA level when compared with the oxidative stress condition induced by 0.5 and 4 mg/ml AGEs. According to the upregulation of RAGE is followed by the activation of the NF-κB pathway, genes expression of RelA, NF-κB1, and NF-κB2, signaling molecules involved in NF-κB pathway, were measured. Preincubated the SH-SY5Y cells with all solvents extracted of *A. lividus* and *A. tricolor* except for petroleum ether *A. tricolor* fraction showed significantly reduced the expression of the RelA gene in the incubated with 0.5 mg/ml AGEs (Figure 41). Whereas NF-κB1 gene expression were significantly decreased in the 0.5 mg/ml AGEs induced cells which were pretreated with all extracts of *A. lividus* and *A. tricolor* except for dichloromethane *A. tricolor* extract (Figure 42). Moreover, preincubation with *A. lividus* and *A. tricolor* petroleum ether extracts and methanol extract of *A. lividus* were significantly decreased NF-κB2 gene expression compared with the control cells that had been exposed to 0.5 mg/ml AGEs as shown in Figure 43. The mechanisms underlying the oxidative stress induced increasing of Aβ generation are unclear at present. In our present experiment, the expressions of BACE1, ADAM10 and PS1 genes in SH-SY5Y cells treatment with AGEs were analyzed as described above. As shown in Figure 44, the pretreatment of herbal extracts showed a significant decrease in BACE1 gene expression with *A. lividus* petroleum ether, dichloromethane and methanol extracts and *A. tricolor* extract using petroleum ether in the cells incubated with 0.5 mg/ml AGEs. In addition, pretreatment with *A. tricolor* petroleum ether fraction as well as dichloromethane and methanol extracts of *A. lividus* also down-regulated BACE1 gene expression in the cells exposed to 4 mg/ml AGEs. We found that PS1 gene expression in the cells which were preincubated with the both plant extracts of all solvents produced a significantly decreasing when compared with 0.5 mg/ml AGEs exposed cells as shown in Figure 45. While, the expression of ADAM10 gene was not found a significantly changing in the herbal extracts treated cells (Figure 46). For neuroinflammation, as we previously presented that exposure of SH-SY5Y cells up from 0.5 mg/ml AGEs significantly induced TNF-α, IL-6 and IL-1 genes expression as shown in Figure 38. After preincubating the SH-SY5Y cells with *A. lividus* and *A. tricolor* petroleum ether and methanol extracts, we found that TNF-α

gene expression significantly decreased compared with the cells exposed to 0.5 mg/ml AGEs as shown in Figure 47. In addition, pretreatment with *A. lividus* methanol extract and *A. tricolor* petroleum ether extract were also significantly down-regulated TNF-α gene expression in 4 mg/ml AGEs induced cells. Regarding the effect of both plants of petroleum ether and dichloromethane extracts, significantly reduced the expression of the IL-6 gene in SH-SY5Y cells exposed to 0.5 mg/ml AGEs (Figure 48). The suppression of IL-1 gene expression was found after the 0.5 mg/ml AGEs exposed cells were preincubated with *A. tricolor* petroleum ether extract (Figure 49).
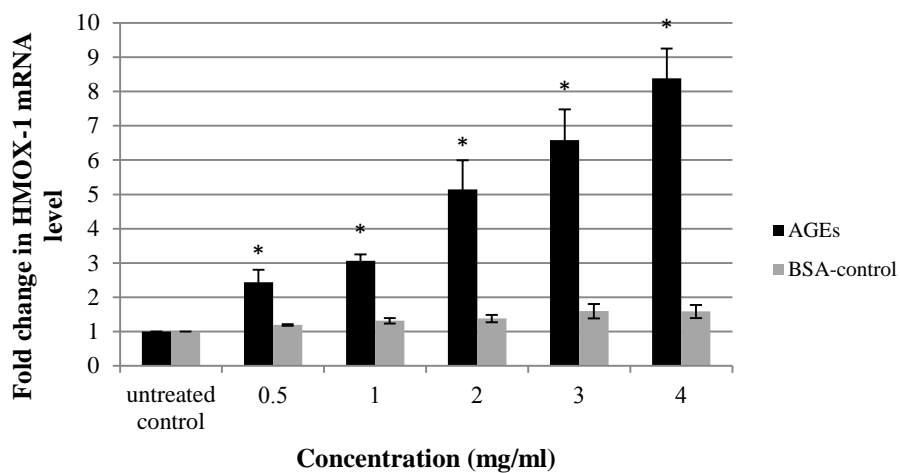
Table 23. Effect of AGEs on the expression of interested genes at 24 h in SH-SY5Y cells treatment using qPCR.

HMOX-1	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	2.44 ± 0.36	3.06 ± 0.19	5.15 ± 0.85	6.58 ± 0.90	8.39 ± 0.87
BSA control	1	1.19 ± 0.03	1.31 ± 0.08	1.38 ± 0.11	1.59 ± 0.21	1.58 ± 0.19
RAGE	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	2.11 ± 0.07	4.43 ± 0.09	5.97 ± 0.91	7.48 ± 1.03	8.15 ± 0.74
BSA control	1	1.14 ± 0.13	1.04 ± 0.07	1.03 ± 0.71	1.01 ± 0.04	1.06 ± 0.10
RelA	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	2.64 ± 0.26	5.75 ± 0.81	6.13 ± 0.50	$7.68 + 0.24$	11.11 ± 0.97
BSA control	1	1.02 ± 0.09	1.17 ± 0.21	1.10 ± 0.04	1.35 ± 0.35	1.24 ± 0.65
NF-κB1	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	2.64 ± 0.27	5.80 ± 0.70	7.94 ± 0.53	10.29 ± 0.36	14.06 ± 0.49
BSA control	1	1.18 ± 0.09	1.17 ± 0.22	1.11 ± 0.02	1.03 ± 0.12	1.08 ± 0.21
NF-κB2	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	1.76 ± 0.21	2.80 ± 0.55	4.69 ± 0.47	5.19 ± 0.82	7.90 ± 1.02
BSA control	1	1.10 ± 0.06	1.17 ± 0.19	1.31 ± 0.12	1.35 ± 0.07	1.53 ± 0.13
BACE1	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	1.72 ± 0.15	3.48 ± 0.32	5.28 ± 0.20	6.92 ± 0.70	6.93 ± 0.07
BSA control	1	1.13 ± 0.12	1.15 ± 0.21	1.16 ± 0.27	1.08 ± 0.06	1.24 ± 0.29
PS1	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	1.67 ± 0.34	2.45 ± 0.18	3.46 ± 0.36	5.43 ± 0.67	5.70 ± 1.11
BSA control	1	0.98 ± 0.22	$1.31 + 0.42$	1.06 ± 0.22	1.00 ± 0.17	1.13 ± 0.04

ADAM10	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	1.06 ± 0.13	1.14 ± 0.07	1.05 ± 0.08	1.13 ± 0.06	1.35 ± 0.04
BSA control	1	1.10 ± 0.03	1.01 ± 0.08	1.21 ± 0.21	1.15 ± 0.11	1.05 ± 0.17
TNF-α	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	1.67 ± 0.21	3.19 ± 0.39	4.27 ± 0.73	4.87 ± 0.11	7.50 ± 0.30
BSA control	1	1.01 ± 0.01	1.01 ± 0.01	1.01 ± 0.01	1.01 ± 0.01	1.01 ± 0.01
IL-6	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	2.16 ± 0.14	4.00 ± 0.73	7.85 ± 1.33	12.26 ± 1.00	16.63 ± 1.28
BSA control	1	1.03 ± 0.02	1.02 ± 0.01	1.03 ± 0.02	1.03 ± 0.02	1.03 ± 0.03
IL-1	Relative changing gene expression (fold)					
Concentration (mg/ml)	0	0.5	1	2	3	4
AGEs	1	5.58 ± 0.48	9.26 ± 0.34	11.48 ± 1.04	36.95 ± 1.42	48.97 ± 4.26
BSA control	1	1.76 ± 0.90	1.60 ± 0.66	1.80 ± 0.86	1.87 ± 1.12	2.04 ± 1.41

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A

B
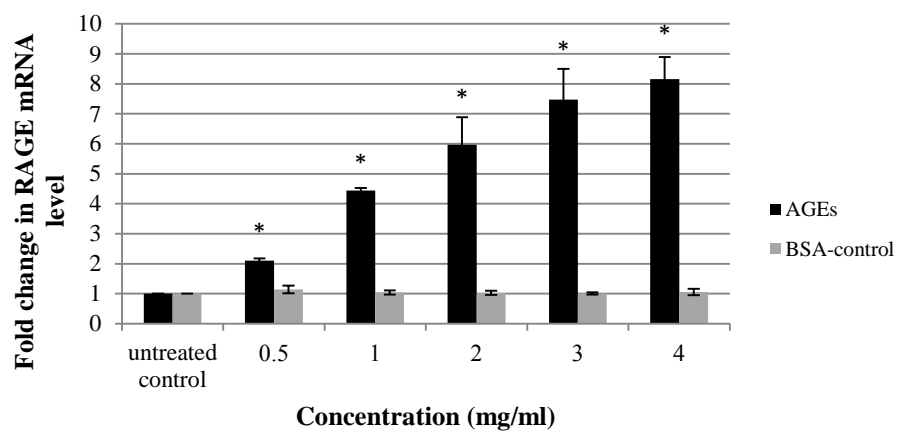
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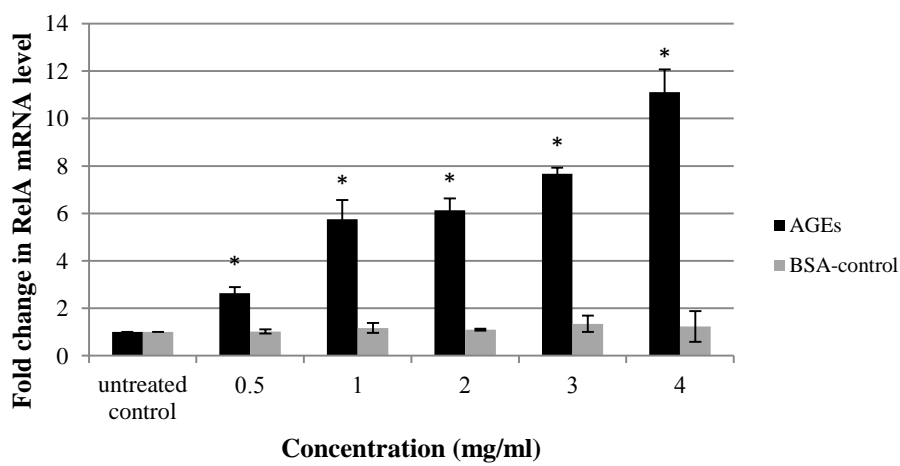
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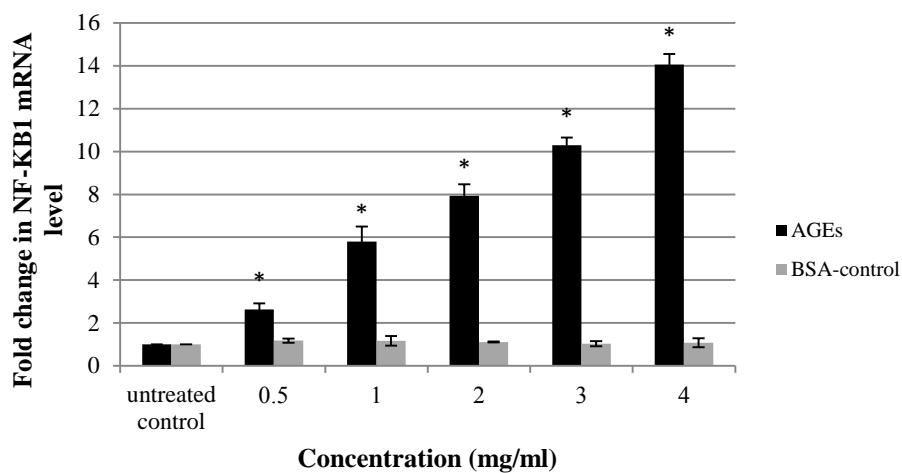
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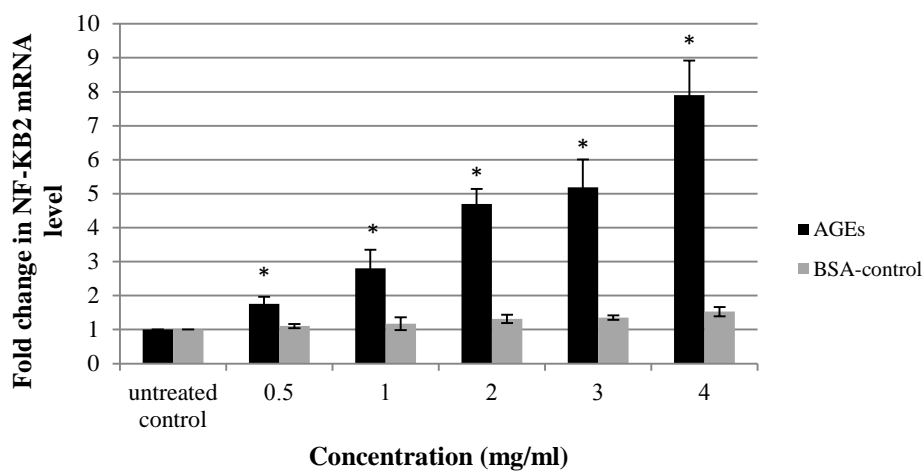
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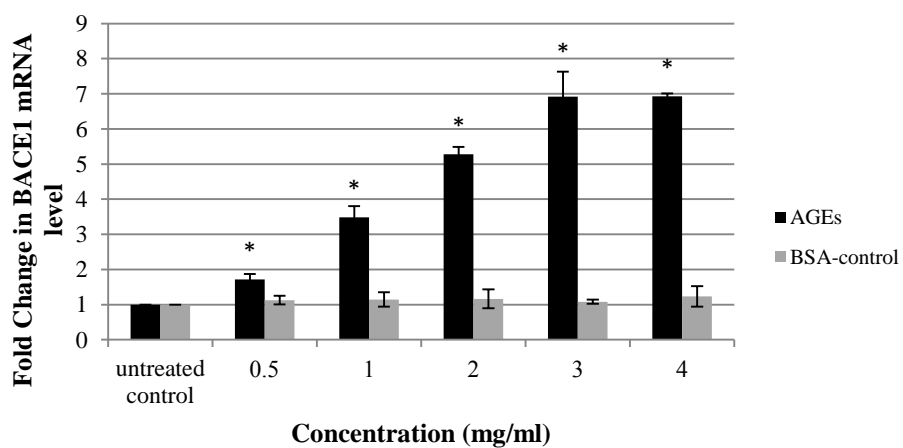
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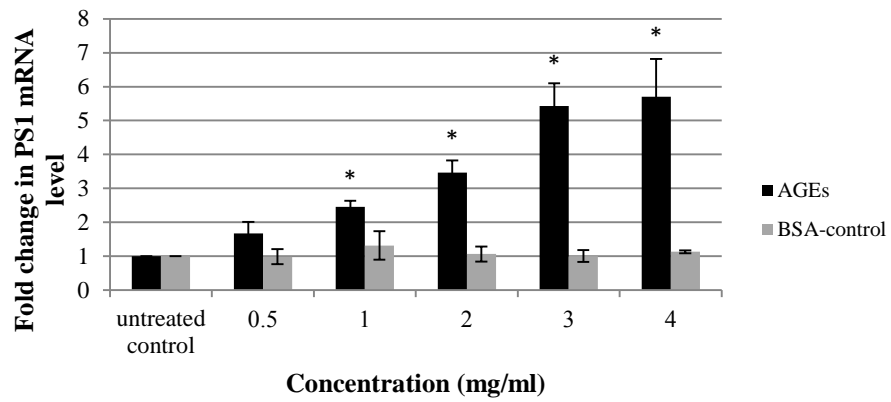
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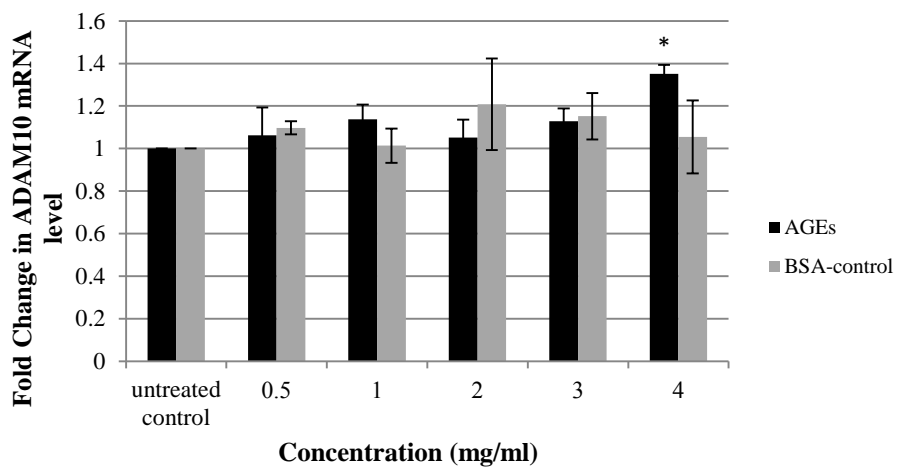
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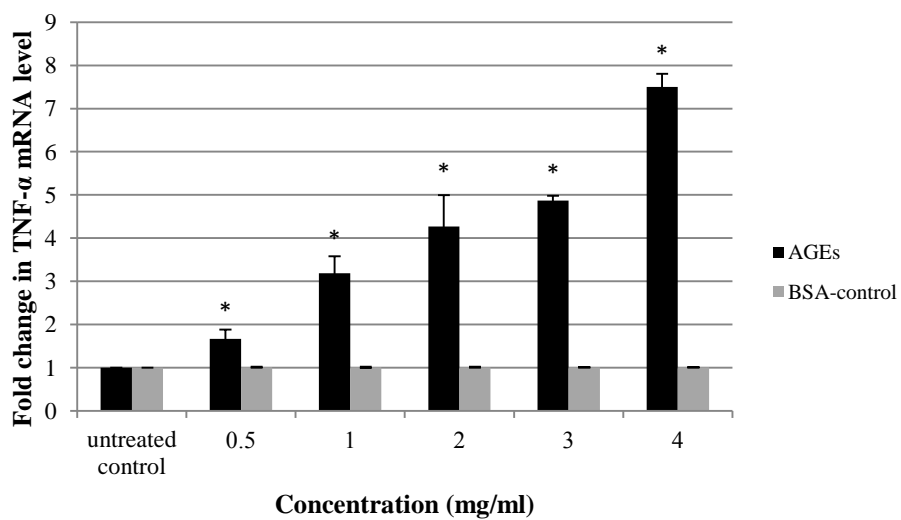
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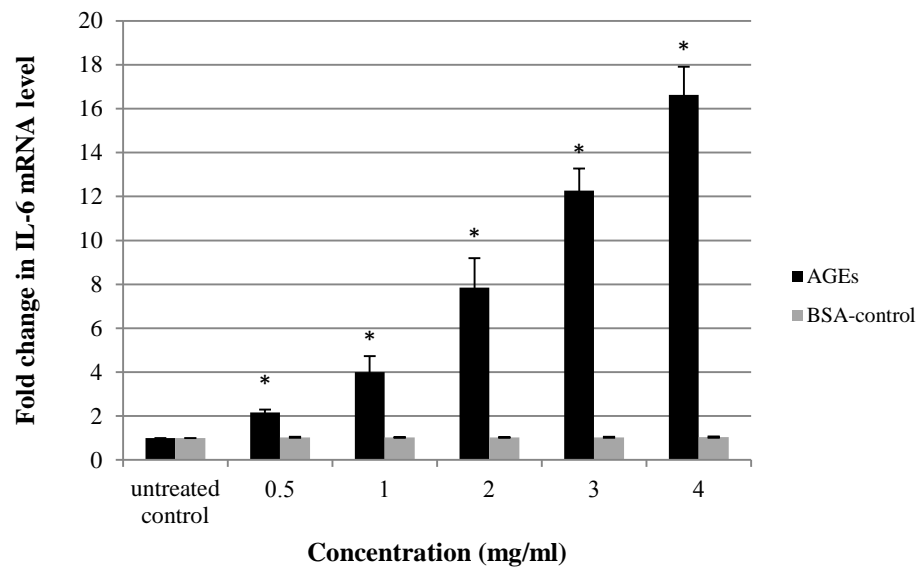
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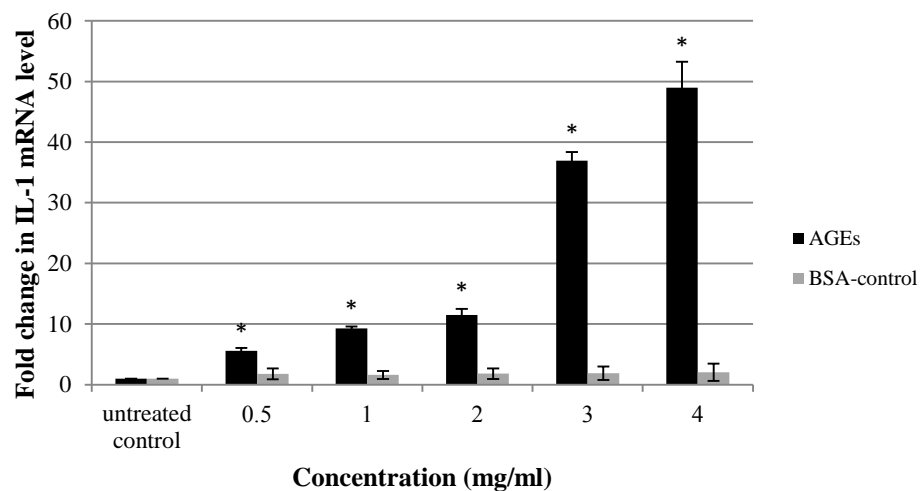
Figure 38. Effect of AGEs on the expression of interested genes in SH-SY5Y cells. SH-SY5Y cells were incubated with 0.5 to 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis; (A) HMOX-1; (B) RAGE; (C) RelA; (D) NF-κB1; (E) NF-κB2; (F) BACE1; (G) PS1; (H) ADAM10; (I) TNF-α; (J) IL-6 and (K) IL-1. Various concentrations of AGEs and BSA compared with untreated control. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by $*P < 0.05$ when compared with untreated cells.

A

A. lividus petroleum ether extract

B

A. lividus dichloromethane extract

C

A. lividus methanol extract

D

A. tricolor petroleum ether extract

E

A. tricolor dichloromethane extract

F
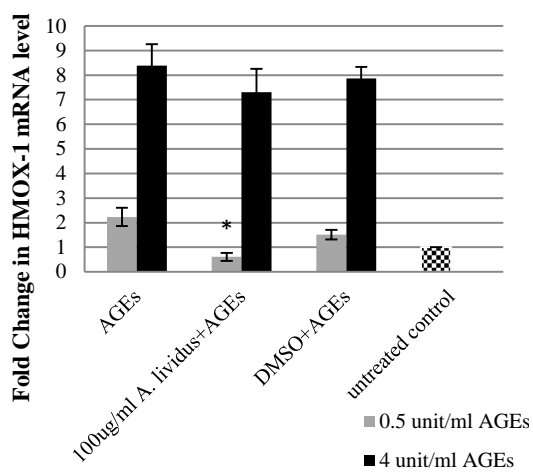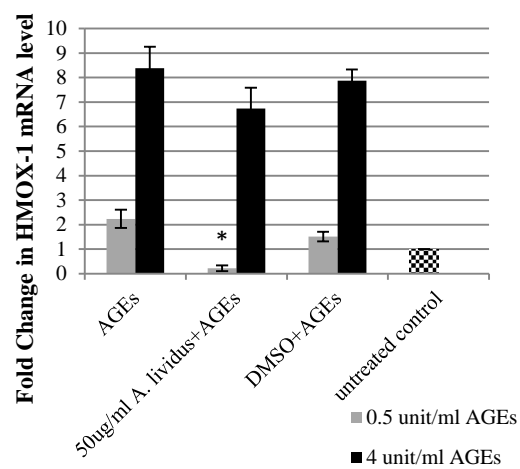
A. tricolor methanol extract

Figure 39. Effect of herbal extracts on HMOX-1 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.

A

A. lividus petroleum ether extract

B

A. lividus dichloromethane extract

132

Figure 40. Effect of herbal extracts on RAGE mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; **(E)** *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.

A

A. lividus petroleum ether extract

B

A. lividus dichloromethane extract

C

A. lividus methanol extract

D

A. tricolor petroleum ether extract

E

F

A. tricolor **dichloromethane extract**

A. tricolor **methanol extract**

Figure 41. Effect of herbal extracts on RelA mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.

A

B

A. lividus **petroleum ether extract**

A. lividus **dichloromethane extract**

Figure 42. Effect of herbal extracts on NF-κB1 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.
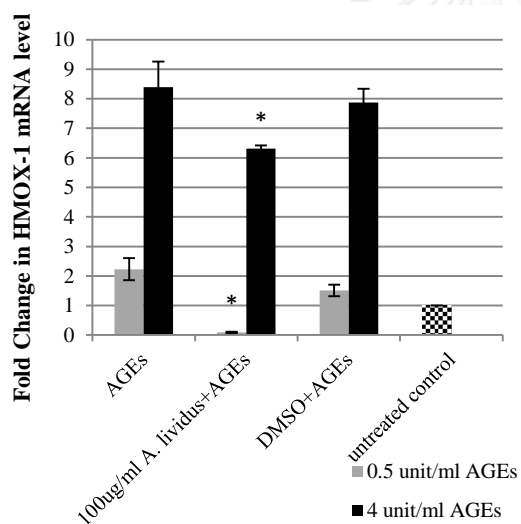
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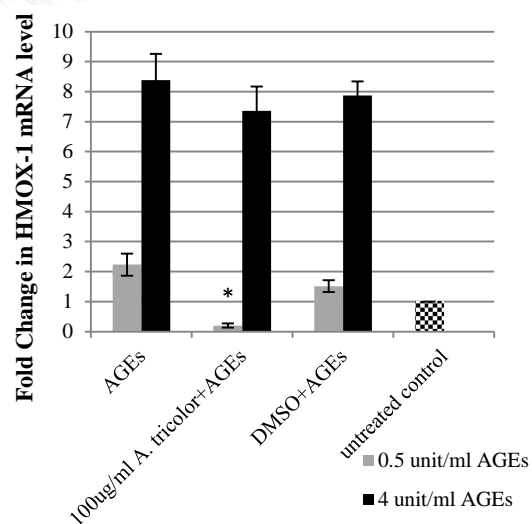**A. lividus petroleum ether extract**

B

A. lividus dichloromethane extract

C

A. lividus methanol extract
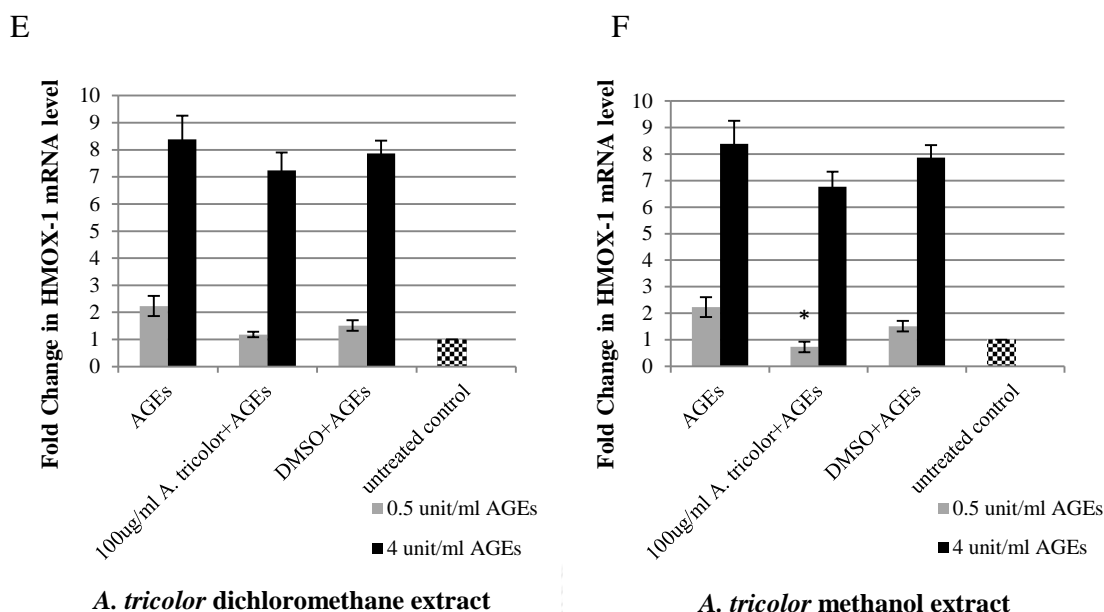
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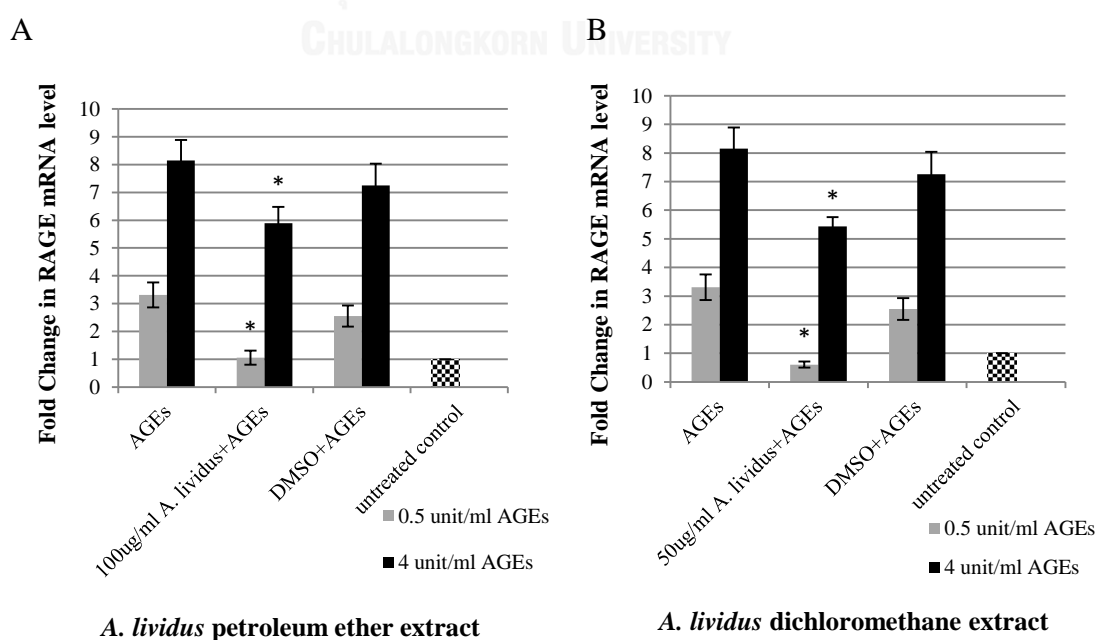**A. tricolor petroleum ether extract**

137

Figure 43. Effect of herbal extracts on NF-κB2 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.
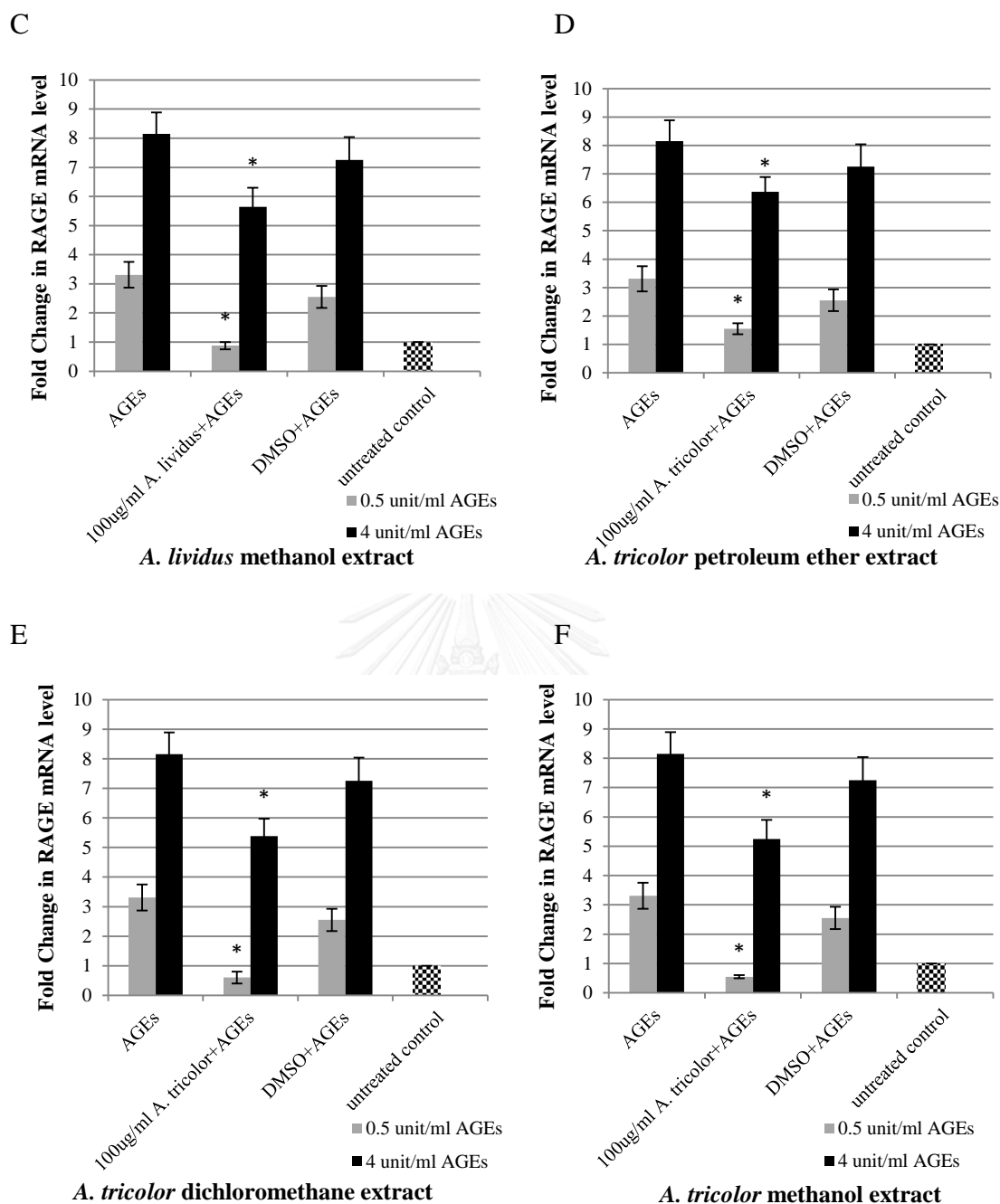
A

A. lividus petroleum ether extract

B

A. lividus dichloromethane extract

C

A. lividus methanol extract

D

A. tricolor petroleum ether extract

Figure 44. Effect of herbal extracts on BACE1 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.
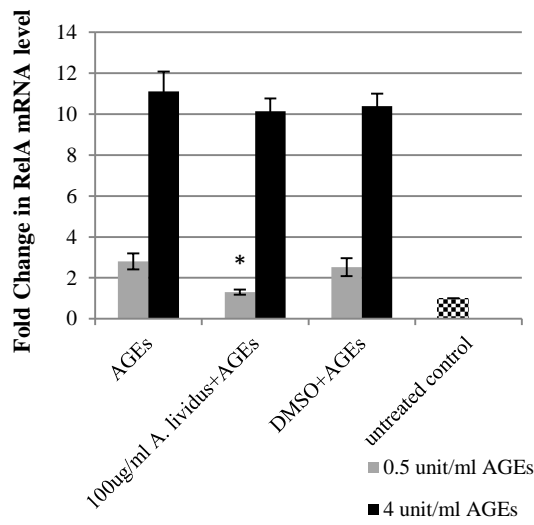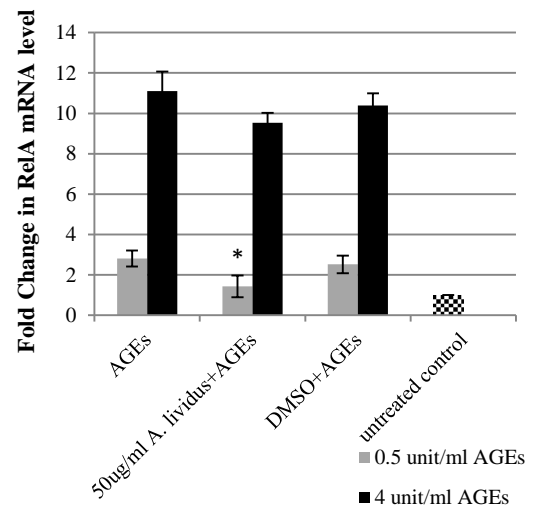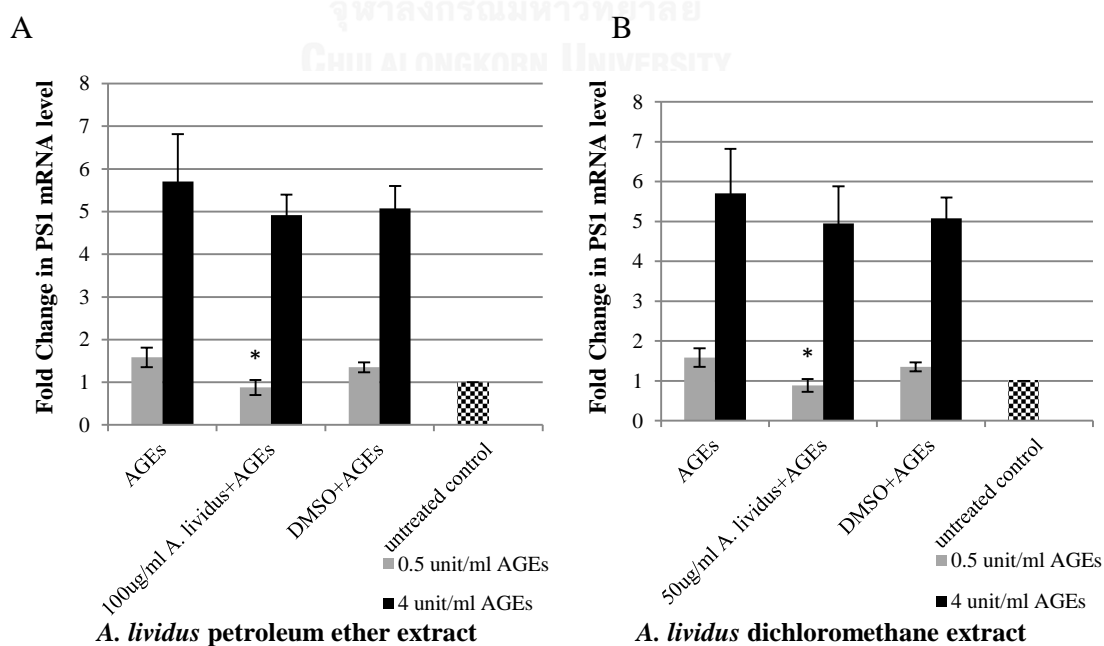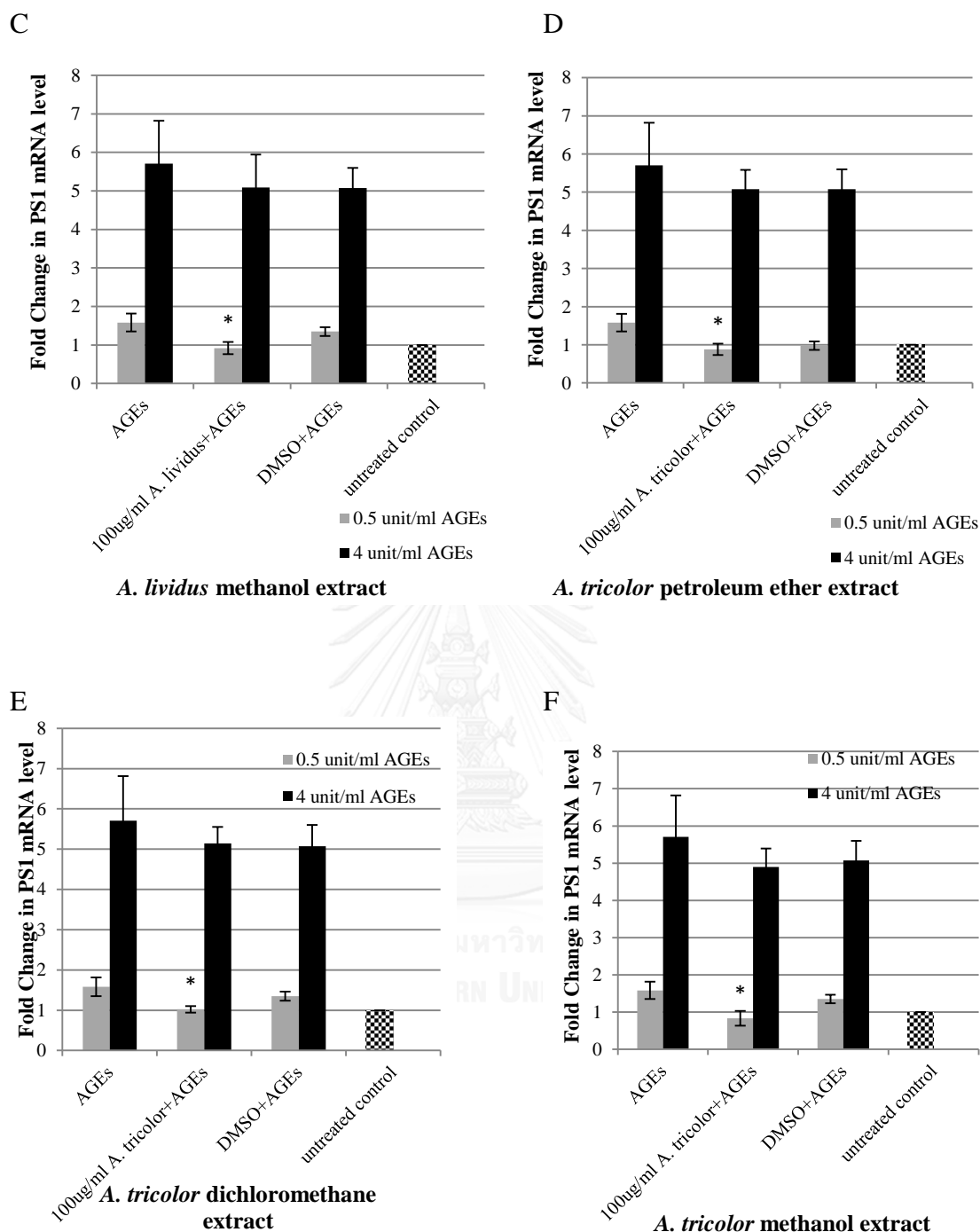
Figure 45. Effect of herbal extracts on PS1 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean

(SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.
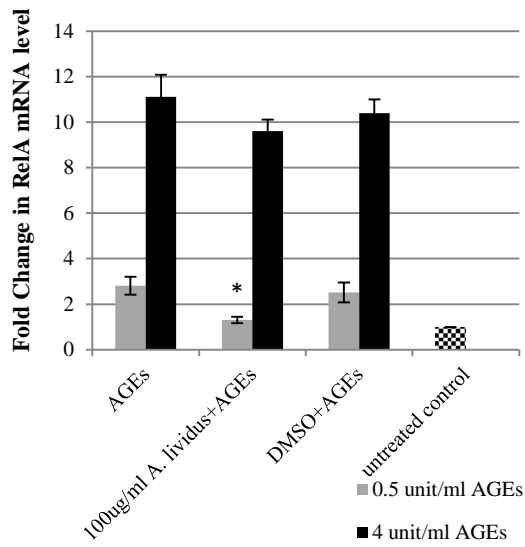

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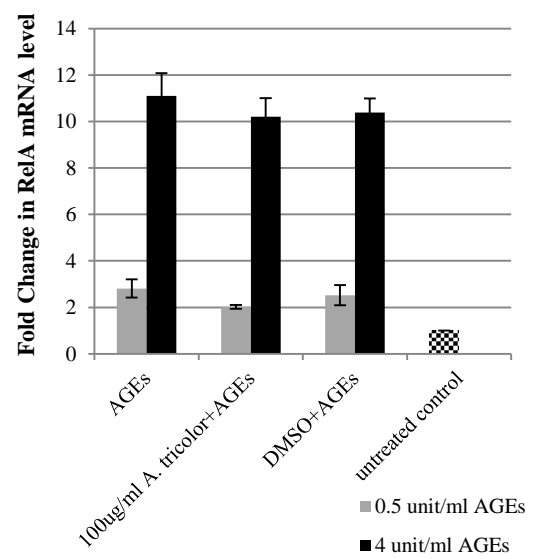*A. lividus* petroleum ether extract

B

A. lividus dichloromethane extract

C

A. lividus methanol extract

D

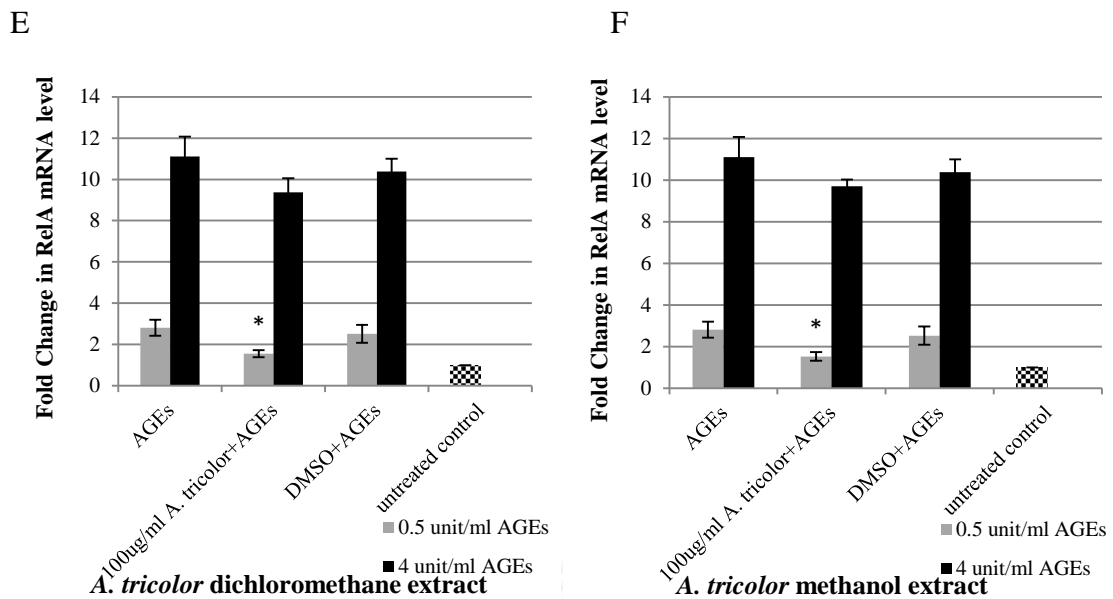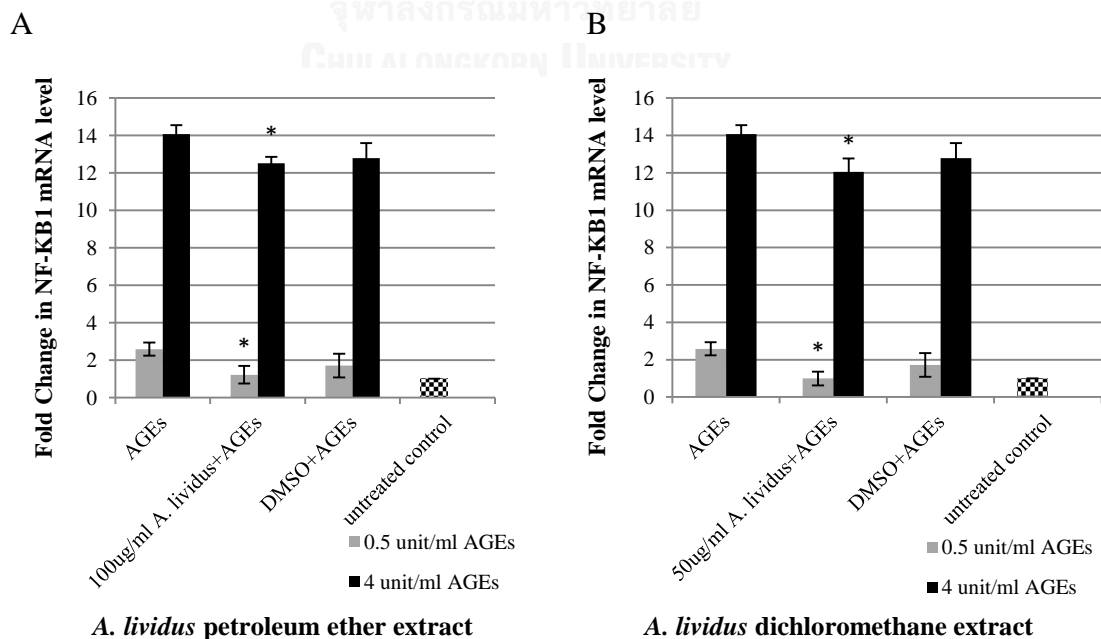*A. tricolor* petroleum ether extract

142

E
F
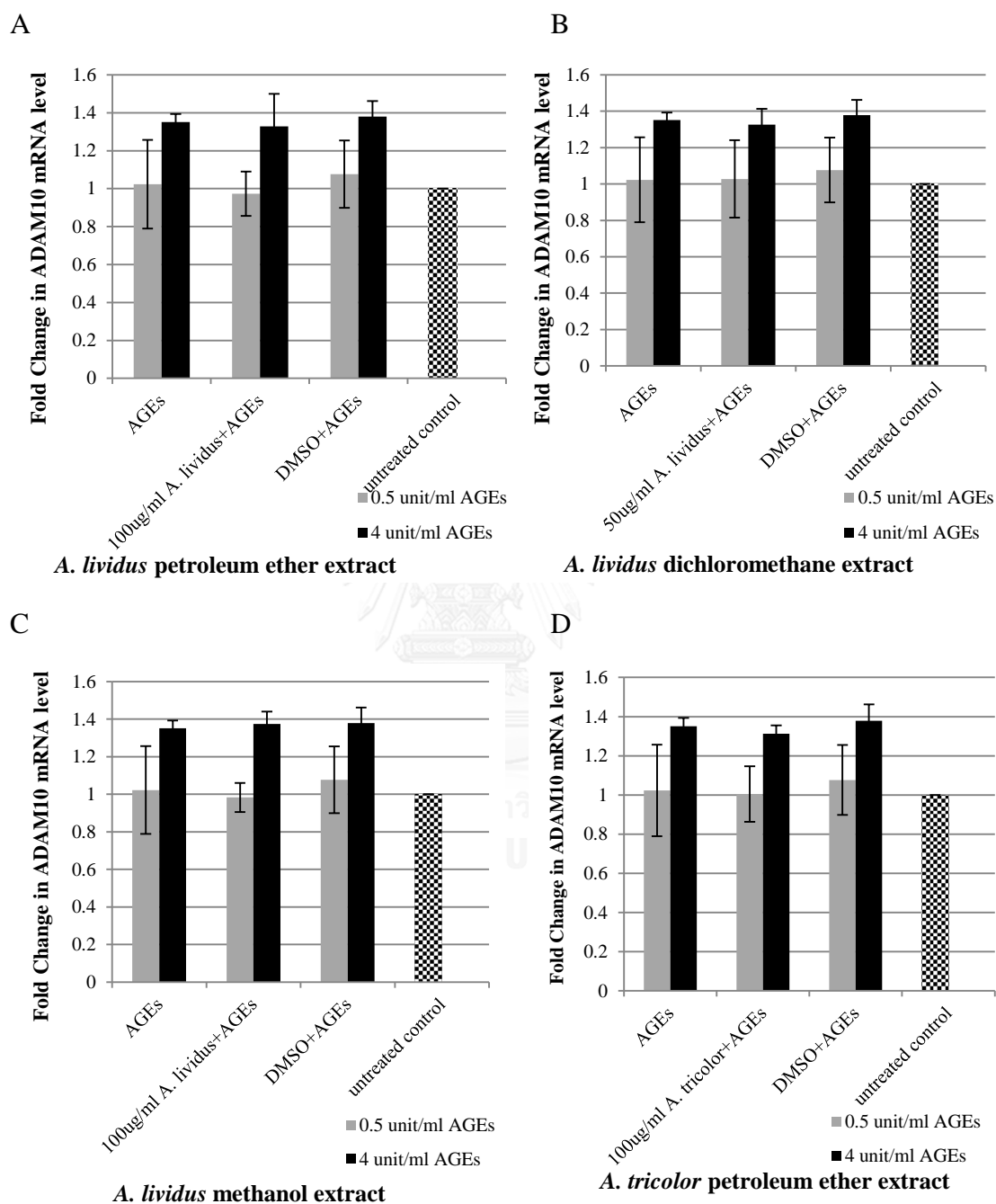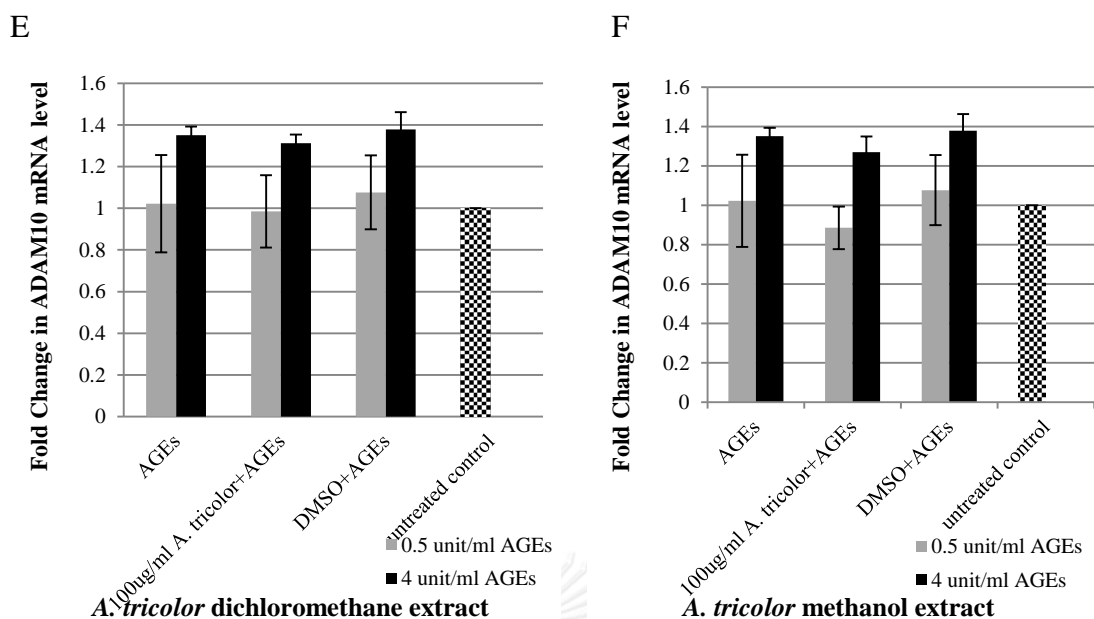
Figure 46. Effect of herbal extracts on ADAM10 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.

A
B
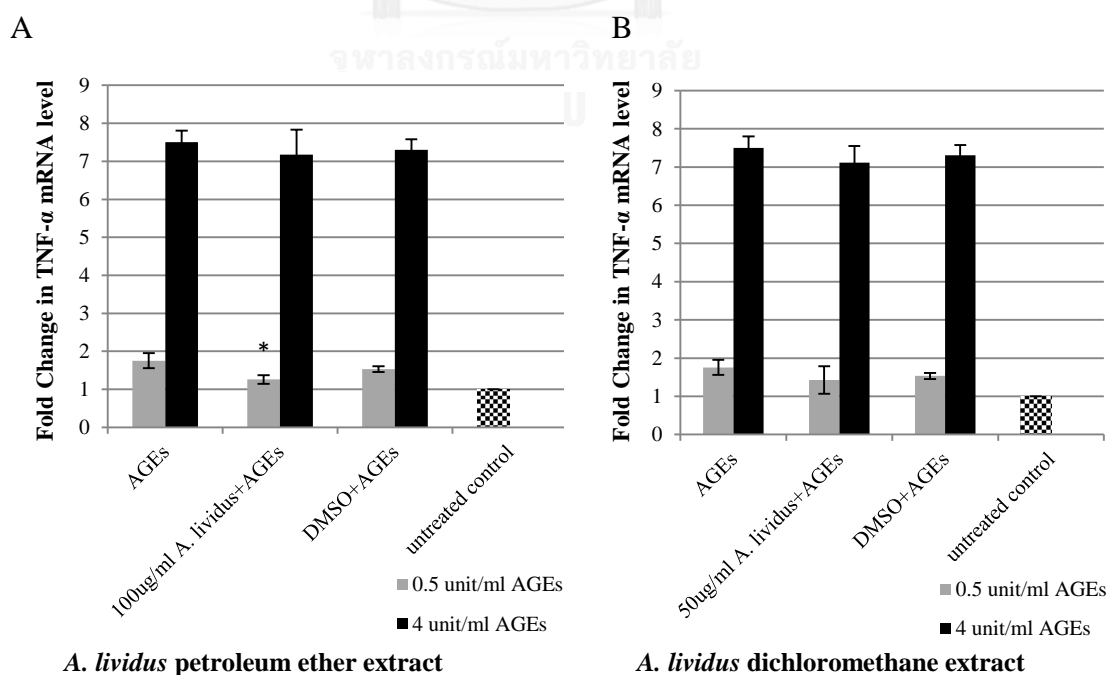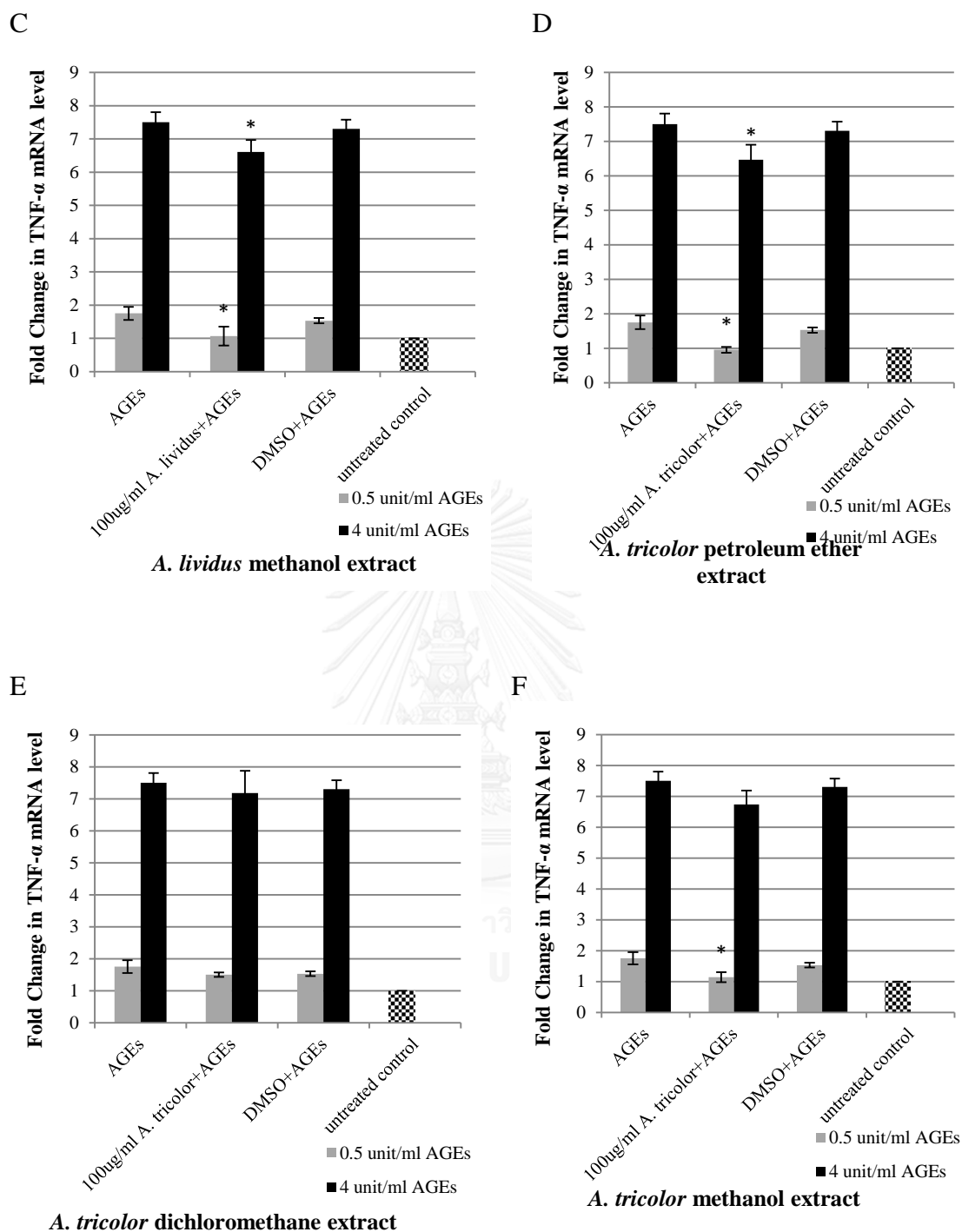
Figure 47. Effect of herbal extracts on TNF-α mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean

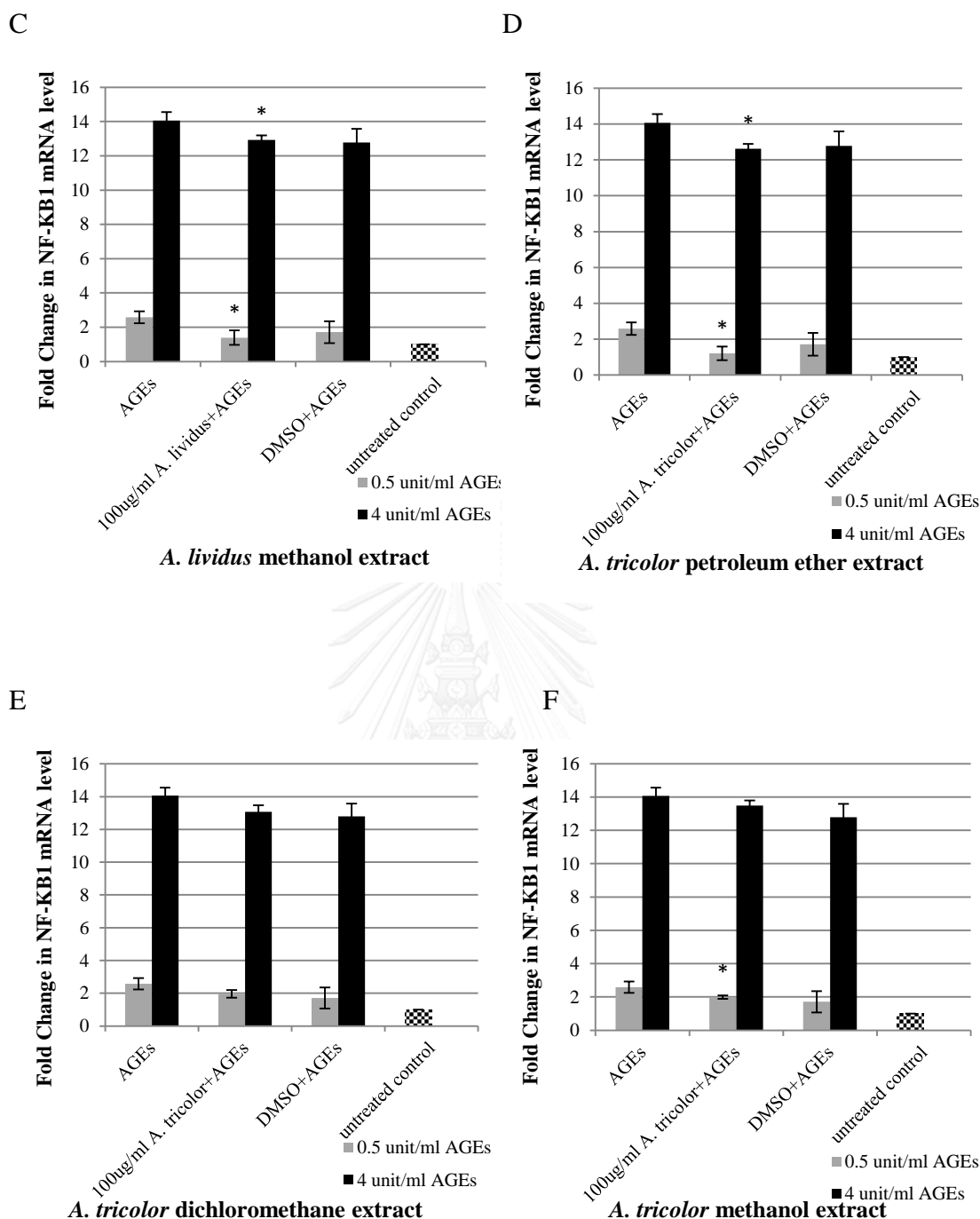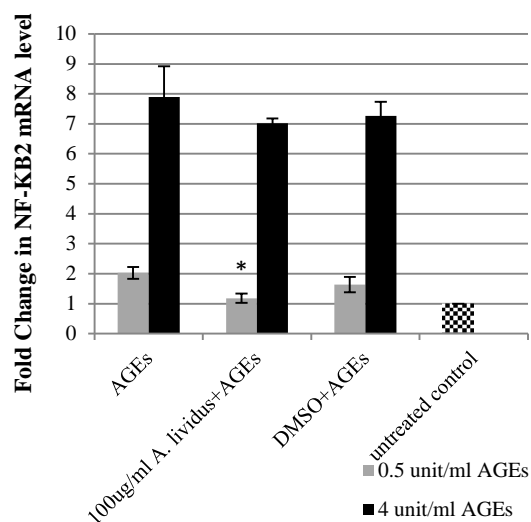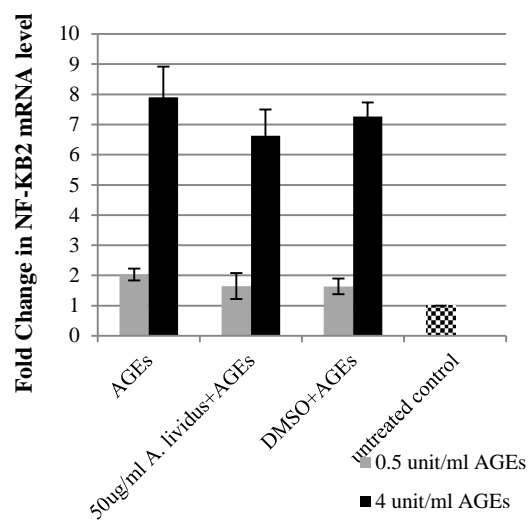(SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.

A

A. lividus petroleum ether extract

B

A. lividus dichloromethane extract

C

A. lividus methanol extract

D

A. tricolor petroleum ether extract

E

A. tricolor dichloromethane extract

F

A. tricolor methanol extract

Figure 48. Effect of herbal extracts on IL-6 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.
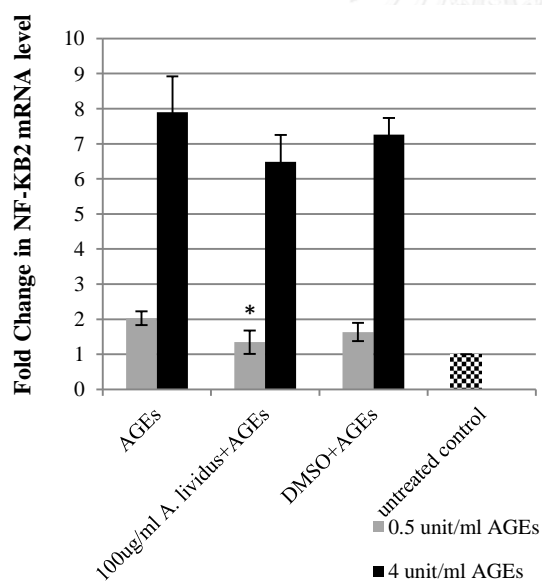
A

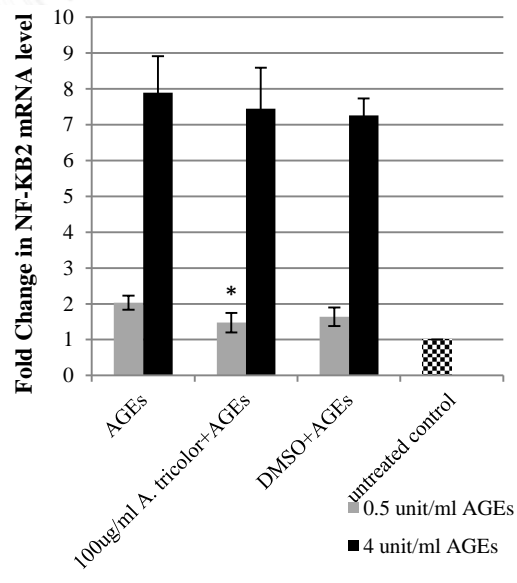**A. lividus petroleum ether extract**

B

A. lividus dichloromethane extract

C

A. lividus **methanol extract**

D

A. tricolor **petroleum ether extract**

E

A. tricolor **dichloromethane extract**
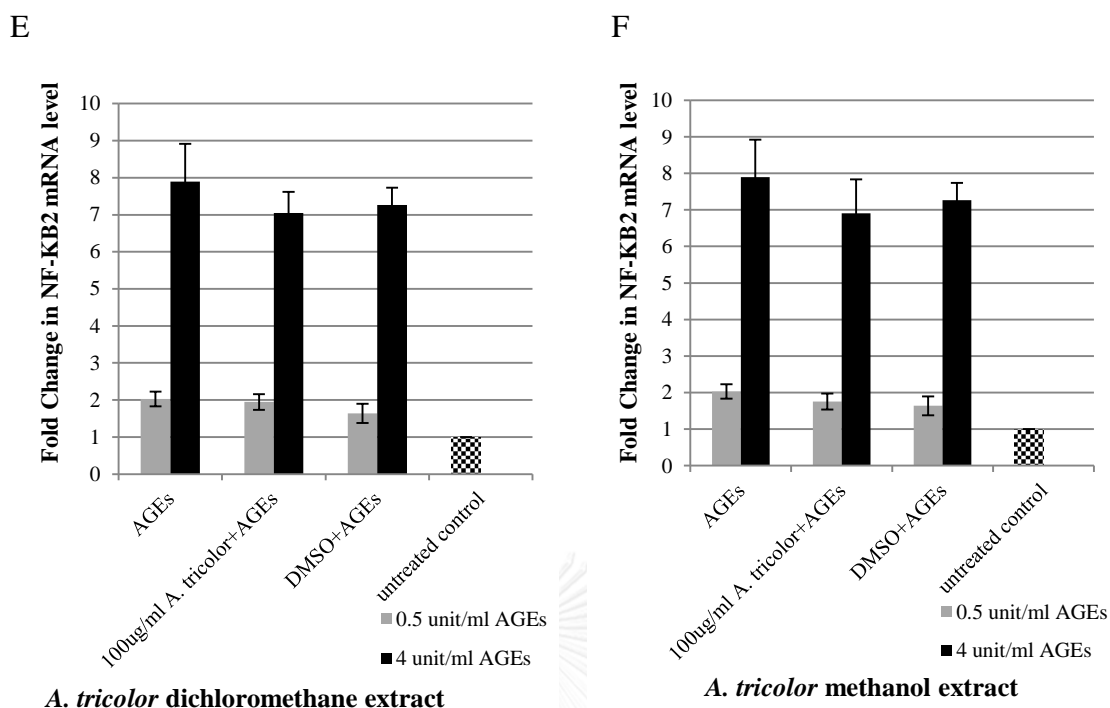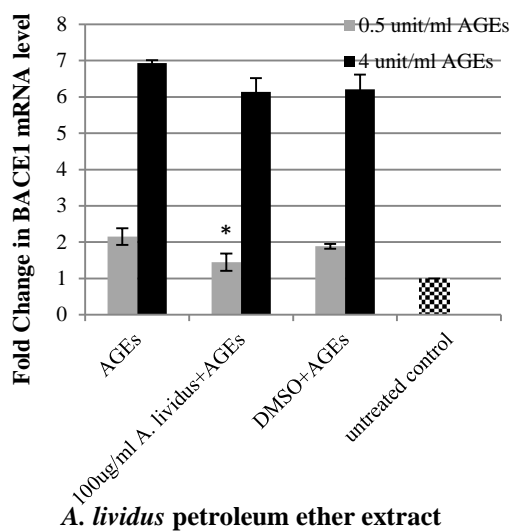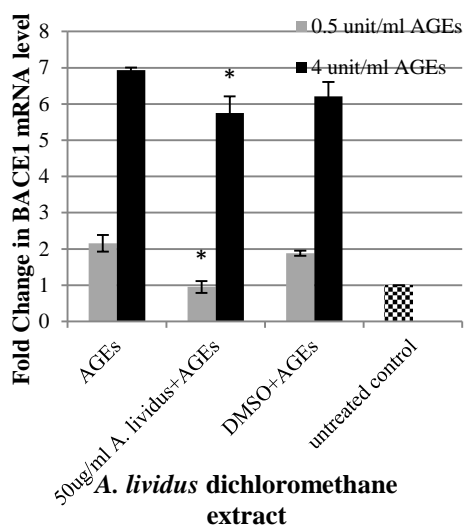
F

A. tricolor **methanol extract**
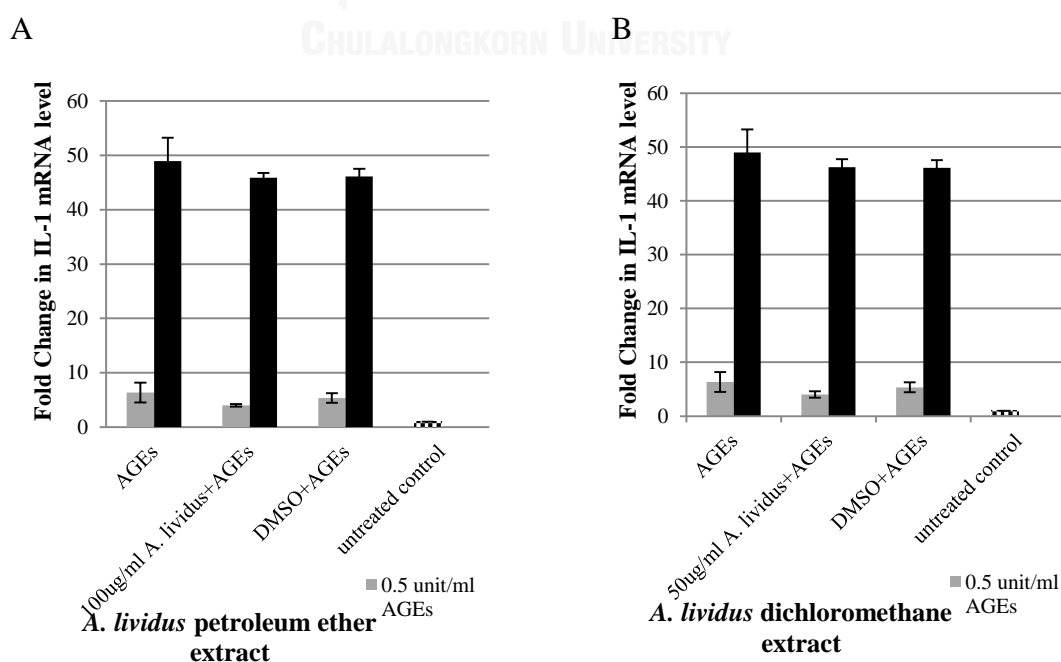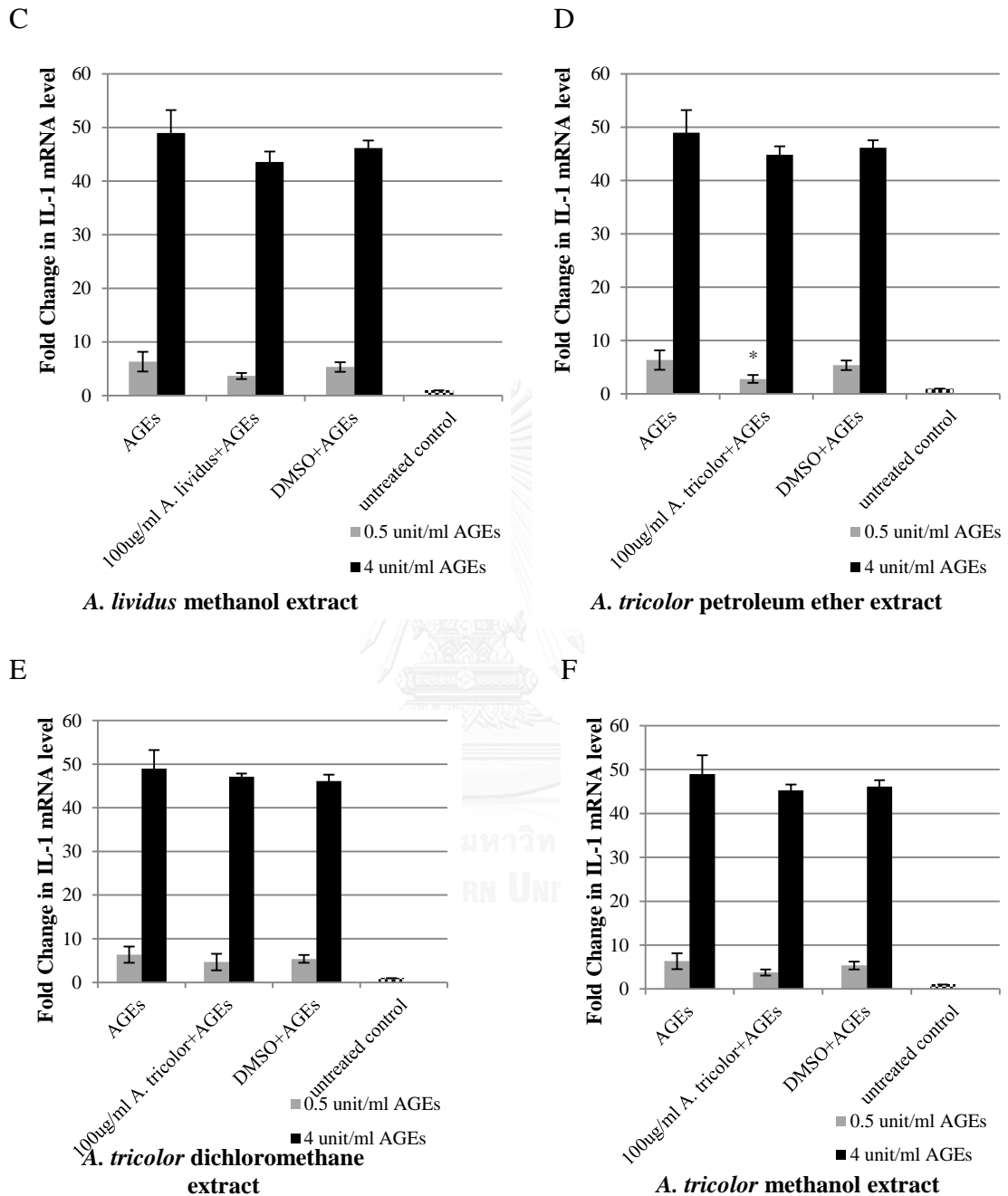
Figure 49. Effect of herbal extracts on IL-1 mRNA level in SH-SY5Y cells treatment with AGEs. SH-SY5Y cells were preincubated with (A) *A. lividus* petroleum ether; (B) *A. lividus* dichloromethane; (C) *A. lividus* methanol; (D) *A. tricolor* petroleum ether; (E) *A. tricolor* dichloromethane and (F) *A. tricolor* methanol extracts for 24 h. The cells were further incubated with 0.5 and 4 mg/ml AGEs for 24 h and gene expression levels were determined by qPCR analysis. The gene expression of GAPDH was used for

normalization. Values are expressed as means with their standard error of the mean (SEM). (N = 3) Significant differences are indicated by *P < 0.05 when compared with untreated cells.
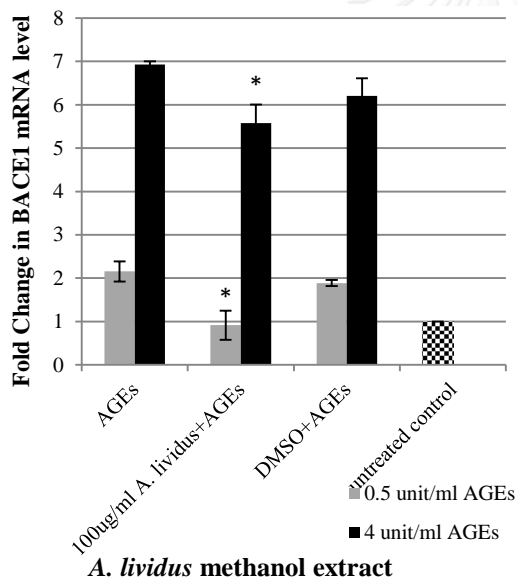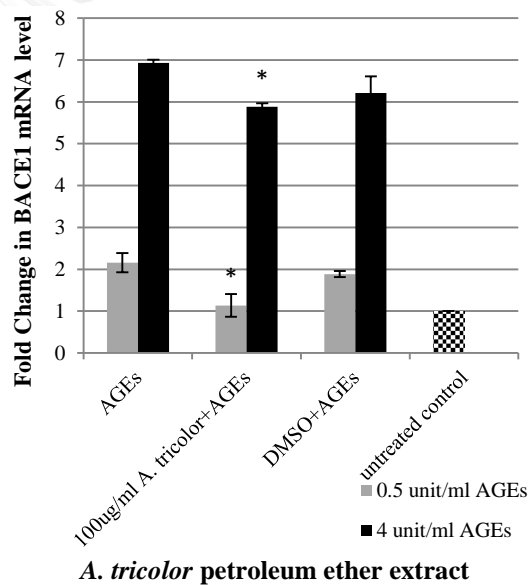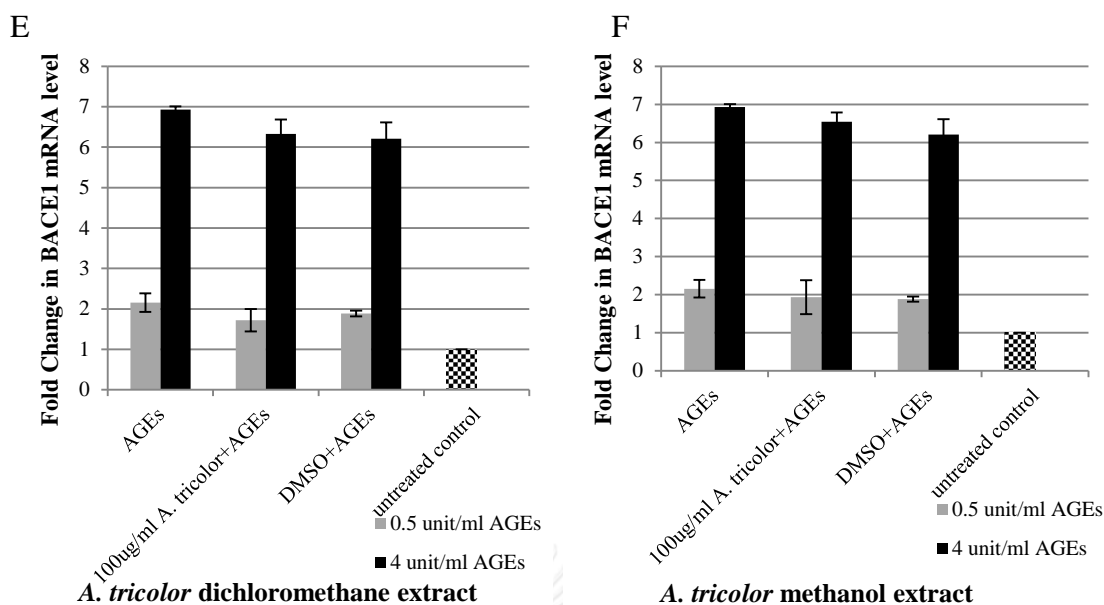
CHAPTER IV

DISCUSSION

Alzheimer's disease (AD) is the most common dementing disorder of late life. There might be various different stimuli that occur in the early stage of the disease, however they seem to converge on a few characters in the late stage, characterized by inflammation and neurodegeneration [212, 524]. It has recently become clear that advanced glycation endproducts (AGEs) involve in physiological brain aging and in an accelerated fashion in AD [246, 525]. AGEs are a group of compounds that are combinations of sugars and proteins and other large molecules followed by a cascade of glycation that affect an alteration of structure and function of tissue proteins [8]. AGEs participate in many of the neuropathological and biochemical features of AD such as extensive protein crosslinking, induction of oxidative stress and neuronal cell death. In AD patients, AGEs can be detected in pathological deposits such as amyloid plaques [235, 526] and neurofibrillary tangles [246]. The stability of proteins that constitute the long-lived intracellular neurofibrillary tangles and extracellular amyloid plaques suggests that they would be ideal substrates for glycation, ultimately resulting in the formation of AGEs. Moreover, AGEs have been demonstrated to cause oxidative stress that pathophysiological effects have been found at the cellular and molecular level [12, 16]. Several studies have shown that oxidative stress can increase in the early stages of AD, suggesting that this is an early event in the development of AD pathogenesis [17]. Since AGEs can interact with cell surface receptors, such as RAGE, then produces reactive oxygen species (ROS), particularly superoxide and hydrogen peroxide, it was inferred that AGEs could exert cytotoxic effects on cells [20]. Oxidative stress and AGEs initiate a positive feedback loop, where normal age-related changes develop into a pathophysiological cascade. RAGE may contribute AD pathogenesis by influencing transport of amyloid beta (Aβ) into the brain [527, 528] or by manipulating inflammatory mechanisms [529, 530]. The activation of RAGE by many of its ligands, including AGEs and Aβ, results in the release of proinflammatory mediators such as free radicals and cytokines by promoting nuclear factor-κB (NF-κB) activation and enhance extracellular matrix accumulation [531-533]. The pathological consequence of RAGE interaction with AGEs and other possible RAGE ligands can activate several intracellular pathways, leading to the induction of oxidative stress and a broad spectrum of signaling, possibly based on the intensity and duration of stimulation. The diverse signaling pathways may be including p21ras, erk1/2, MAPKs, p38 and SAPK/JNK MAPKs, PI3K, and the JAK/STAT pathway. The downstream consequence of pathways is the activation of key transcription factors, nuclear factor-κB (NF-κB) which in turn causes induction of molecules with damaging actions on the cells [534].

AGEs formation is not very specific, as it is not directed by enzymes, between free amino acid groups of protein, mostly lysine, arginine, and cysteine residues, and carbonyl group of reducing sugar such as glucose [535] leading to formation of

reversible structure called Schiff's bases. This structure can have further rearrangement, through the formation of enaminol intermediate, into the more stable ketoamine termed Amadori products. These products undergo further autoxidation which leads to release of some free radicals, generation of dicarbonyl intermediates and subsequent formation of AGEs [536, 537] which takes several days to weeks to complete process. However, we have concerned on the *in vitro* preparation of AGEs. Since the results are dependent on the stimulation of SH-SY5Y cells with AGEs prepared *in vitro*, it is very important that we prepare our AGEs properly. Since AGEs have been implicated in the chronic complications of diabetes mellitus and have been reported to play an important role in the pathogenesis of AD [538]. Previously, Biao X. and collaborators showed that serum concentration of AGEs is approximately 0.2 mg/ml in diabetic patients [539]. In the present study, AGEs were prepared by glycation of protein with glucose in a concentration that corresponding found in the circulation of diabetic patients ($\geq$7 mmol/l) [540]. As glucose has been thought to play a primary role in the Maillard reaction and albumin has been also presented in the circulation at relatively high concentration (35–50 g/l) and has been prone to glycation *in vivo* [541] as well as it has been the commonly used for protein-AGEs adduct studies *in vitro* [55, 542-544]. Therefore, we prepared AGEs by incubating endotoxin-free bovine serum albumin (BSA) with D-glucose in a phosphate buffer at pH 7.4 at 37°C in the dark as described previously [243, 508, 509] with minor modification. However, control BSA was also produced in a similar manner but without reducing sugars. Chemical modifications of proteins by reducing sugars can alter the structure, function, and turnover of proteins to formation of a heterogeneous group of mostly fluorescent and brown products [8]. Maillard or browning reactions are among the major non-enzymatic pathways contributing to protein damage, and autoxidation of sugars as well as oxidative degradation of Amadori product during these reactions enhance the chemical modifications of proteins by ROS [545, 546]. The extent of protein glycation was estimated by measuring the brownish color and autofluorescence intensity. Brown staining is characteristic for protein-AGEs adducts, indicating that albumin is modified upon glucose incubation. The present study revealed that the color of BSA incubated with D-glucose tended to brown during the incubation period and after 12 weeks they were intense brown. In addition, previous studies of AGEs formation have monitored the increase in fluorescence arising from browning glycation products. The fluorescent cross-linking AGEs can be produced by dicarbonyl or glycoxidation products that arise from free sugar, from the initial Schiff bases, and from Amadori and other intermediates [541, 544, 547]. In this work we chose to assess for formation of AGEs by monitoring the production of fluorescent products at excitation and emission maximum of 335 and 460 nm, respectively, as previously explained [548]. Based on the fluorescence property, our result demonstrated that exposure of 10 mg/ml BSA to 7 mmol/l D-glucose for 12 weeks could enhance the fluorescence intensity almost five fold compared to BSA control. Surprisingly, we also detected an increase in fluorescence intensity of BSA in our BSA control preparation, which was treated identically but without the addition of glucose, when compared with non-incubated BSA. Next we tested the ability of synthesized AGEs on cell viability in human neuroblastoma SH-SY5Y cells. Incubation of these cells with AGEs-BSA resulted in massive cell death after 48 h exposure while BSA-control incubated cells showed slightly cell death. These results demonstrated that synthesized AGEs, in contrast to BSA-control, were cytotoxic

to cultural cells tested, similar to what was observed in previous reports [12, 542, 543, 549]. Next, we tested the ability of AGEs prepared by incubating BSA with D-glucose on cellular oxidative damage. Since the mechanism by which AGEs is thought to cause cell toxicity is mainly through an increase in oxidative stress [7, 20]. Formation of ROS is associated with the oxidation of sugars and Amadori products. This process begins with the production of superoxide radicals by transition metals-catalyzed autoxidation of the sugars and proteins bound Amadori products, followed by dismutation of superoxide anion radicals to hydrogen peroxide and then can be further catalyzed and converted to of lethal hydroxyl radicals by the metal-catalyzed via the Fenton reaction [8]. An accumulation of these ROS leads to oxidative stress. Moreover, AGEs can also bind to their receptor, RAGE, expressed on the cell surface. Binding and stimulation of RAGE with AGEs leads to intracellular signal transduction through the key target NF-κB. There have been reported that activation of RAGE by AGEs caused oxidative stress and activation of NF-κB via activation of the p21ras and the mitogen-activated protein (MAP) kinase signaling pathway [20, 35]. Here we showed that exposure AGEs to the neuroblastoma SH-SY5Y cell increased oxidative stress resulting in an increased of intracellular fluorescent ROS dye DCFH-DA, increased generation of TBARS which is a byproducts of lipid peroxidation during oxidative stress as well as upregulation the expression of the HMOX-1, an indicator of cellular oxidative stress. These findings were in accordance with previous findings that using other cell lines [16, 44, 550]. Moreover, engagement of RAGE with AGEs has been reported to increase expression of RAGE itself in a variety of cells [551-553]. Thus, AGEs-RAGE interaction can increase oxidative stress, then over-expression of RAGE creates positive feedback loops that allows to sustained activation of the RAGE downstream pathway such as NF-kB transcription factor [242, 554]. In our present experiment, the expressions of RAGE and NF-kB members including RelA, NF-κB1 and NF-κB2 were upregulated when the cells were exposure to AGEs. This finding indicates that induction of RAGE/NF-κB axis by AGEs elicits oxidative stress generation. In agreement with our finding Schmidt A.M., et al., also found that AGEs induced oxidant stress resulted in generation of TBARS, induction of HMOX mRNA, and activation of the transcription factor NF-KB. Each of these AGEs-induced changing of indicative of cellular oxidant stress was blocked by prevention of access of AGEs with antibodies to RAGE [529]. These data are consistent with the concept that AGEs, after interaction with their cellular receptors RAGE, are responsible for induction of oxidant stress and activation of NF-KB.

Oxidative stress is one of the key pathological events in AD because it contributes to membrane damage, cytoskeletal alterations and cell death [555]. Oxidants and oxidative products have been reported to increase the expression of amyloid precursor protein (APP) [556] and Aβ levels in brain cells [557, 558]. Aβ is produced via sequential proteolytic cleavages of APP by β-secretase, also known as beta-site APP cleaving enzyme 1 (BACE1), and γ-secretase, a multiprotein complex which is made up of presenilin 1 or 2 (PS1 or PS2), nicastrin, anterior pharynx defective (APH) and presenilin enhancer 2 (PEN2). BACE1 cleaves APP at the N-terminal end, producing a 99 amino acid APP C-terminal fragment, which is further cleaved within the transmembrane domain by γ-secretase, resulting in the release of Aβ peptides [3, 165, 559, 560]. On the other hand, cleavage of APP by a α-secretase precludes the formation of toxic Aβ peptides [561]. There have been demonstrated that in conditions

involved oxidative stress including ischemia [562], hypoxia [563], and in cultured neuronal cells exposed to hydrogen peroxide [282] or the lipid peroxidation product 4-hydroxy-2,3 nonenal (HNE) [281] elevated the expression and activity of BACE1, the β-secretase enzyme which involved in Aβ production. In addition, it has been found recently that ischemia and hypoxia [564] and in cultured neuronal cells exposed to hydrogen peroxide and $FeCl_2$ [282] also increased γ-secretase activity that responsible for the cleavage of APP for generation of the Aβ peptides [564]. Our data also provided evidence for a modulation of amyloid precursor protein metabolism in SH-SY5Y human neuroblastoma cells exposed to AGEs related with up-regulation of BACE1 (β-secretase) and PS1 (γ-secretase) while up-regulation of ADAM10, α-secretase when incubated with a high level of AGEs. These finding contrasted with other study in which oxidative stress decreased expression of enzymes with α-secretase activity [282]. The possible explanation is that α- and β-secretase appear to compete for the initial cleavage of APP, but have opposite effects on Aβ generation. Specifically, α-secretase cleavage precludes Aβ formation and is considered to be part of the non-amyloidogenic pathway in APP processing [561]. Thus, an increase in α-secretase cleavage is considered as a therapeutic approach for AD [565]. It has been reported that overexpressed of ADAM10 could counteract apoptotic signaling and promote synapse formation [566]. However, the molecular mechanisms regulating α-secretase cleavage remain only partly understood. Consideration the involvement of oxidative stress and increasing the expression and activation of β- and γ-secretase, there have found the elevation of BACE1 and PS1 expression/activity [286, 287, 567] and the increasing of transcription factors NF-κB in AD brains [568]. Therefore, it is possible that the increased oxidative stress in AD brains may initiate the activation of a cascade of redox-sensitive cell signal pathways including NF-KB, which promotes the expression of BACE1 and PS1, eventually enhancing the production of Aβ and the deterioration of cognitive function in AD. In addition, the promoter and 5′ untranslated region of BACE1 gene contain binding sites for multiple transcription factors including NF-κB, activation of which by oxidative stress may in turn enhance BACE1 expression [569]. Taken together, AGEs can be enhance oxidative stress, may impair neuronal function and further exacerbate neuronal oxidative damage, contributing to the pathological development of AD through RAGE-mediated activation of the activation of redox-sensitive signaling pathways such as NF-κB.

Since AGEs-RAGE interaction has been shown to activate a signal transduction pathway involving the transcription factor NF-κB. The NF-κB pathway regulates gene transcription for generation of pro-inflammatory cytokines such as IL-1α, IL-6 and tumor TNF-α [67]. Previous studied, they found that the activation of RAGE by AGEs results in the release of proinflammatory mediators such as free radicals and cytokines [570]. In this present study, we found that proinflammatory cytokine gene in cells which were incubated with AGEs increased genes expression of TNF-α, IL-6 and IL-1. In agreement with our finding, Dukic-Stefanovic S., et al., found that AGEs from chicken egg albumin could induce TNF-α and IL-6 production [571], Berbaum K., et al. also found that AGEs induced expression of selected cytokines and chemokines in murine cell lines determined with a cytometric bead array [530]. These data collectively showed that the AGEs, prepared by incubation BSA with D-glucose, can induce oxidative stress, inflammation and cell toxicity in SH-SY5Y cells, therefore it was suitable for further experimental analyses.

Nowaday, there are six medications approved by the US Food and Drug Administration (FDA) for AD which are unable to prevent or reverse the disease progression but are only modestly efficacious [215]. Since the pathogenesis of AD is complex as such, the inhibition of the cause characterized by the deposition of amyloid plaques and formation of neurofibrillary tangles of the disease, has not been the only intervention target for AD [460]. Inflammation and oxidative stress are also play an important role during the development of AD [572]. Alternatives, including anti-inflammatory and anti-oxidative drugs, have also been developed as therapeutic strategies for AD [572, 573]. Therefore, other more effective treatments are urgently needed. An herbal medicine which demonstrated some interesting therapeutic properties have place for alternative medicine for AD. The present study demonstrates that extract of *A. lividus* and *A. tricolor* possessed antioxidant activities depending on extracting solvents. These finding agreed with previously study that antioxidant capacity depends on the solvent used [574]. A great number of *in vitro* methods have been developed to measure antioxidant capacity of foods and biological samples either as pure compounds or as plant extracts. Most of the assays employ the same principle of scavenging of a synthetic colored radical or redox-active compound by antioxidant activity of a biological sample and is monitored by spectrophotometer. One approach is based on an electron transfer and involves reduction of a colored oxidant such as ABTS and DPPH assay. The commonly used antioxidant assays along with an appropriate standard such as Trolox equivalent antioxidant capacity (TEAC) or vitamin C equivalent antioxidant capacity (VCEAC) can be used as positive control [575]. The data from antioxidant assays describes the ability of redox molecules in biological sample to scavenge free radicals. This provides information of the antioxidants present in a biological sample and may be useful to study the potential health benefits of antioxidants on oxidative stress-mediated diseases. *In vitro* methods to characterize antioxidant activity used in this study were including the most popular being ABTS and DPPH assay. These methods are popular due to their high speed and sensitivity. It is essential to use more than one method to evaluate antioxidant capacity of plant extracts because of the complex nature of phytochemicals [576]. Moreover, these assays are based on different strategies, different advantage/disadvantage and provide different information about the ROS interaction. In specification, the ABTS or TEAC (Trolox equivalent antioxidant capacity) assay is based on the ability of the antioxidants to scavenge a blue/green ABTS•+ radical cation, which is applicable to both hydrophilic and lipophilic antioxidant systems. On the other hand, the DPPH assay is based on the reduction of a purple DPPH• to 1,1-diphenyl-2-picryl hydrazine, which uses a radical dissolved in organic media and is, therefore, applicable to hydrophobic systems [577]. Both assays are convenient in their application and thus most popular; nevertheless they are limited as they use non physiological radicals [578]. Results showed that the extracts derived from methanol fraction appeared to have the greater radical-scavenging activity than those from dichloromethane and petroleum ether in both measurement methods. In addition, the methanol extract of *A. tricolor* possessed the highest antioxidant capacity in the ABTS radical scavenging assay, while the methanol extract of *A. lividus* had the most scavenging activity in the DPPH radical scavenging assay. The explanation of these result from the recent data indicated that the difference between antioxidant activity determined by these two assays was found in highly pigmented foods such as cherry, spinach, plums and red cabbage [579]. Nevertheless,

the results showed high correlation between antioxidant capacity in the ABTS and DPPH assay. The data from present study, however, had limitation because the antioxidant capacity from ABTS and DPPH assay were *in vitro* models and did not assess all of the antioxidant activities in foods. The findings of the present study suggest that the method and solvent used for preparation of the extracts affect the physical-chemical properties and possibly also the biological activities of the herbal extracts. Our results also showed that both *A. lividus* and *A. tricolor* displayed potent antioxidant properties in accord to previous observations [493, 580].

Next, we investigated the ability of herbal extracts to prevent AGEs induced toxicity in neuronal cell. To evaluate protective effect of herbal extracts on the cell survival, proliferation and damage of neuroblastoma SH-SY5Y cells, time-course and concentration-dependent experiments were conducted to characterize the neurotoxicity effects of *A. lividus and A. tricolor* in SH-SY5Y cells which detected by trypan blue staining, MTS and LDH, respectively. Time and dose dependent inducing cell death suggested the herbal extracts were attributed to their cytotoxicity. However, all of these effects were observed at relatively high extract concentrations that are not likely relevant biologically environment. It is well known that Amaranthus comprises several active constituents like alkaloids, flavonoids, glycosides, phenolic acids, steroids, amino acids, terpenoids, lipids, saponins, betalains, b-sitosterol, stigmasterol, linoleic acid, rutin, catechuic tannins and carotenoids [489]. Studies on these pure compounds such as alkaloids, flavonoids, phenolic acids, steroids and betalain pigments revealed protection against cytotoxicity on neuronal cells [581-585]. These data suggested that the active compound in Amaranthus may be responsible for protection neuronal cells against cytotoxicity. However, due to the fact that these plant extracts were crude extracts which usually composed of a combination of various type of bioactive compounds or phytochemicals with different polarities and some of them may not be responsible for the activity of interest [586]. Besides, in crude extracts, the activity of some compounds may be due to compound interaction; this action can be synergistic when the compound interaction causes an increase in the effects or antagonistic when the compound interaction causes a decrease in the effects [587]. The loss of activity in the crude extract possibly due the active compounds in the crude extract are very little but possess high activity so it showed activity at a increased concentration, or the active compounds in the crude extract may be in very high amount but less active, then it also showed activity at an increased concentration. A further study is needed to identify any active compounds in particular solvent extracts that showed positive results in order to assess which chemical constituents responsible for the activity.

Considerable attention has been paid to the toxicity of AGEs. AGEs generally cause DNA damage and oxidative stress leading to death of neuronal cells [71]. Recent study has shown that low concentrations of AGEs cause neurotoxic and inhibit regeneration neuron's neuritis whereas high doses of AGEs directly induce neuronal cell death [588]. In the present study we exposed SH-SY5Y cells to a relatively high concentration of AGEs which were shown to cause cell damage (as indicated by approximately 50 % increasing of LDH release and 55 % reduction of ability of cell proliferation) and cell death (as indicated by approximately 55 % reducing in cell survival staining) by 48 h post-AGEs incubation. Since *in vitro* assays were shown that *A. lividus* and *A. tricolor* in each solvents possessed different antioxidant activities. It

is likely that these make a contribution toward neurotoxicity induced by AGEs observed in this study. In addition, the result suggested both *A. lividus* and *A. tricolor* methanol extracts showed the most protective effects against AGEs induced toxicity. This might be explained by the greater radical-scavenging activity of the herb extracts derived from methanol fraction observed in chemical assay above.

In general, AGEs toxicity occurs through an oxidative pathway [589]. It is conceivable that protection from oxidative damage was linked to the antioxidant properties of the herbal extracts. The antioxidant properties of *A. lividus* and *A. tricolor* to protect neuronal cells from oxidative damage were further confirmed by determination of intracellular ROS scavenging activity. ROS are also known to perturb redox homeostasis which considered to play a role in an alteration of normal physiological processed and proposed to be a general pathological mechanism of chronic neurodegenerative diseases [590]. Indeed, it has been shown that natural compounds that have an antioxidant capacity or free radical scavenging activity can inhibit toxin induced cellular oxidative damage. Several studies have shown that resveratrol found in wine [591], rutin (bioflavonoid) found in apple [592, 593], epigallocatechin-3-gallate (catechin) found in green tea [594], quercetin, catechin, caffeic acid, phytic acid and betaine [595, 596] have been shown to exert protective effect against cellular oxidative damage in different cell lines. The present study demonstrated that *A. lividus* and *A. tricolor* extracts possessed intracellular ROS scavenging activity. Different pattern between intracellular ROS scavenging activity of *A. lividus* and *A. tricolor* in each solvent were detected. However, both *A. lividus* and *A. tricolor* derived from methanol fraction showed maximum protection against intracellular ROS.

Among the organs in the human body, a brain takes vulnerability to oxidative abuse [597, 598]. One factor that contributes to oxidative damage in the brain is high content of polyunsaturated fatty acids in the membranes and low levels of enzymatic and nonenzymatic antioxidants [599]. The peroxidation of membrane lipids is the most important consequences of the generation of free radical in the brain. Lipid peroxidation is initiated by free radical attack on membrane polyunsaturated fatty acids leading to their transformation and fragmentation to alkanes and reactive aldehyde compounds. The measurement of thiobarbituric acid (TBARS) is commonly used to monitor lipid peroxidation and oxidative stress *in vitro* and *in vivo* [600]. Evaluation of the effect of AGEs *in vitro* showed a significant increase in TBARS levels in SH-SY5Y cell exposed to AGEs. The decreases in the TBARS values were followed by the preincubation of *A. lividus* and *A. tricolor* extracts. Moreover, methanol extract of both *A. lividus* and *A. tricolor* showed the maximum diminishing the TBARS concentration. The ability of the methanol fraction to inhibit the process of lipid peroxidation may be due to the free radical scavenging activities of its phytochemical components, as earlier reported by Olorunnisola O.S., et al. [601]. Several studies suggested that plant extracts could reduce lipid oxidation by incorporating natural antioxidants to attenuate free radicals [602]. The results obtained in the present work confirmed the data found in literature and indicating a considerable effect of natural extracts such as barks of cinnamon (*Cinnamomum iners*), buds of clove (*Syzygium aromaticum* Linn), rhizomes of ginger (*Zingiber officinale* Rosc.), leaves of green tea (*Camellia sinensis*) , leaves of thyme

(*Thymus vulgaris* Linn.) [603] and leaves of rosemary (*Rosmarinus officinalis*) [602] on the inhibition of lipid oxidation.

Several of natural antioxidant compounds have been shown to exhibit direct regulatory effects on endogenous antioxidant enzyme systems. Heme oxygenase (HMOX or HO) is the rate-limiting enzyme for catabolism of the pro-oxidant heme. HMOX-1 is an inducible isoform in response to stress such as oxidative stress, hypoxia, heavy metals, and inflammatory stimuli [604, 605]. In this study, we showed that AGEs enhanced genes expression of HMOX-1 and NF-κB in SH-SY5Y. Preincubation of *A. lividus* and *A. tricolor* extracts attenuated HMOX-1 and NF-κB expression in AGES-stimulated SH-SY5Y cells. In agreement with our finding Rocejanasaroj A. found that *Thunbergia laurifolia* extract down-regulated the HMOX-1 expression in HepG2 cells [606]. However, these findings contrast with other studies in which increasing HMOX-1 expression seems to be protective [607-609]. HMOX-1 is up-regulated by NF-κB [102-104] and other transcription factors in response to stimuli. Study in other cell types challenged with AGEs have shown that upregulation of HMOX-1 expression involved with Nrf2 pathway which is in part mediated by the activation of intracellular protein kinase cascades [550] including an increase in phosphorylation of p38MAPK, ERK and JNK [610, 611]. The interaction between Nrf2 and NF-κB is interesting because numerous phytochemicals that have antioxidant property suppress NF-κB signaling and activate the Nrf2 pathway [612]. The mitogen-activated protein kinase (MAPK) contributes to both the Nrf2 and NF-κB pathways. NF-κB competes with Nrf2 for binding to the transcriptional coactivator CREB-binding protein (CBP) and also promotes the binding of the corepressor histone deacetylase 3 (HDAC3) to ARE. Thus, NF-κB may be a negative regulator of the Nrf2 pathway [613]. However, in this study we focused primarily on protective effects from antioxidant property of the herbal extracts on AGEs induced oxidative stress involving NF-κB and have not characterized the potential involvement of other transcription factors such as Nrf2 in the induction of the antioxidant gene HMOX-1. Thus, the discrepancy of the result might be come from other effects *A. lividus* and *A. tricolor* extracts on regulation of HMOX-1 expression. Other explanation was NF-κB activation. Since it is well known that the expression of NF-κB target genes typically promotes cellular survival. One of the most important ways is antioxidant NF-κB targets in which NF-κB influences ROS levels by increased expression of antioxidant proteins such as superoxide dismutase or HMOX-1. Another way is pro-ROS NF-κB targets in which NF-κB regulate transcriptional pro-oxidant targets because NF-κB is important in inflammation. Some enzymes that promote the production of ROS are also regulated as its targets such as NADPH oxidase or inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2) [74]. Therefore preincubation with *A. lividus* and *A. tricolor* might be modulate the NF-κB response through NF-κB-mediated inhibition of these target genes and these genes might be attenuate ROS damage as well as signaling to promote survival. A further study is needed to identify underlying mechanism. Taken together, these results suggest that *A. lividus* and *A. tricolor* extracts exerts anti-oxidative effects in the AGEs treated SH-SY5Y cells.

In general, AGEs toxicity occurs through at least three mechanisms: 1.) interaction with the receptor for AGEs (RAGE); 2.) tissue deposition; and 3.) *in situ* glycation [589]. Receptor for AGEs (RAGE) is a multiligand member of the

immunoglobulin superfamily of cell surface receptors. The multi-ligand nature of RAGE is highlighted by its ability to bind to various ligands such as AGEs, S-100/calgranulins, high motility group protein B1 (amphoterine), Aβ peptides and β-sheet fibrils [614, 615]. The engagement of RAGE by AGEs has been demonstrated to mediate various intracellular pathways, including phosphoinositide 3-kinase/AKT, mitogen-activated protein kinase, and nuclear factor NF-kB [616]. AGEs impair cell functions, such as proliferation, migration, apoptosis and adhesion. Stimulation of RAGE results in inducing cellular oxidant stress via activation of the transcription factor nuclear factor-κB (NF-κB) and subsequent transcription of many proinflammatory genes [20, 243]. As such, the AGE-RAGE interaction is thought to play a pivotal role in inflammation, atherosclerosis, nephropathy, neurodegeneration, cancer, diabetes and its complications [617-619]. Studies have shown that RAGE activation is strongly responsible for the pathogenicity associated with AGEs in many signaling pathways [36, 620]. In the present study, we found AGEs upregulated RAGE, NF-κB and proinflammatory cytokines expression. This upregulation may cause an increase in transduction signals following stimulation by AGEs and this may leads to the activation of the inflammatory response, including the production of cytokines. Thus, the inhibition of AGEs formation, blockade of the AGE-RAGE interaction, and suppression of RAGE expression or its downstream pathways may contribute to the therapeutic value for the treatment. Several studies have demonstrated that the expression of RAGE can be inhibited by clinical drugs and natural compounds including antihypertensive drugs, Telmisartan and Candesartan [47, 621], antihyperlipidemic agents (statins) [622], antidiabetic agents (thiazolidinediones) [623], vitamin A [624], selenium [625], n-3 polyunsaturated fatty acids [626] and several extracts from Chinese traditional medicines, such as *Ginkgo biloba* extract [627] and *Panax notoginseng saponins* [628], *Litsea japonica* (Thunb) [629] and *Fimbristylis ovata* Kern. [630]. This suggests that reduced expression of RAGE as a promising therapeutic method of these drugs. The present study showed that pretreatment with *A. lividus* and *A. tricolor* extracts caused the downregulation of TNF-α, IL-1β and IL-6 proinflammatory cytokines genes expression and RAGE expression. In addition, these herbal extracts suppressed the NF-κB expression. These data suggest that the herbal extracts can downregulate the AGEs-mediated inflammation. Furthermore, increase in the RAGE and NF-κB expression on SH-SY5Y cells with concomitant augmentation of inflammatory cytokines by AGEs suggests that AGEs may stimulate inflammatory processes at least in part by RAGE associated with NF-κB signaling in neuronal cells. In addition, our present data of decreased RAGE expression and inflammatory cytokines following exposure to *A. lividus* and *A. tricolor* extracts indicates that the herbal extracts suppresses inflammation at least in part by decrease in RAGE expression. Our results are partly in line with Eunjin S., et al. who demonstrated combination of medicinal herbs, KIOM-79, can modulate inflammatory responses through the suppression of AGEs/RAGE/NF-κB activation [631]. In addition, our findings are consistent with recent studies in other cell lines where some medicinal plants or active constituents in many medicinal plants could modulate the intracellular signaling pathways initiated by AGEs and subsequently inhibiting the effects of RAGE thereby suppressing the downstream pathways activation and decreasing the multiple inflammatory disorders. Abdol K.S., et al. demonstrated PBMCs isolated from 20 type 2 diabetes mellitus patients treated with garlic (*Allium sativum*) in presence or absence

of glycated albumin. The results revealed that glycated albumin increased RAGE expression and proinflammatory cytokines secretion. Treatment with garlic extract reduced TNF-α and IL-1β secretion and RAGE expression. These data indicated that modulation of RAGE expression may be one possible reason for the garlic effects on proinflammatory cytokines secretion [632] and Zhengyu Z., et al. also examined human cell line ECV304, derived from urinary bladder carcinomacells treated with AGEs in the presence hyperoside, a flavonoid compound. The results demonstrated that hyperoside inhibited JNK activation and promoted cell proliferation. Furthermore, hyperoside could inhibit RAGE expression in AGEs-stimulated cells. These results suggested that hyperoside may inhibit JNK activation and promote cell proliferation by downregulating RAGE [633]. Taken together, we concluded that *A. lividus* and *A. tricolor* extracts reduced RAGE expression in the SH-SY5Y exposed to AGEs. The decreasing RAGE activation thereby suppressed NF-κB activation and subsequently decreasing the expression of multiple proinflammatory cytokines. Therefore treatment with *A. lividus* and *A. tricolor* extracts exerts anti-inflammatory effects on the SH-SY5Y treated with AGEs that may help in the prevention and delay of AGEs-induced neuroinflammation.

The pathogenesis of AD is complex therefore, the anti-oxidative and anti-inflammatory not be the only intervention target. Alternatives, including intervention target the inhibition of Aβ production and aggregation, have also been developed as therapeutic strategies for AD [572, 573]. Up to now, given the lack of effective long-term therapies for AD, there is a persistent need to discover a new molecules or approaches with disease-modifying properties. Currently, four approaches for the prevention and modification of AD are applied; 1.) anti-amyloid deposition strategy (up-regulation of α-secretase and down-regulation of β- and γ-secretase); 2.) acetylcholinesterase and butyrylcholinesterase activity inhibition; 3.) neuroprotective treatment and 4.) neurorestorative/neuroregenerative therapies [634]. Recently, several studies have reported a potential therapeutic interest for plant extracts either in clinical or in preclinical tests, on several pathological features related for the prevention or cure of AD [635-637]. Efficient natural active compounds have been isolated, acting on several targets involved in AD pathogenesis, and some of them have already shown promising activity in clinical trials such as the *Ginkgo biloba* extract (EGb761) is formally approved and registered in Germany and in Belgium as a drug against AD [637, 638].

Since Aβ formation an APP proteolysis process is fundamental in neurodegenerative pathological mechanism in the AD development [3, 639, 640]. The APP proteolysis includes physiological processing (non-amyloidogenic pathway) and pathological processing (amyloidogenic pathway). The APP proteolysis catalyzed by α- and γ-secretase generating a non-cytotoxic soluble fragments sAPPα peptide as opposed to the proteolytic cleavage of APP by β- and γ-secretase secretases yields in the production of a secreted APP derivative sAPPβ and Aβ peptides of different lengths [3, 165, 639-643]. Therefore the anti-amyloidogenic properties of plant materials that influence on the APP processing via modulation of secretases may offer a new promising therapeutic strategy for diminishing Aβ and amyloidosis in AD. There are strong evidences pointing the role of β- and γ-secretases as a susceptibility factor of brain amyloidosis and may be an excellent therapeutic target for the prevention and

more effective therapy of AD [644]. It is conceivable that increasing β-and γ-secretases expression could lead to the increased generation of neurotoxic Aβ peptide and trigger neuronal dysfunction [645, 646]. Thus, reduction of Aβ generation via the interference of β- and γ-secretases may be a major drug target for AD. It is well known that the major component of amyloid plaques is Aβ peptide, which is generated by the sequential cleavage of APP via β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) and γ-secretase [176]. BACE1 can cleave APP at the first amino acid of the Aβ domain and is important for the production of Aβ peptides [647]. BACE1, therefore, is the rate-limiting enzyme for Aβ peptide generation [648]. BACE1 expression is regulated by a number of transcription factors positively or negatively regulation including peroxisome proliferator-activated receptor-γ (PPARγ) [649], nuclear factors such as NF-κB [650] and cAMP response element binding protein (CREB) [651].

According to previous results, the inhibition of β-secretase represents a potential therapeutic target in AD treatment. The plant biologically active compounds such as isophthalamides [652], myricetin [653], cardiofolioside, azadirachtin, berberine, kutkin, α- and β-caryophyllene, β-caryophyllene oxide, piperine [654] and EGCG from the leaf of green tea [655, 656] can acts as a modulator for β-secretase [657]. Previously, Ran G., et al. showed that fuzhisan in ginseng root attenuated the amyloidogenic pathway by decreasing the expression of BACE1 via the upregulation of SIRT1 expression but not related to the SIRT1-PPARγ-PGC-1α pathway [658]. In agreement with Qi W., et al. found that inhibiting the processing of amyloidogenic APP pathway as well as the expression of BACE1 by treatment with triptolide, a major active compound extracted from *Tripterygium wilfordii* Hook.f., both *in vivo* and *in vitro* [659]. In this study, we have shown that *A. lividus* and *A. tricolor* extracts have the ability to decrease BACE1 expression in AGEs treated SH-SY5Y cells. Recently Wang H., et al. reported that inflammation and oxidative stress can upregulate the expression of BACE1 [660]. Further, Marwarha G., et al. have demonstrated that the transcription factor NF-κB positively regulates BACE1 transcription [661]. Our data also showed that *A. lividus* and *A. tricolor* extracts reduced the expression of NF-κB in SH-SY5Y cells exposed to AGEs. This evidence suggests that *A. lividus* and *A. tricolor* extracts-mediated downregulation of BACE1 expression might result from the suppression of the transcriptional activity of NF-κB. The detailed molecular mechanism on the effects of *A. lividus* and *A. tricolor* extracts on BACE1 need further research.

Taking a preventive point of view, only a few studies isolated from natural products have been focused on the modulation of γ-secretase. Presenilin I (PS1), which is a member of the aspartic protease family, has been identified as the catalytic subunit of the γ-secretase complex [662, 663]. Previous studies revealed that mutations in PS1 have been linked to familial AD (FAD). Thus, PS1 is a potential target in the design of drugs against AD. It is suggested that natural products with γ-secretase modulating properties may reduce Aβ generation which presenting an interesting therapeutic potential for AD [664]. Data demonstrating that active phytochemical compounds such as beauveriolide III [665], luteolin [666] and epigallocatechin gallate [656] were effective to inhibit γ-secretase activity. In addition, other plant extracts with γ-secretase modulating property have been identified such as Chinese Hop flower extracts [667], *Pterocarpus erinaceus* kino extract [668], *Varthemia iphionoides* and *Anchusa strigosa*

extracts [669]. This is the first report to determine γ-secretase modulating property on *A. lividus* and *A. tricolor* extracts. Our study found that *A. lividus* and *A. tricolor* extracts showed a decreasing in PS1 expression in the oxidative stress environment induced by AGEs, suggesting that these herbal extracts might be attenuate Aβ production from amyloidogenic APP processing by decreasing PS1, ultimately conferring neuroprotection. These results suggests that treatment of *A. lividus* and *A. tricolor* extracts inhibit the processing of amyloidogenic APP by both downregulate the expression of BACE1, β-secretase and PS1, a catalytic subunit of γ-secretase complex.

According to previous reports, the non-amyloidogenic pathway is involved in increasing neuronal plasticity, enhancing synaptic signaling and reducing neuronal susceptibility to cellular stress [643]. There is a strong belief that α-secretase upregulation may be an important target for the prevention and more effective therapy of AD. Thus, recent studies are directed to the discovery of plant substances that can increase the concentration and activity of α-secretase in brain and, increased generation of neuroprotective sAPPα [670]. Several members of the disintegrin and metalloproteinase (ADAM) family have been proposed to be physiologically active α-secretases including ADAM9, 10, and 17. However, that ADAM10 has been demonstrated the highest α-secretase activity *in vivo*. Recent research has been suggested that the upregulation of ADAM10 could be a potential therapeutic target for the treatment of AD because ADAM10 has a potential neuroprotective role that can promote the non-amyloidogenic pathway [671]. Recently, a number of studies on herbal plants have been reported that phlorotannins, curcumin, cardiofolioside, azadirachtin, berberine, and kutkin have the ability to modulate α-secretase, favoring neuroprotection [657, 672]. Mei Z., et al. demonstrated that cryptotanshinone (CTS) from root of *Salvia miltiorrhiza* has been caused of decreasing the Aβ40 and Aβ42 fragments levels, increasing the release of the sAPPα fragments through upregulating of α-secretase activity that promote APP metabolism toward the non-amyloidogenic products pathway. Moreover, it was shown that CTS could induce ADAM10 protein possessing an α-secretase activity that promote non-amyloidogenic processing of APP in cortical neurons [673]. Consistant with a epigallocatechin gallate (EGCG) from the leaf of *Camellia sinensis* (greentea) has also shown the therapeutic potential of compounds that may promote the non-amyloidogenic pathway of APP by enhancing the processing of APP to elevate sAPPα through α-secretase activity *in vitro* and *in vivo* [674, 675]. In addition, Obregon D.F., et al. demonstrated that EGCG increased gene expression of ADAM10 in both cultured neuronal and microglial cells [676]. Here we did not detect a significant change in the mRNA level of ADAM10 in SH-SY5Y that had been treated with *A. lividus* and *A. tricolor* extracts. The findings suggested that in the AGEs treated cells, *A. lividus* and *A. tricolor* extracts could not induce ADAM10 expression. The possible explanation of this conflict may be due to its anti-inflammatory and anti-oxidative effects of these herbal extracts; however, the molecular mechanisms by which *A. lividus* and *A. tricolor* extracts inhibits APP processing through anti-inflammatory and anti-oxidative pathways requires additional studies.

Taken together, these data suggest that the molecular mechanisms by which *A. lividus* and *A. tricolor* extracts might also inhibit APP processing are mainly owing to the inhibition of BACE1 and PS1 expression in anti-inflammatory- and antioxidative-

mechanisms, *A. lividus* and *A. tricolor* extracts therefore, confers protection against the effects of AD and are emerging as a promising therapeutic candidate drug for AD.

CHAPTER V

CONCLUSION

An increasing number of studies have reported novel therapeutic interventions for neurodegenerative diseases. Natural compounds have become an increasingly attractive option to treat neurodegenerative diseases because there is growing evidence that these nutritional constituents have potential adjunctive therapeutic effects be it protective or restorative on various neurodegenerative diseases. In spite of the diversity of the neurodegenerative diseases, oxidative stress due to excessive production and release of ROS has been proposed to be a general pathological mechanism of major chronic neurodegenerative diseases including AD. An accumulation of ROS leads to oxidative stress which can induce cell damage and promote inflammation. AGEs which are cross linked structures formed as irreversible byproducts from the cascade of glycation formed by the reaction of sugars with amino acid side chains can induce oxidative stress and affect an alteration of structure and function of tissue proteins leading to protein modification and dysfunction. AGEs have also been implicated in many neurodegenerative diseases including AD.   Recently report has revealed that Amaranthus has a beneficial effect on the cognitive performance from AD and dementia. However, the underlying mechanism of Amaranth extract on the beneficial effects of AD remains largely unknown. So far no work has been carried out in the neuroprotective effect of Amaranth extract on AGE-induced neurotoxicity. This is a first report of its kind.

The present study designed to investigate two kinds of vegetable which are native and wildly used in Thailand: *Amaranthus lividus* and *Amaranthus tricolor*. The leaves of Amaranthus constitute a rich source of antioxidants and possess medical properties. Screening by *in vitro* method, we found that scavenging capabilities of *A. lividus* and *A. tricolor* extracts from leaves in petroleum ether, dichloromethane, and methanol by the ABTS and DPPH assay. The antioxidant activities were found to be the highest in the methanol fraction of both kinds of plant. We next test the protective effect of these herbal extracts on AGES induced oxidative damage.

Firstly, AGEs were synthesized *in vitro* by incubation D-glucose with BSA at 37°C in the dark for 12 weeks and then were sight to assess the cytotoxic effect of AGEs in SH-SY5Y cells. We used the intracellular fluorescent ROS dye DCFH-DA to examine the level of ROS in the SH-SY5Y cells after treated with un-effective doses of AGEs. The results revealed that the cells exposed to AGEs exhibited increased DCF fluorescence in a dose dependent manner suggesting AGEs increased intracellular ROS. In order to confirm that the increased intracellular ROS were resulted from AGEs, measurement of TBARS using MDA assay was used to examine the byproducts of lipid peroxidation during oxidative stress. The data was indicated that generation of TBARS occurred when SH-SY5Y cells were incubated with AGEs and was dependent on the

AGEs concentration. AGEs have been demonstrated to cause cell toxicity in SH-SY5Ycells. The results demonstrated that AGEs increased cell toxicity in a concentration and time dependent manner which were shown to cause approximately 50 % increasing of LDH release, 55 % reduction of cell survival staining and 55 % reduction of ability of cell proliferation by 48 h post-AGEs incubation. We then investigate the protective effects of the herbal extracts on AGES induced oxidative damage. We found all three different extracts of *A. lividus* and *A. tricolor* were effective at reducing oxidative stress in the SH-SY5Y cells in response to AGEs. As well as there was a significant decrease in TBARS formation upon pretreatment the herbal extracts. In addition, the results indicated that *A. lividus* and *A. tricolor* extracts were capable in a dose dependent manner to attenuate the neuron cell toxicity caused by AGEs treatment.

We assessed the effects of AGEs on the expression of oxidative gene, HMOX-1, AGEs receptor (RAGE), NF-κB signaling network biomarkers including RelA, NF-κB1 and NF-κB2 as well as secretases cleavage of the APP including β-secreatase (BACE1), α-secretase (ADAM10) and presenilin1 (PS1) of γ-secretase complex and proinflammatory cytokines including TNF-α, IL-6 and IL-1. The marker gene for oxidative stress, HMOX-1, enabled a quantitative assessment of the ability of the herbal extracts to overcome oxidative stress induced by AGEs. The data showed that the gene expression level of HMOX-1 was found to be increased in oxidative stress condition in a dose-dependent manner upon incubation with AGEs. We found that HMOX-1 gene expression which was preincubated with *A. lividus* and *A. tricolor* extracts was significantly decreased and was able to bring these back to near normal levels, suggesting their antioxidative role in SH-SY5Y cell lines. The roles of AGEs and its receptor, RAGE in AD has resulted in increased scientific focus. The binding of AGEs to RAGE activates oxidative stress and activates redox-sensitive transcription factors such as NF-κB. In our present study, the expressions of RAGE, RelA, NF-κB1 and NF-κB2 in SH-SY5Y cells incubated with AGEs showed a significant increase a dose dependent manner. Interestingly, *A. lividus* and *A. tricolor* extracts could significantly down-regulated of these genes. Previous studies have shown that a pathologic mechanism of AGEs through its contribution to Aβ accumulation. The mechanisms underlying the oxidative stress induced increasing of Aβ generation are unclear at present. In our present experiment, the expressions of BACE1 and PS1 genes in SH-SY5Y cells treatment with AGEs were significantly increased in a dose-dependent manner. Whereas ADAM10 expression was unchanged when incubated with various concentrations of AGEs except for incubation with a high level of AGEs. The pretreatment of *A. lividus* and *A. tricolor* extracts showed a significant decrease in BACE1 and PS1 mRNA levels while the expression of ADAM10 gene were not found a significantly changing. As AGEs can cause oxidative stress and then induce cell damage and promote inflammation. The present study also presented consistant data that exposure of SH-SY5Y cells to AGEs significantly induced TNF-α, IL-6 and IL-1 genes expression in a dose dependent manner. After preincubating the cells with *A. lividus* and *A. tricolor* extracts, we found that TNF-α, IL-6 and IL-1 genes expression were significantly decreased.

The result indicates that these plants possess antioxidant activity, anti-oxidative damage against AGEs and anti-inflammatory activity as well as anti-Aβ production.

The neuroprotective effects of these plants may be associated with their inhibitory actions via the RAGE/NF-kB pathway. However, additional studies are necessary to elucidate the downstream signaling pathways for the exact mechanisms involved in the AGEs signaling to gain more insight in the potential benefit of Amaranthus extracts. The present data may provide a new to the possibility of using these herbal extracts for potential therapy of AD.

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APPENDIX

LISTS OF ABBREVIATIONS

ΔΔCt	Delta-delta-Ct
°C	Degrees celsius
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromole
ABTS	2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AD	Alzheimer's disease
AGEs	Advanced glycation endproducts
APP	Amyloid precursor protein
Aβ	Amyloid-beta
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
cm^2	Square centimeter
CO_2	Carbon dioxide
Ct	Cycle threshold
DCFH-DA	2'-7'-Dichlorodihydrofluorescein diacetate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EU	Enzyme unit

FBS	Fetal serum albumin
g	Standard gravity
h	Hour
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
IL	Interleukin
l	Liter
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
MEM/EBSS	Minimum essential medium with Earle's balanced salts
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimole
mRNA	Messenger RNA
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappaB
nm	Nanometer
PBS	Phosphate buffered saline
pH	Hydrogen ion concentration
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RAGE	Receptor for advanced glycation endproducts
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RT	Room temperature
SC	Scavenging activities
SEM	Standard error of the mean
TBARS	Thiobarbituric acid reactive substances
TNF	Tumor necrosis factor
VCEAC	Vitamin C equivalent antioxidant capacity
w/v	Weight/volume
w/w	Weight/weight

VITA

My name is Warisa Amornrit. I was born on December 22, 1982, in Bangkok, Thailand. I graduated with bachelor degree (Medical Technology, second class honors) from Faculty of Allied Health Science, Chulalongkorn University and obtained master degree (Immunology) from Faculty of Medicine Siriraj Hospital, Mahidol University. Then I furthered my study at Faculty of Allied Health Science, Chulalongkorn University in doctoral degree program. My field of study is biochemistry and molecular biology of plant extract for possible use in Alzheimer's disease therapy.