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Neuroprotective effect of *Aster yomena* (Kitam.) Honda against hydrogen peroxide-induced oxidative stress in SH-SY5Y cells

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Abstract Oxidative stress is one of the contributors of neurodegenerative disorders including Alzheimer's disease. According to previous studies, *Aster yomena* (Kitam.) Honda (AY) possesses variable pharmacological activities including anti-coagulant and anti-obesity effect. In this study, we aimed to determine the neuroprotective effect of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda (EFAY) against oxidative stress. Therefore, we carried out 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide, lactate dehydrogenase (LDH), and 2',7'-dichlorofluorescin diacetate assays in SH-SY5Y neuronal cells treated with hydrogen peroxide (H_2O_2). H_2O_2-treated control cells exhibited reduced viability of cells, and increased LDH release and reactive oxygen species (ROS) production compared to normal cells. However, treatment with EFAY restored the cell viability and inhibited LDH release and ROS production. To investigate the underlying mechanisms by which EFAY attenuated neuronal oxidative damage, we measured protein expressions using Western blot analysis. Consequently, it was observed that EFAY down-regulated cyclooxygenase-2 and interleukin-1β protein expressions in H_2O_2-treated SH-SY5Y cells that mediated inflammatory reaction. In addition, apoptosis-related proteins including B-cell lymphoma-2-associated X protein/B-cell lymphoma-2 ratio, cleaved caspase-9, and cleaved-poly (ADP-ribose) polymerase protein expressions were suppressed when H_2O_2-treated cells were exposed to EFAY. Our results indicate that EFAY ameliorated H_2O_2-induced neuronal damage by regulating inflammation and apoptosis. Altogether, AY could be potential therapeutic agent for neurodegenerative diseases.

Keywords Apoptosis · Blotting · Cell culture techniques · Hydrogen peroxide · Oxidative stress · Western

Introduction

As the average life expectancy of the world population is increasing, the incidence of neurodegenerative diseases is also increasing in parallel. According to the World Health Organization, in 2016, about 50 million people lived with dementia and the number is estimated to reach 152 million in 2050 [1]. Neurodegenerative diseases are characterized by progressive dysfunction of neuronal system, in which damaged neurons lead to the decline of cognitive function [2]. Although the mechanism of pathogenesis in neurodegenerative diseases has not been fully revealed, oxidative stress is considered as an important risk factor [3]. Oxidative stress is generated in living organisms when the homeostasis between the production of free radicals and antioxidant defense system gets out of balance [4]. Previous studies demonstrated that oxidative stress exerts cellular damage including DNA injury, inflammation, and apoptosis of brain cells including neuronal, glial, and cerebrovascular cells [5-7]. High concentrations of reactive oxygen species (ROS) can cause damage to cellular lipids, proteins, and DNA because of strong reactivity [8]. Hydrogen peroxide (H_2O_2) is generated in mitochondria during oxygen consumption and reacts with ions of iron or copper to

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form hydroxyl radical (·OH), which can cause direct damage to proteins and DNA [9,10]. Furthermore, the brain is particularly susceptible to the action of ROS. The brain has high polyunsaturated fatty acids (PUFAs) content and ROS preferentially oxidizes PUFAs resulting in lipid peroxidation [11,12]. These facts imply that the brain produces a large amount of ROS compared to other organs and ROS can induce oxidative damage in the brain.

Aster yomena (Kitam.) Honda (AY) is a perennial plant, widely distributed in Asia. It is used as traditional medicine and edible herb in Korea. According to previous studies, AY possesses variable pharmacological activities such as anti-coagulant and anti-obesity effect [13,14]. It has been demonstrated that AY showed radical scavenging effect through *in vitro* assays against 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl (·OH), and superoxide (O_2^-) radicals [15]. According to Bae and Kim [16], ethyl acetate fraction of AY (EFAY) was the most effective fraction with antioxidative activity compared to other extract and fractions and it was believed that there were abundant active compounds in EFAY, with strong pharmacological activities [16]. Nevertheless, until date, none of the studies have examined the neuroprotective effect of EFAY against oxidative stress in neuronal cells. Hence, we aimed to investigate the protective effect of EFAY on H_2O_2-induced oxidative damage in SH-SY5Y neuronal cells.

Materials and Methods

Materials

Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin solution, fetal bovine serum (FBS), and trypsin-EDTA solution were obtained from Welgene Inc. (Daegu, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT) was purchased from Bio Basic Inc. (Toronto, Canada). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was from Sigma Chemical Co. (Saint Louis, MO, USA). Radioimmunoprecipitation (RIPA) buffer was purchased from Elpis Biotech. (Daejeon, Korea) and polyvinylidene fluoride (PVDF) membrane was obtained from Millipore Co. (Billerica, MA, USA). Primary and secondary antibodies were purchased from Cell Signaling Tech. (Beverly, CA, USA), Calbiochem Co. (San Diego, CA, USA), Bioss Inc. (Beijing, China), and Santa Cruz Biotech. (Santa Cruz, CA, USA).

Preparation of sample

AY was obtained from Gurye-gun (Jeollanam-do, Korea) and verified by Dr. K. Choi, Korea National Arboretum, Korea. Voucher specimen (LEE 2016-01) was deposited at Department of Plant Science and Technology, Chung-Ang University, Anseong, Korea. The leaf part of AY (1,645 g) was extracted with methanol (MeOH) at 65-75 °C and refluxed at the same time. After 8 times of repeated extraction, 393.9 g of AY MeOH extract was obtained. The partitioning of MeOH extract was conducted using n-hexane, dichloromethane, ethyl acetate, and n-butanol. Consequently, 4.2 g of EFAY was obtained.

Cell culture

The SH-SY5Y cell line was obtained from KCLB (Korean Cell Line Bank, Seoul, Korea). SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS and 100 units/mL penicillin-streptomycin. The humidified incubator was set at 37 °C and 5% CO_2/95% air. When the adherent cells reached approximately 80% confluence, they were harvested using 0.05% trypsin-EDTA in phosphate buffered saline (PBS, pH 7.4).

MTT assay

Harvested SH-SY5Y cells from T-75 flask were seeded at the density of 5×10^4 cells/mL in 96-well plate. After incubation for 24 h, the cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h and H_2O_2 (300 μM) was added followed by 24 h incubation. Subsequently, the cells were incubated with MTT solution (1 mg/mL) for 3 h and later solubilized by DMSO. The absorbance of each well was read at 540 nm [17].

Lactate dehydrogenase (LDH) release assay

LDH release assay was carried out employing an LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan). SH-SY5Y cells were harvested from T-75 flask and plated at a density of 5×10^4 cells/mL in 96-well plate followed by 24 h incubation. The cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h. Subsequently, H_2O_2 (300 μM) was added and the cells were incubated for 24 h. The supernatant was mixed with a reaction solution in a 96-well plate, and then incubated for 30 min at room temperature. The amount of LDH release was measured using a microplate reader at 490 nm.

DCFH-DA assay

SH-SY5Y cells were plated in 96-well black plate at a density of 5×10^4 cells/mL and incubated for 24 h. Before treating with H_2O_2 (300 μM) for 24 h, EFAY (2.5, 5, and 10 μg/mL) was pretreated with the cells for 2 h. After incubation with DCFH-DA (80 μM) for 30 min, fluorescence was measured at excitation - 480 nm and emission - 535 nm for 60 min.

Western blot analysis

Proteins were extracted using RIPA buffer supplemented with 1X protease inhibitor cocktail. These proteins were electrophoresed on 10-13% sodium dodecyl sulfate-polyacrylamide gels and then transferred to PVDF membranes. The membranes were blocked with 5% skim milk in PBS with tween 20 for 50 min and incubated overnight at 4 °C with primary antibodies. The primary antibodies used were: β-actin (1:1000; Cell Signaling Tech.); COX-2 (1:1000; Calbiochem Co.); IL-1β (1:1000; Bioss Inc.); cleaved caspase-9 (1:1000; Cell Signaling Tech.); cleaved PARP

(1:500; Cell Signaling Tech.); Bax (1:500; Santa Cruz Biotech.); and Bcl-2 (1:500; Santa Cruz Biotech.). After incubation with appropriate HRP-conjugated secondary antibodies for 1 h, immunoreactive proteins were visualized using a chemiluminescent imaging system (CoreBio, Seoul, Korea).

Statistical analysis

The results of this study are shown as the mean ± standard deviation (SD). The significance of data was determined by one-way analysis of variance (ANOVA) and Duncan's multiple range test for multiple comparisons. Statistical significance was considered when p was less than 0.05.

Results

Effect of EFAY on cell viability in SH-SY5Y cells treated with H_2O_2

The cell viability was measured to determine the protective effect of EFAY on H_2O_2-treated SH-SY5Y cells by performing MTT assay. As illustrated in Fig. 1, H_2O_2 reduced cell viability to 40.75% compared with 100% of normal cells. However, when SH-SY5Y cells were treated with EFAY, cell viability was restored to up to 45.50% at the concentration of 10 μg/mL.

Effect of EFAY on LDH release in SH-SY5Y cells treated with H_2O_2

LDH release assay was used to evaluate the protective effect of EFAY on neuronal damage induced by H_2O_2 in SH-SY5Y cells. A significant increase in the amount of released LDH was observed in H_2O_2-treated cells compared with normal cells (57.37%), as shown in Fig. 2. Meanwhile, the treatment with EFAY significantly decreased the release of LDH compared to control cells, indicating inhibition of neuronal cytotoxicity induced by H_2O_2 in SH-SY5Y cells.

Effect of EFAY on ROS production in SH-SY5Y cells treated with H_2O_2

In order to investigate the neuroprotective effect of EFAY against ROS production, DCFH-DA assay was carried out employing H_2O_2-treated SH-SY5Y cells. Figure 3 shows the effect of EFAY on H_2O_2 mediated production of ROS in SH-SY5Y cells (100%) compared with the non-treated group (79.38%). Treatment with 10 μg/mL of EFAY resulted in a decrease in ROS production by 85.99%. The results demonstrated that ROS production caused by H_2O_2 was blocked by EFAY with subsequent reduction in neuronal cell death.

Effects of EFAY on inflammation-related protein expressions in H_2O_2-induced SH-SY5Y cells

To evaluate the effect of EFAY on inflammatory processes in H_2O_2-treated SH-SY5Y cells, Western blot analysis was performed. As shown in Fig. 4, protein levels of interleukin-1β (IL-1β) and cyclooxygenase-2 (COX-2) were increased in the presence of H_2O_2 as compared to normal cells, indicating that H_2O_2 induced inflammation in neuronal cells. However, treatment with EFAY significantly down-regulated protein levels of IL-1β and COX-2 compared with H_2O_2-treated control cells. In particular, EFAY

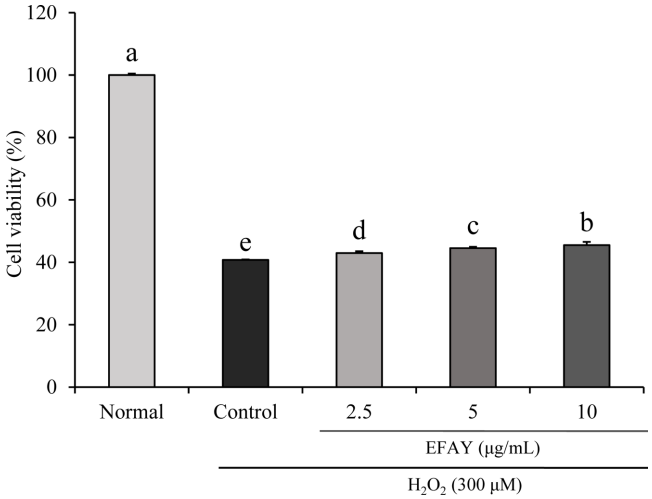

Fig. 1 Effect of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda on cell viability in SH-SY5Y cells treated with H_2O_2. SH-SY5Y cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h and H_2O_2 (300 μM) was added followed by 24 h incubation. H_2O_2-untreated cells served as a normal group. H_2O_2-treated cells served as a control group. Values are mean ± SD. [a~e]Means with the different letters are significantly different (p <0.05) by Duncan's multiple range test

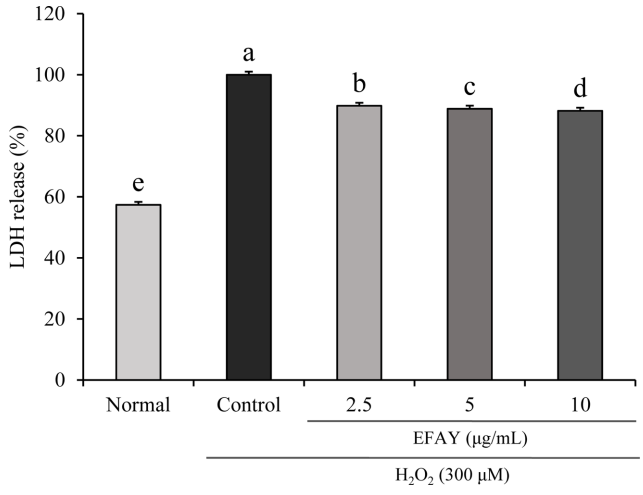

Fig. 2 Effect of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda on LDH release in SH-SY5Y cells treated with H_2O_2. SH-SY5Y cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h and H_2O_2 (300 μM) was added followed by 24 h incubation. H_2O_2-untreated cells served as a normal group. H_2O_2-treated cells served as a control group. Values are mean ± SD. [a~e]Means with the different letters are significantly different (p <0.05) by Duncan's multiple range test

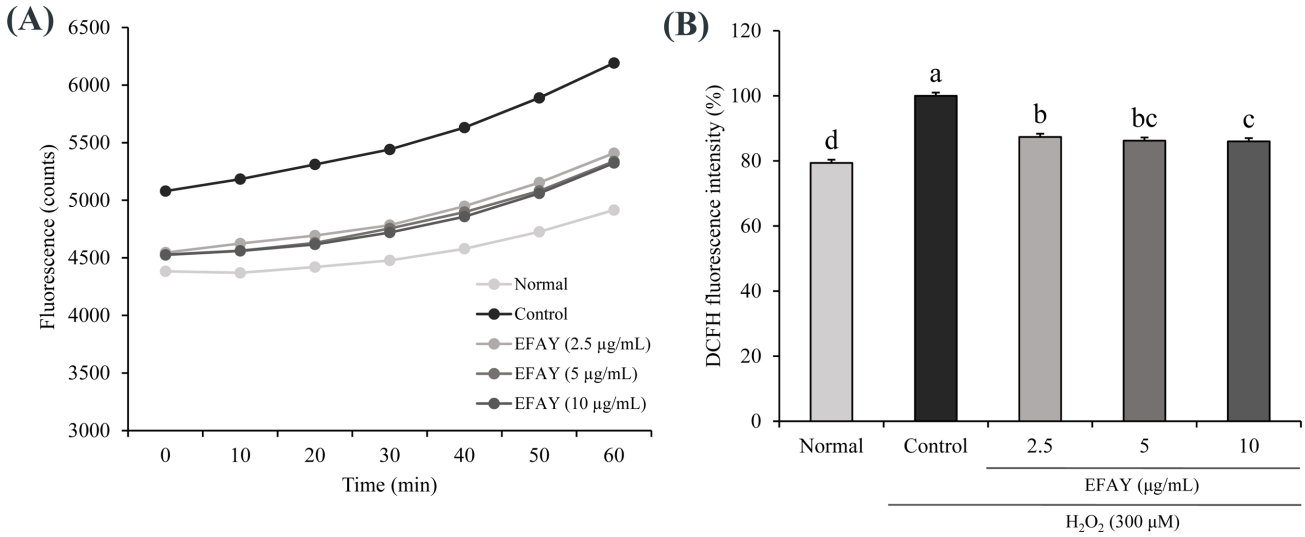

Fig. 3 Effects of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda on ROS production in SH-SY5Y cells treated with H_2O_2. (A) Change of ROS fluorescence during 60 min; (B) The intensity of ROS fluorescence at 60 min. SH-SY5Y cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h and H_2O_2 (300 μM) was added followed by 24 h incubation. H_2O_2-untreated cells served as a normal group. H_2O_2-treated cells served as a control group. Values are mean ± SD. [a~d]Means with the different letters are significantly different (p <0.05) by Duncan's multiple range test

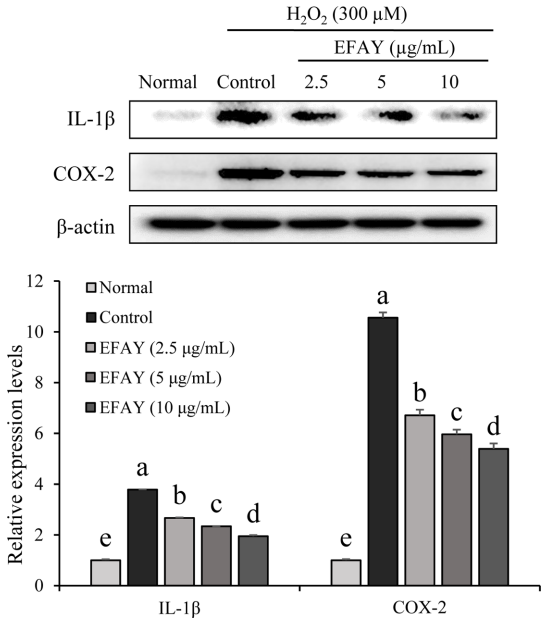

Fig. 4 Effects of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda on inflammation-related protein expressions in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h and H_2O_2 (300 μM) was added followed by 24 h incubation. β-actin was used as the loading control. H_2O_2-untreated cells served as a normal group. H_2O_2-treated cells served as a control group. Values are mean ± SD. [a~e]Means with the different letters are significantly different (p <0.05) by Duncan's multiple range test

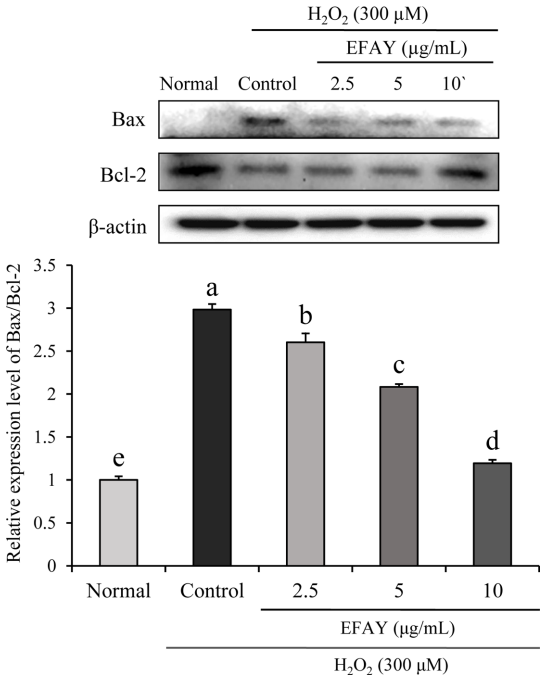

Fig. 5 Effect of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda on the level of Bax/Bcl-2 in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h and H_2O_2 (300 μM) was added followed by 24 h incubation. β-Actin was used as the loading control. H_2O_2-untreated cells served as a normal group. H_2O_2-treated cells served as a control group. Values are mean ± SD. [a~e]Means with the different letters are significantly different (p <0.05) by Duncan's multiple range test

effectively reduced COX-2 protein expression in H_2O_2-treated SH-SY5Y cells. These results suggested that EFAY protected H_2O_2-induced neuronal inflammation by down-regulating IL-1â and COX-2 expressions.

Effects of EFAY on apoptosis-related protein expressions in H_2O_2-induced SH-SY5Y cells

We investigated the effect of EFAY on apoptosis pathway in SH-

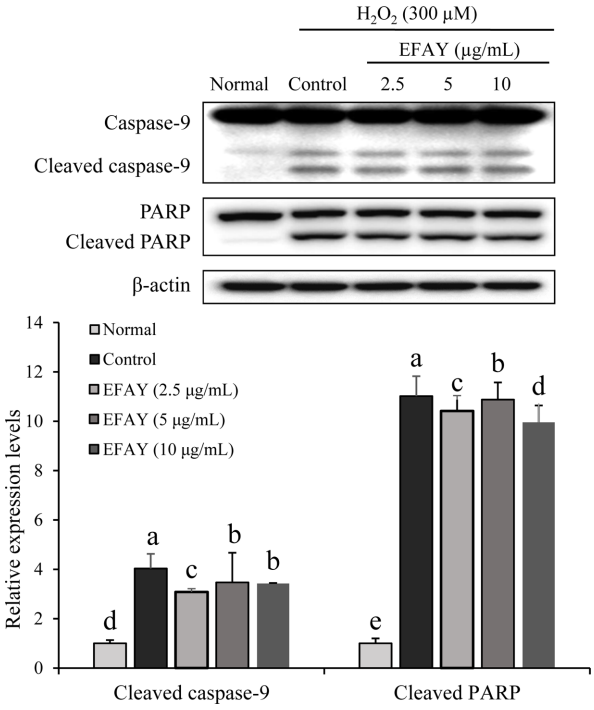

Fig. 6 Effects of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda on apoptosis-related protein expressions in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were treated with EFAY (2.5, 5, and 10 μg/mL) for 2 h and H_2O_2 (300 μM) was added followed by 24 h incubation. β-Actin was used as the loading control. H_2O_2-untreated cells served as a normal group. H_2O_2-treated cells served as a control group. Values are mean ± SD. $^{a-e}$Means with the different letters are significantly different (p <0.05) by Duncan's multiple range test

SY5Y cells under oxidative stress induced by H_2O_2. Figure 5 illustrates that H_2O_2 significantly up-regulated B-cell lymphoma-2-associated X protein (Bax)/B-cell lymphoma-2 (Bcl-2) ratio. Furthermore, the protein levels of cleaved caspase-9 and cleaved poly (ADP-ribose) polymerase (PARP) were down-regulated compared to the normal cells, as can be seen in Fig. 6. However, treatment with EFAY significantly down-regulated Bax/Bcl-2 ratio and up-regulated cleaved caspase-9 and cleaved PARP protein levels. These results indicated that the treatment of H_2O_2-induced damaged SH-SY5Y cells with EFAY suppressed apoptosis pathway by modulating Bax/Bac-2 ratio, cleaved caspase-9, and cleaved PARP.

Discussion

Neurodegenerative diseases are characterized by abnormal deposition of specific proteins in the brain, which could cause neuronal dysfunction and cell death [18]. For example, in the brain of a patient with Alzheimer's disease, amyloid beta plaque and the neurofibrillary tangles are commonly observed as pathological markers [19]. The major lesion of Parkinson's disease is selective degeneration of dopaminergic neurons around substantia nigra [20]. Previous studies demonstrated that the modification and abnormal deposition of proteins in the brain are closely related to oxidative stress resulting from overgeneration of ROS [21]. Therefore, the accumulation of oxidized protein is dependent upon the balance between protein oxidation and degradation of the oxidized protein. A battery of ROS, including free radicals (such as ·OH and O_2^-) and non-radical oxygen derivatives (such as H_2O_2 and $ONOO^-$), contributes in maintaining the balance by promoting protein oxidation [22]. H_2O_2, one of the oxygen derivatives, is generated from O_2^-, catalyzed by superoxide dismutase in the mitochondria. When the concentration of H_2O_2 exceeds that of antioxidant enzymes, H_2O_2 undergoes ferrous ion-catalyzed cleavage process by the Fenton reaction to produce ·OH [23]. The ·OH is one of the critical free radicals in the body because it could indiscriminately damage targets and mediate tissue damage consequently [24]. H_2O_2 can also induce neuronal cell death in SH-SY5Y cells *via* inflammatory and apoptotic processes [25,26]. Thereby, SH-SY5Y cells are widely used to study the pathogenesis of neurodegeneration. It was reported that H_2O_2 activated nuclear translocation of nuclear factor-kappa B (NF-κB) in SH-SY5Y cells and it up-regulated COX-2 and inducible nitric oxide synthase (iNOS) in the inflammatory pathway [26]. Our previous study also demonstrated that H_2O_2 activated apoptosis pathway in SH-SY5Y cells and several therapeutic agents reduced the cell death [5].

In our previous study, AY exhibited antioxidant effects by suppressing generation of free radicals including DPPH, ·OH, and O_2^- radicals [15]. It has been demonstrated that phenolic compounds from AY also had antioxidant activity. The phenolic compounds in AY are esculetin, caffeic acid, and apigenin [27]. Esculetin showed antioxidant activity by regulating glutathione system and lipid peroxidation in liver supernatants from male C57BL/6J mice [28]. Caffeic acid had antioxidative effects on intestinal ischemia-reperfusion injury in rats [29]. Especially, apigenin showed neuroprotective effects from H_2O_2-induced oxidative damage in SH-SY5Y cells [30]. However, the protective effect of AY from oxidative stress in neuronal cells has not been revealed yet. Therefore, we examined whether EFAY has a neuroprotective effect against oxidative stress that is induced by treating SH-SY5Y cells with H_2O_2.

It has been observed that the treatment of SH-SY5Y cells with H_2O_2 induced cellular toxicity and subsequently cell death [31]. We confirmed that H_2O_2 suppressed cell viability of SH-SY5Y cells based on MTT assay. According to previous study, ethanol extract from AY (EEAY) showed no cytotoxicity at concentration up to 500 μg/mL [32]. EEAY decreased adipocyte differentiation in the 3T3-L1 cells by suppressing adipogenic transcriptional factors without cytotoxicity at the concentration of 200 μg/mL [13]. Furthermore, our previous study unveiled that EFAY also reduced adipogenesis at a concentration up to 100 μg/mL [33]. Therefore, EFAY was treated under 100 μg/mL SH-SY5Y cells,

that had both no cytotoxicity and beneficial effect against oxidative stress. Furthermore, cellular damage by the cytotoxic stimulus, results in leaking of LDH, an enzyme normally found in cytosol into the extracellular fluid because of the disturbed cell membrane [34]. Our data showed that the release of LDH was increased in H_2O_2-treated SH-SY5Y cells and reversed by EFAY treatment in oxidative stress-induced SH-SY5Y cells. These findings revealed that EFAY exhibited a cytoprotective effect in SH-SY5Y cells against neuronal damage induced by H_2O_2.

Previous studies stated that treatment with H_2O_2 generates ROS (e.g., ·OH), which is one of the triggers leading to apoptotic signaling in SH-SY5Y cells [35,36]. The production of ROS could be monitored by measuring fluorescence emission following the conversion of DCFH-DA into DCF in the presence of ROS in cells [37]. Our results showed that H_2O_2 increased the ROS production in SH-SY5Y cells. However, EFAY significantly decreased the levels of ROS that were generated by H_2O_2. We previously confirmed antioxidative effect of AY by DPPH, ·OH, and O_2^- assay [15]. These data indicated that EFAY inhibited H_2O_2-induced oxidative stress by reducing ROS production in SH-SY5Y cells.

In the central nervous system (CNS), the up-regulation of IL-1β is a patterned response after CNS insults such as infection and trauma [38]. IL-1β stimulates NF-κB translocation into the nuclear region, which increases pro-inflammatory gene expressions including COX-2 and iNOS [39]. COX-2 is considered as a major regulator of the inflammatory process, and neuronal inflammation, which is a hall mark of cognitive dysfunction [40]. Many studies reported that COX-2 participated in prostaglandin synthesis, which mediated pathogenic mechanism such as inflammatory response [41,42]. In the brain, COX-2 also produces prostaglandins and subsequently induces neuronal toxicity, which can lead to neurological diseases [43,44]. Previously, it has been demonstrated that treatment of H_2O_2 activated phosphorylation of NF-κB at p65 in cells induced the productions of COX-2 and IL-1β [26]. In the present study, it was confirmed that H_2O_2 up-regulated the protein expressions of IL-1β and COX-2 in SH-SY5Y cells, suggesting that H_2O_2 activated inflammatory reaction in SH-SY5Y cells. On the contrary, treatment with EFAY down-regulated the expressions of IL-1β and COX-2. Earlier, it has been revealed that several phenolic compounds present in EFAY dysregulated interleukin-6 production in TNF-α stimulated MG-63 cells, proposing their role as anti-inflammatory agents [45]. Furthermore, EFAY lowered ovalbumin- and LPS-induced inflammation by down-regulating NF-κB and iNOS in RAW 264.7 cells [46]. These findings suggested that EFAY ameliorated H_2O_2-induced neuronal inflammation by regulating IL-1β and COX-2 expression.

When cells are exposed to cellular stress, pro-apoptotic BH3-only proteins directly activate pro-apoptotic proteins (e.g., Bax and Bak) and inactivate anti-apoptotic proteins (e.g., prosurvival Bcl-2-like proteins), leading to indirect activation of Bax and Bak. The activated Bax is oligomerized and translocated to the mitochondrial outer membrane, resulting in the release of cytochrome C [47]. Bcl-2 is one of the prosurvival Bcl-2-like proteins and protects apoptosis by interfering with the release of cytochrome C from mitochondria [48,49]. The released cytochrome C leads to caspases activation that plays a key role at the various stages of the apoptotic process [50]. Caspase-9, one of the caspase family, subsequently promotes downstream caspases and finally activates PARP leasing to ultimate cell death [51]. Excessive oxidative stress leads to neuronal apoptosis and H_2O_2 has been used as an inducer of oxidative stress in SH-SY5Y cells [52]. Previous studies demonstrated that treatment with H_2O_2 led to the formation of apoptotic features such as decreased Bax/Bcl-2 ratio, activation of the caspase cascade, and DNA fragmentation in SH-SY5Y cells [31,53,54]. Our results showed that H_2O_2-mediated neuronal apoptosis was activated in SH-SY5Y cells by up-regulating Bax/Bcl-2 ratio, whereas EFAY treatment down-regulated Bax/Bcl-2 ratio. Furthermore, up-regulated protein expressions of cleaved caspase-9 and cleaved PARP were observed subsequent to treatment of SH-SY5Y cells with H_2O_2. However, treatment with EFAY also down-regulated cleaved caspase-9 and cleaved PARP expressions, suggesting that EFAY exerts a protective effect against apoptosis in H_2O_2-treated neuronal cells. In addition, isoquercitrin, one of the active compounds of EFAY acts as an antioxidant and regulates apoptosis-related protein expressions of Bcl-2 and cleaved PARP in H_2O_2-treated RGC-5 cells [55]. Isoquercitrin was also reported to inhibit H_2O_2-induced apoptosis of EA.hy926 cells by mediating the PI3K/Akt/GSK3β signaling [56]. These results indicated that EFAY exerts a protective effect on the apoptotic effect against H_2O_2-induced neuronal damage in SH-SY5Y cells by down-regulating Bax/Bcl-2 ratio and caspase cascades.

Our findings proposed that EFAY recovered the cell viability and diminished release of LDH by suppressing the production of ROS against H_2O_2-mediated neuronal damage in SH-SY5Y cells. EFAY attenuated inflammation-related protein expressions including IL-1β and COX-2. Moreover, EFAY alleviated apoptosis by regulating Bax/Bcl-2 ratio, cleaved caspase-9, and cleaved PARP protein expressions. Based on the obtained results, the inhibitory effect of EFAY on inflammation and apoptosis pathways is apparent. In this study, we investigated neuroprotective effect of AY *via* inflammatory and apoptosis pathway. According to previous study, AY inhibited HO-induced growth inhibition in RAW 264.7 cells, which was associated with expression of nuclear factor erythroid 2-related factor-2 (Nrf-2) and heme oxygenase-1 (HO-1) [56]. Nrf-2 and its downstream mediator, HO-1, are important molecules in host defense against oxidative injury. Nrf-2 is a cytoprotective factor regulating the expression of genes coding for antioxidant proteins such as HO-1 [57]. HO-1 exerts beneficial effects through the elimination of toxic heme and production of biliverdin, iron ions, and carbon monoxide [58]. Since Nrf-2/HO-1 pathway is confused under neurodegenerative disease such as AD, the improvement of Nrf-2/HO-1 by AY

treatment leads to neuronal protection. Taken together, AY protects neuronal cells against oxidative stress and is proposed as an effective natural agent for the prevention of neurodegenerative disorders.

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