The Protective Effect of Amber Extract on Neurodegenerative Diseases

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Luo Yuening

The Protective Effect of Amber Extract on Neurodegenerative Diseases

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Luo Yuening

Supervisor: Prof. Kazuichi SAKAMOTO

Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8572, Japan

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Abbreviations

Aβ, amyloid-β (1-42)

AD, Alzheimer's disease

PD, Parkinson’s disease

6-OHDA, 6-Hydroxydopamine

BACE1, β-site amyloid precursor protein cleaving enzyme 1

LC3, Microtubule-associated protein 1A/1B-light chain 3

APP, Amyloid-β precursor protein

MAP, Microtubules Associated Proteins

FBS, Fetal bovine serum

MMP, Mitochondrial membrane potential

RA, All-trans-retinoic acid

DMSO, Dimethyl sufoxide

SDS, Sodium dodecyl sulfate

BHT, Butylated hydroxytoluene

SOD1, Superoxide dismutase 1

SOD2, Superoxide dismutase 2

CAT, Catalase

DPPH, 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazino radical

DCFDA, 2',7'-dichlorodihydrofluorescein diacetate

ERK, Extracellular signal-regulated kinase

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

ROS, Reactive oxygen species

DMEM/F12, Dulbecco's modified eagle medium/nutrient mixture F-12

Abstract

1. Background and Objective

The population in the world was over 7.8 billion in 2019. Among them, the population aged 60 years and over is expected to exceed 1 billion in 2019 and 2.1 billion by 2050 (World population ageing 2019). Currently, in some countries, like Japan, Switzerland, and South Korea, the average life expectancy is over 80 years old. Ageing had become a fundamental to some diseases, such as hearing loss, cataracts, diabetes, neurodegenerative diseases, etc. Unlike other diseases, almost no effective treatments or drugs are available for neurodegenerative diseases nowadays. In previous studies, some traditional Chinese medicinal plants, have been reported had effect on neurodegenerative diseases in vivo and vitro.

Here, I focused on amber, which is a fossil plant resin. Amber is commonly used as jewelry and decorative object. However, the medicinal properties of amber, such as mental stability, stoppage of bleeding, wound healing, and diuresis, have been mentioned in the book from thousand year ago in China. Furthermore, in previous studies, amber extract also has anti-allergic effects and melanin pigment reduction. In our laboratory, we had clarified that amber extract has physiological effects such as anti-inflammatory and reduction of fat accumulation. Although amber has been used to induce mental stability in humans for thousands of years, no research evidence is available to date. Therefore, this study aimed to investigate the protective effects of amber extract on neurodegenerative diseases.

In this study I focused on Alzheimer's disease and Parkinson's disease. Alzheimer's disease (AD), an irreversible and progressive brain disorder, is characterized by a slow impairment of memory and thinking abilities. Eventually, the patients lose their ability to perform basic daily tasks. Parkinson’s disease (PD) is the second most common progressive neurodegenerative disease after Alzheimer’s disease and is characterized by movement disorders such as resting tremor, bradykinesia, rigidity, and postural instability. Both of these diseases have a strong relative with ageing.

Therefore, in this study I used amyloid-β to establish an Alzheimer's disease model and used 6-OHDA to establish a Parkinson's disease model and aimed to elucidate the protective effects of amber extracts and the mechanism.

2. Results and discussion

2-1. The protect effect on Alzheimer's disease model in vitro

I used amyloid-β (1-42) and SH-SY5Y cell to establish an Alzheimer's disease model. The result show that cell viability was significantly increased in the amber extract group than in the amyloid-β (1-42) group. Thus, it can be concluded that amber extract protected the cells from amyloid-β (1-42)-induced cytotoxicity. Then, apoptosis assay used to analyze cell apoptosis and cleaved caspase 3 protein level has also been detected. The increased apoptosis cell and cleaved caspase 3 due to amyloid-β (1-42) were decreased by amber extract. This is suggested that amber extract can protect cell from amyloid-β (1-42)-induced cell apoptosis. Then, anti-apoptosis and apoptosis relative gene, Bcl-2, and Bax mRNA levels were measured. The expression of Bcl-2 decreased in amyloid-β (1-42) group and increased in amber extract group compare with amyloid-β (1-42) group. However, the expression of Bax showed no significant difference among the groups. Additionally, the mRNA ratio of Bax/Bcl2 was significantly increased in the amyloid-β (1-42) group, and it was decreased in amber extract group. Then, ROS generation was measured. ROS generation was increased in amyloid-β (1-42) group. However, the increased ROS were decreased in amber extract group. The results of ROS, Bcl-2, and Bax mRNA levels demonstrated that amber extract reduces apoptosis via the ROS-mediated mitochondrial pathway.

The BACE1 protein level increased in the amyloid-β (1-42) group and decreased in amber extract group. These results suggest that amber extract can downregulate amyloid-β (1-42)-induced BACE1 expression. In addition, autophagy relative proteins Beclin1 and LC3 have also been measured, amyloid-β (1-42) increased the protein levels of Beclin-1 and LC3 II. The protein levels of Beclin-1 and LC3 II were higher in the amber extract group than in the amyloid-β (1-42) group. Furthermore, the protein ratio of LC3 II/LC3 I show no different between amyloid-β (1-42) group and control group but increased in the amber extract group. It is suggested that amber extract can promote autophagy. These

results revealed that amber extract may protect neuronal cells from the toxicity of amyloid β.

2-2. The protect effect on Parkinson's disease model in vitro

I used 6-OHDA and SH-SY5Y cell to establish a Parkinson's disease model. The result of cell viability and apoptosis show that amber extract reduced 6OHDA-induced cell apoptosis. Then, ROS generation and phosphorylated ERK protein levels were measured. ROS generation and phosphorylated ERK protein levels were increased in 6-OHDA group. However, the increased ROS generation and phosphorylated ERK protein levels were decreased in amber extract group. These results suggest that amber extract regulates cell death via the ROS and ERK pathways.

Autophagy relative proteins Beclin1 and LC3 have also been measured. 6-OHDA decreased the protein levels of Beclin-1, but no effect on LC3 II. However, the protein levels of Beclin-1 and LC3 II were increased in the amber extract group. Furthermore, the protein ratio of LC3 II/LC3 I show no different between 6-OHDA group and control group but increased in the amber extract group. Which suggest that amber extract can promote autophagy. These results revealed that amber extract may protect neuronal cells from the toxicity of 6-OHDA.

These results indicate that amber can be potentially used as a novel treatment and prophylactic candidate for neurodegenerative diseases.

General Introduction

1.1 Aging and Diseases

According to worldwide research results by United Nations, the worldwide population in 2019 was 7.8 billion, of which about 1 billion of the population were over 60 years old. And in 2050, the worldwide population will increase to 9.7 billion, and the population aged 60 and over will reach approximately 2.1 billion(ONU 2019; ONU 2017). Usually, in humans, physiological aging is started by the age of 60. As shown in this data, it is not only the population but also the proportion of aging people were increased. It was approximately 1/8 in 2019 and increased to 1/5 in 2050. In other words, in 2050, one in five people will be over 60 years old. In addition, the aging rate of the country tends to be higher in developed countries and lower in developing countries(ONU 2019). For example, Japan entered an aging society in the 1970s (the population aged 65 and over exceeded 7% of the total population), and in 2007 it entered a super-aging society (the population aged 65 and over exceeded 21% of the total population), and in nowadays Japan had the most highest proportion of aging people in the world(Muramatsu et al. 2011) .

Entering an aging society effect a lot of things, such as enormous expenses for medical problems, etc. According to data from the Ministry of Health, Labor and Welfare, in 2019, Japan's medical expenses were 44389.5 billion yen, with an average of 351,800 yen per person, but the average number of aging people aged 65 and over was 754,200 yen per person, and the average number of aging people aged 75 and over was 930,600 yen per person, 2.65 times than the average.

Some diseases are more likely to occur in older ages. For example, Hearing loss, Cataracts, Diabetes, Cardiovascular diseases, Cancer, Neurodegenerative diseases, etc. and prevention of these age-relative diseases can lead to a healthy old age.

Neurodegenerative diseases are diseases in which neuronal cells undergo progressive degeneration and finally lead to death. The basic structure of the nervous system is neurons. Neurons usually do not replicate or replace themselves in humans. This means if they are damaged or dead, they cannot be replaced by the body. Moreover, unlike other diseases, the causes of neurodegenerative diseases are still unclear, and almost no treatment has been developed that can slow or stop the progression of the disease. Neurodegenerative diseases have several different kinds, such as, diseases that impair cognitive function (Alzheimer's disease, etc.), diseases that unable to exercise smoothly (Parkinson's disease, etc.), diseases that weaken muscles (Amyotrophic lateral sclerosis (ALS)), Diseases that difficult to balance the body (Spinocerebellar degeneration (SCD)).

In this study, I focused on age relative neurodegenerative diseases, Alzheimer's disease and Parkinson's disease.

1.2 Amber

In recent years, some Chinese traditional drugs have been proven to be neuroprotective. For example, Ginkgo, a maidenhair tree fruit, has been reported to have a protective effect against Alzheimer's disease and Parkinson's disease (Shi et al. 2009; Yin et al. 2021). Compare with the chemical substances, Chinese traditional drugs show the benefit on multifunctionality, less cost and few side effects.

Another traditional drug that has been used for a long time in China is amber, which is a fossil plant resin. Amber is commonly used as jewelry and decorative object. In the book, Lei's Treatise on the Preparation of Medicinal Substances (Lei Gong Pao Zhi Lun, around C.E. 5th century), the medicinal properties of amber, such as mental stability, stoppage of bleeding, wound healing, and diuresis, have been mentioned. Recently, research on amber is being extensively conducted, and its antiallergic action (Maruyama et al. 2018), suppression of melanin production, and promotion of collagen production have been reported (Suzuki et al. 2020). Although amber has been used to induce mental stability in humans for thousands of years, no research evidence is available to date.

Therefore, in this study, I aimed to investigate the protective effects of amber extracts on neurodegenerative diseases, Alzheimer's disease and Parkinson's disease, in vitro.

1.3 Aim for this study

1. Used amyloid-β to establish an Alzheimer's disease model and aimed to elucidate the protective effects of amber extracts and the mechanism.

2. Used 6-OHDA to establish a Parkinson's disease model and aimed to elucidate the protective effects of amber extracts and the mechanism.

Chapter I. Role of amber extract in protecting SHSY5Y cells against amyloid β1-42-induced neurotoxicity

2.1. Introduction

Alzheimer's disease (AD), an irreversible and progressive brain disorder, is characterized by a slow impairment of memory and thinking abilities. Eventually, the patients lose their ability to perform basic daily tasks. According to a worldwide survey conducted in 2006, the number of patients with AD was 26.6 million. By 2050, a four-fold increase in the number of patients is predicted, i.e., 1 in every 85 people worldwide will be living with the disease (Brookmeyer et al. 2007). Risk factors of the development of Alzheimer's disease are including old age, family history, head injury, gender, etc., but the most important risk factor is age. The prevalence of Alzheimer's disease is approximately 5.3% at the age of 65-74 and increases to 34.6% at the age of 85 and older(Mount et al. 2006; Anon 2021). Nevertheless, few treatment options are currently available for AD.

Amyloid-β precursor protein (APP) is a transmembrane protein that is enriched in neuronal tissues. When APP is cleaved by β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and γ-secretase, amyloid-β is generated (Bi et al. 2019), this is called the amyloidogenic pathway. In a normal brain, a balance exists between the production and clearance of amyloid-β. However, examination of the brain of patients with AD revealed abnormal aggregates and amyloid plaques (Roychaudhuri et al. 2009). Initially, a theory, called the amyloid cascade hypothesis, was proposed; this theory stated that the formation of neurofibrillary tangles, also known as amyloid plaques, in the brain is triggered by the deposition of amyloid-β, ultimately causing the death of the neuronal cells (Hardy et al. 2002). This theory has been supported by many researchers since its proposition. The amyloid hypothesis has become an important model to understand the etiology of AD, leading to the development of potential treatments. Later, an oligomer

hypothesis was also reported. According to this hypothesis, a small aggregation of amyloid-β (3 to n molecules) in neuronal cells causes more damage than the amyloid plaques, which is commonly speculated to be a critical cause of AD (Walsh et al. 2002).

Tau protein aggregation has also been found in the brain of patients with AD. Tau protein is a type II Microtubules Associated Proteins (MAP), mainly located in axons. In a normal condition, tau protein interacts with motor proteins and is hard to aggress, which is one kind of soluble protein. However, in the pathological condition, Tau protein can self-assemble into insoluble structures, also known as the paired helical filaments (PHFs)(Goedert 1999; Toral-Rios et al. 2020; Jouanne et al. 2017).

Many studies have reported the role of autophagy in amyloid-β degradation (Cho et al. 2014). Autophagy is a lysosome-dependent process that degrades proteins to release energy, indicating that autophagy might play an important role in AD (Uddin et al. 2018). According to the proposed hypothesis, treatment strategies targeting amyloid-β have been developed. One treatment strategy is to suppress the production of amyloid-β by inhibiting the catalytic activity of BACE1, and the other is to eliminate the aggregated amyloid-β by inducing autophagy.

The effects of many therapeutic substances on AD have previously been reported. Among them, the substances derived from plants have attracted attention (Zeng et al. 2019). Some particular traditional Chinese drugs, such as Huperzia serrata (Qian Ceng Ta), Rhizoma anemarrhenae (Zhimu), Ginkgo biloba (Ginkgoaceae), etc., show the potential ability in AD's therapy (Pei et al. 2020; Yan et al. 2007). Compared to chemical substances, traditional Chinese drugs/herbs have several advantages such as multifunctionality, few side effects, and prolonged effects. For example, in previous studies, Polygala tenuifolia (Wang et al. 2019) and Ginkgo biloba extract (Shi et al. 2009), the traditional Chinese medicinal plants, have been reported that suppress the production of amyloid-β and promote autophagy to protect neuronal cells from amyloid β-induced apoptosis. In the clinical trials using traditional Chinese medicine, the cognitive functions of AD patients have been significantly improved (Yu 2012; Dong et al. 2016).

Another traditional drug that has been used for a long time in China is amber, which is a fossil plant resin. Although amber has been used to induce mental stability in humans for thousands of years, no research evidence is available to date. Therefore, in this study, I aimed to investigate the protective effects of amber extracts on amyloid-β-induced neuronal cell apoptosis.

2.2. Materials and Methods

2.2.1 Materials

Amyloid-β1-42 (human) was purchased from the PEPTIDE INSTITUTE, INC(Osaka, Japan). Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mitotracker Red was purchased from Thermo Fisher(Waltham, MA, USA) Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT, USA). Penicillin, streptomycin, DPPH, and all-trans-retinoic acid (RA) were purchased from Wako (Tokyo, Japan).

Apoptosis/Necrosis detection kit (blue, green, red) and DCFDA/H2DCFDA Cellular ROS Assay Kit were purchased from Abcam (Cambridge, UK). Antibodies (BACE1, Beclin, caspase 3, cleaved caspase 3, and LC3A/B XP) and LumiGLO reagent were purchased from Cell Signaling Technology (Danvers, MA, USA). RNAiso Plus was purchased from Takara Bio (Shiga, Japan). The THUNDERBIRD SYBR qPCR Mix was purchased from TOYOBO (Tokyo, Japan).

2.2.2 Amber/Amyloid β (1-42)

Baltic amber (Kaliningrad, Russia) was crushed, powdered, and extracted in 50% ethanol at 40 ℃ for 1 h with stirring and double filtration. The extracted solution was depressurized and freeze-dried to form a powder (Kohaku Bio Technology Co. Ltd, Tsukuba, Japan). Amber extract powder was dissolved in dimethyl sufoxide (DMSO), and the mixture was stored at -80 ℃. Amyloid-β (1-42, Peptide Institute, Osaka, Japan) was dissolved in DMSO, and the resulting solution was stored at -80 ℃.

2.2.3 Cell culture

SHSY5Y cells were cultured in DMEM/F12 medium with 10% FBS, penicillin, and streptomycin, and subsequently, the medium was replaced by DMEM/F12 medium containing 10 μM RA and 1% FBS. The cells were incubated for 6 days, and the medium

was changed every 3 days. After 6 days of differentiation, cells were pre-treated with amber extract for 24 h, and following this, they were treated with amyloid-β and amber extract for another 24 h. Cells were maintained in a humidified atmosphere under 5% CO_2 at 37 °C.

2.2.4 MTT assay

Cell viability was measured using the MTT assay. The medium was replaced with 90% DMEM/F12 and 10% MTT, and the cells were cultured at 37 °C for 4 h. Subsequently, 10% sodium dodecyl sulfate (SDS) was added, and the mixture was kept at room temperature overnight. The absorbance was measured at 570 nm using a microplate reader (BioTek, Tokyo, Japan).

2.2.5 Apoptosis assay

The apoptosis/necrosis detection kit (blue, green, red) was used to measure apoptotic/necrotic/live cells. The cells were washed by buffer, and then replaced into Apoptosis buffer, for 20 mins at r.t., then, washed in buffer. The images were captured using a fluorescence microscope (KEYENCE, Tokyo, Japan).

2.2.6 DPPH assay

DPPH was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in EtOH as a concentration of 0.04 mg/ml. Vitamin C and BHT were dissolved in 70 % EtOH to concentration of 1 mg/ml and 2 mg/ml separately, amber solution was diluted into 2mg/ml by 70 % EtOH.

Vitamin C was diluted to 10, 20, 50, 100, 200 μg/ml, BHT and Amber were diluted to 100, 200, 500, 1000 and 2000 μg/ml. 20 μl of solution from Vitamin C, BHT and amber added into 96-well plate, following this, 180 μl of DPPH solution was added in each well. Then, incubation at room temperature for 30 min in Dark. The absorbance was measured at 517 nm using a microplate reader (BioTek, Tokyo, Japan).

20 μl of 70 % EtOH + 180 μl EtOH was considered as blank, 20 μl of 70 % EtOH +

180 μl DPPH was considered as positive control. The raw data was calculated as:

scavenging activity = (1 – Sample OD/Positive control OD) × 100 %

2.2.7 Reactive oxygen species (ROS) assay

DCFDA/H2DCFDA Cellular ROS Assay Kit was used to measure intracellular ROS concentration. The cells were washed by buffer, and then replaced into DCFDA, for 20 mins at 37℃. Then, washed in buffer. The images were captured using a fluorescence microscope (KEYENCE, Tokyo, Japan).

2.2.8 Mitochondrial membrane potential(MMP) assay

Mitotracker red Assay Kit was used to measure mitochondrial membrane potential(MMP). The medium was replaced by Mitotracker red solution, for 30 mins at 37℃. Then, replaced the solution with fresh medium. The images were captured using a fluorescence microscope (KEYENCE, Tokyo, Japan).

2.2.9 Western blotting

The cells were collected via centrifugation, washed twice with cold phosphate-buffered saline, and lysed using RIPA buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 0.1% SDS, 0.5% Na-deoxycholate, and protein inhibitor). After electrophoresis and transfer, the membrane was incubated with primary antibodies, including BACE1, Beclin, caspase 3, cleaved caspase3, and LC3 A/B XP. Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. The LumiGLO reagent was used to detect HRP. The bands were captured using an AE-9300 Ez-capture MG system (Atto Corporation, Tokyo, Japan). Protein expression levels were quantified using the ImageJ software.

2.2.10 Real-time PCR

Total RNA was extracted using RNAiso Plus, and 1 μg RNA was reverse transcribed using the PrimeScript™ RT reagent kit with gDNA eraser (Takara Bio).

Thermal Cycler Dice Real-Time System Lite (Takara Bio) was used for PCR amplification. Subsequently, 5 μL THUNDERBIRD SYBR qPCR Mix was added to the PCR tube along with 0.5 μL forward primer, 0.5 μL reverse primer, and 4 μL cDNA sample. The PCR amplification protocol was as follows: 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 30 s, followed by 95 ℃ for 15 s, 60 ℃ for 30 s, and 95 ℃ for 15 s.

Relative gene expression was normalized using β-actin gene expression.

The primer sequences used were:

Table1. The primer sequences used in this expriment

	Forward	Reverse
Bax	TCTGACGGCAACTTCAACTG	GGAGGAAGTCCAATGTCCAG
Bcl-2	ATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGGTACTCAGTC
SOD1	CAGTGCAGGTCCTCACTTTA	CCTGTCTTTGTACTTTCTTC
SOD2	TACGTGAACAACCTGAACGT	CAAGCCATGTATCTTTCAGTTA
CAT	AGGGGCCTTTGGCTACTTTG	ACCCGATTCTCCAGCAACAG
BACE1	TCTGTCGGAGGGAGCATGAT	GCAAACGAAGGTTGGTGGT
Beclin-1	CCATGCAGGTGAGCTTCGT	GAATCTGCGAGAGACACCATC
LC3	AACATGAGCGAGTTGGTCAAG	GCTCGTAGATGTCCGCGAT
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

2.2.11 Data analysis

All experiments were repeated at least thrice. The results are expressed as the mean ± SD. The one-way ANOVA post hoc tukey's test was used to compare data among the groups. $P < 0.05$ was considered statistically significant.

2.3. Results

2.3.1 Amber extract protects against Aβ (1-42)-induced cytotoxicity in SHSY5Y cells

Cell viability was measured using the MTT assay. Low concentrations of amber extract (below 15 µg/mL) showed no cytotoxicity. However, 20 and 25 µg/mL amber extract-treated groups showed significantly decreased cell viability compared to the control group (Fig. 1A).

Significant decrease were observed in the viability of cells treated with 1, 2, 4 µM amyloid-β1-42, then 2µM amyloid-β1-42 have been choosed in the further experiment (Fig. 1B).

A significant decrease was observed in the viability of cells treated with 2 µM amyloid-β1-42 as compared to that in the control group, and treatments with 5, 10, and 15 µg/mL amber extract showed a protective effect against amyloid-β1-42-induced cytotoxicity (Fig. 1C).

2.3.2 Amber extract decreases Aβ (1-42)-induced apoptosis in SHSY5Y cells

Cells treated with 2 µM amyloid-β1-42 showed a significant increase in cell death as compared to the control group cells, and the level of apoptosis was lower in the amber extract-treated than in the amyloid-β1-42 group (Figs. 2A & B). To confirm and investigate the signaling pathway of apoptosis, the level of cleaved caspase 3 protein was detected (Fig. 3A & B) and the mRNA levels of Bax and Bcl2 were also detected (Figs. 3C & D).

Compared with the levels in the control group, Bcl-2 mRNA levels were significantly decreased, and cleaved caspase 3 protein levels were increased in the amyloid-β1-42 group, suggesting that amyloid-β1-42 induces apoptosis through the caspase pathway.

A significant increase in Bcl-2 mRNA level and decrease in cleaved caspase 3 protein level were observed in the amyloid-β1-42 and amber extract-treated groups compared to

those in the amyloid-β1-42 group. Additionally, the expression of Bax showed no significant difference among the groups. However, the mRNA ratio of Bax/Bcl2 was significantly increased in the amyloid-β1-42 group, and it was decreased after amber extract treatment (Fig. 2E), suggesting that amber extract has an inhibitory effect on apoptosis.

2.3.3 Amber extract decreases Aβ (1-42)-induced ROS level in SHSY5Y cells

Then intracellular ROS were measured. Cells treated with 2 μM amyloid-β1-42 showed a significant increase in ROS levels as compared to the control group cells; however, the increased ROS levels due to amyloid-β1-42 were decreased after amber extract treatment (Figs. 4A & B).

Then, I measured the chemical scavenging activity of amber extract in vitro by DPPH assay. Vitamin C (Vc) and butylated hydroxytoluene (BHT) , two kinds of antioxidant reagent, were used as positive control. In the result, the scavenging effect of amber, in the low concentration of amber (used in cell experiment) show no chemical scavenging activity, but in high concentration of amber, 2000 μg/ml show a little chemical scavenging activity(Fig. 4C).

Subsequently, the expression levels of SOD1, SOD2, and CAT were analyzed (Figs. 3D, E & F). As compared to those in the amyloid-β1-42 group, the mRNA levels of SOD1 and SOD2 were significantly increased in the amber extract-treated group, suggesting that amber extract can promote the antioxidative capacity of cells.

2.3.4 Effect of amber extract on Mitochondrial membrane potential(MMP) in SHSY5Y cells

To confirm the effect of amber extract on the mitochondrial function, mitochondrial membrane potential were measured. Cells treated with 2 μM amyloid-β1-42 showed a significant decrease in MMP as compared to the control group cells; however, the decreased MMP by amyloid-β1-42 were recovery after amber treatment (Figs. 5A & B).

2.3.5 Effect of amber extract on BACE1 expression levels in SHSY5Y cells

To confirm the effect of amber extract on the amyloid-β production pathway, the expression levels of BACE1 mRNA and protein were quantified. Inconsistent with my expectations, BACE1 mRNA levels did not show a significant difference in any group (Fig. 6A). However, the BACE1 protein level significantly increased after amyloid-β1-42 treatment, and it decreased after amber extract treatment (Fig. 6B & C). These results suggest that amber extract can downregulate amyloid-β1-42-induced BACE1 expression.

2.3.6 Effect of amber extract on autophagy-related gene expression in SHSY5Y cells

To confirm the effect of amber extract on autophagy, the mRNA and protein expression levels of autophagy-related genes, Beclin1 and LC3, were measured. I observed that amyloid-β1-42 did not regulate Beclin-1 and LC3 gene expression at the mRNA level whereas amber extract significantly increased their mRNA levels (Figs. 7A & 8A). Unlike the mRNA level, amyloid-β1-42 increased the protein levels of Beclin-1 and LC3-II; the protein levels of Beclin-1 and LC3-II were higher in the amber extract-treated group than in the amyloid-β1-42 group (Figs. 7B, C & 8B, C). Furthermore, I observed no significant difference in the protein ratio of LC3II/LC3I, another marker for autophagy, between the amyloid-β1-42 and control groups. However, the protein ratio of LC3II/LC3I increased in the amber extract group (Fig. 8D). These results suggest that amber extract promotes autophagy in SHSY5Y cells.

2.4. Discussion

Cell viability was significantly increased in the amber extract-treated group than in the amyloid-β1-42 group. Thus, it can be concluded that amber extract protected the cells from amyloid-β1-42-induced cytotoxicity.

Amber is a fossilized plant resin, which has been used in Chinese traditional medicine to stabilize the mind, stop bleeding, and heal wounds. In recent years, many medicinal effects of amber, such as the antiallergic effect (Maruyama et al. 2018), suppression of melanin production, and promotion of collagen production (Suzuki et al. 2020), have been investigated. However, its effect on neuronal cell protection has not been elucidated till date. Therefore, the purpose of this study was to focus on the effects of amber on AD. To clarify this hypothesis, I used amyloid-β to establish an Alzheimer's disease model, and analyzed the effect of amber in this model.

The apoptosis assay was performed to confirm the effects of amber extract on cell death due to amyloid-β1-42. Since the number of apoptotic cells increased due to amyloid-β and decreased due to amber extract, caspase 3 and cleaved caspase 3 levels were measured. Caspase 3 and cleaved caspase 3 play distinct roles in amyloid-β-induced apoptosis (Harada et al. 1999). I observed that the cleaved caspase 3 level increased after amyloid-β1-42 treatment, which was consistent with previous studies (Awasthi et al. 2005; Wang et al. 2009; Zhang et al. 2010), and it decreased after amber extract treatment.

Several studies have shown that ROS is involved in the apoptotic mechanism of amyloid-β (Behl et al. 1994; Cheignon et al. 2018). I observed that intracellular ROS levels increased after amyloid-β1-42 treatment, which was consistent with the findings of previous studies (Wang et al. 2009; Zhang et al. 2010), and these levels decreased after amber extract treatment. SOD is an important antioxidant enzyme, which superoxides to oxygen and hydrogen peroxide. Three types of SOD have been characterized in mammals. SOD1 is found in intracellular cytoplasmic spaces, SOD2 is found in mitochondrial spaces, and SOD3 is found in extracellular spaces (Zelko et al. 2002). Specifically, I

measured the SOD1 and SOD2 levels. Their mRNA levels increased in the amber-treated group, which suggests that amber extract can scavenge ROS by upregulating SOD1 and SOD2, thereby decreasing oxidative stress and apoptosis.

Apoptosis occurs through two different pathways: intrinsic and extrinsic (Ghobrial et al. 2005). Since our result is related to mitochondria and ROS, there is a possibility that it may be related to the intrinsic (mitochondria) pathway. To prove this hypothesis, I measured the mRNA levels of the anti-apoptotic protein Bcl2 and the pro-apoptotic protein Bax. According to the results, the mRNA level of Bcl2 decreased in the amyloid-β1-42 group, but it increased in the amber-treated group. In contrast, Bax mRNA levels increased in the amyloid-β1-42 group and decreased in the amber-treated group at low concentrations. However, at high concentrations, Bax mRNA levels were the same in both groups; no significant difference was observed between the groups. These results prove that amber extract decreases apoptosis via the ROS-mediated mitochondrial pathway.

BACE1 is one of the enzymes that cleaves APP, which is a membrane protein that produces amyloid-β by BACE1 and γ-secretase (Fig.9). An increase in amyloid-β is associated with the development of AD (Bi et al. 2019), thus suggesting the possibility of AD exacerbation by promoting the amyloid-β-APP pathway (Son et al. 2016). Therefore, the regulation of BACE1 is a strategy for AD therapy (Toh et al. 2016). In this study, the effect of amber extract on BACE1 mRNA and protein expression in the amyloid-β-APP pathway was evaluated. I observed that amyloid-β1-42 and amber extract did not affect the mRNA level of BACE1; however, the level of BACE1 protein increased due to amyloid-β1-42; the protein level was decreased due to amber extract in a concentration-dependent manner. These results suggest that amber extract can inhibit the amyloid-β-APP pathway. In several clinical studies, BACE1 inhibitor has been shown to decrease amyloid-β level; however, no pathological improvement has been observed (Vassar 2014; Moussa 2017). Additionally, it has been reported that BACE1 inhibitors may lead to abnormalities in neuronal functions. Hence, it is very important to maintain a balance

between a reduction in BACE1 level and maintenance of neuronal functions. Low concentrations of amber extract maintained a similar BACE1 level in the amber-treated group as in the control group; hence, low concentrations may have a better effect on neurological protection than the high concentration.

In a previous study, amyloid β was shown to induce autophagy (Cho et al. 2014). However, the cell's autophagic response to amyloid-β production may not be able to completely eliminate amyloid-β. Incomplete elimination of amyloid-β leads to neuronal cell apoptosis and eventually results in the onset of AD (Watson et al. 2005). Therefore, the regulation of autophagy is a new possibility for AD treatment. In this study, amyloid-β1-42 increased autophagy in SHSY5Y cells; this observation was consistent with that in previous studies (Singh et al. 2017; Lv et al. 2009). In addition to this, amber promoted autophagy. Amber shows a similar effect on autophagy as other plant extracts, such as tenuifolin (Cao et al. 2016).

Previous studies have shown that overexpression of Beclin-1 can activate autophagy and improve the etiology of neurodegeneration (Swaminathan et al. 2016). However, in acute nutritional deficiency conditions, Bcl-2 can bind to Beclin1 and inhibit autophagy. Bcl2 regulation may help maintain autophagy and prevent cell death from excessive autophagy (Pattingre et al. 2005). In this study, all experiments were conducted in the presence of FBS. Therefore, it can be considered that autophagy may not be inhibited, which is also proved by the result of LC3II ratio. Additionally, amber decreased apoptosis, which suggests that amber may promote autophagy to decrease apoptosis.

In summary, amber extract protected SHSY5Y cells from amyloid-β1-42-induced apoptosis. Additionally, it decreased intracellular ROS level and BACE1 gene expression and promoted autophagy. These results indicate that amber may improve the etiology of neurodegeneration.

Chapter II. Protect effect of amber extract on human dopaminergic cells against 6-hydroxydopamine-induced neurotoxicity

3.1. Introduction

3.1.1 Parkinson's disease

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease after Alzheimer's disease and is characterized by movement disorders such as resting tremor, bradykinesia, rigidity, and postural instability (Hoehn et al. 1967). The prevalence of PD increases with age, affecting approximately 1% of the population having the age of more than 60 and 2%–4% of the population having the age of more than 80 (Pringsheim et al. 2014; Tysnes et al. 2017). Similar to many other neurological disorders, the cause of PD is still not completely understood.

3.1.2 α-synuclein and autophagy

The death of dopaminergic neurons in the substantia nigra pars compacta, striatal dopamine depletion, and presence of α-synuclein aggregates are the neuropathological hallmarks of PD (Dauer et al. 2003; Poewe et al. 2017). Mitochondrial dysfunction, neuroinflammation, and oxidative stress have also been reported to be associated with PD (Exner et al. 2012; Dias et al. 2013; Hirsch et al. 2009). Recently, α-synuclein aggregation has been identified as a therapeutic target for PD (Poewe et al. 2017). A previous study reported that α-synuclein has two types of degradation pathways: proteasomal and autophagic proteolysis (Webb et al. 2003; Dehay et al. 2010). Unlike unfolded proteins, aggregated proteins are usually resistant to unfolding, and their degradation is highly dependent on autophagy (Mizushima et al. 2011). The autophagy enhancer rapamycin has been shown to have a protective effect on PD-related dopaminergic

neurodegeneration (Dehay et al. 2010). According to a previous study, autophagy, which eliminates aggregated α-synuclein, may represent a potential neuroprotective strategy in PD (Zhang et al. 2012; Webb et al. 2003).

3.1.3 6-Hydroxydopamine induced Parkinson's disease model

6-Hydroxydopamine (6-OHDA) is commonly used to develop an in-vitro model of PD (Bové et al. 2005). The structure of 6-OHDA is similar to that of dopamine and it can easily oxidize into hydrogen peroxide and para-quinone (Przedborski et al. 2005). It can enter neuronal cells and generate intracellular reactive oxygen species (ROS), ultimately causing neuronal cell death (Blum et al. 2000).

In previous study show that amber extract can upregulation the SOD1 and SOD2 mRNA level and finally decreased the ROS level. Then, I wonder, amber extract may also have the potential ability on 6-OHDA induced neuronal cell death. In this study, I aimed to investigate the protective effects of amber extracts against 6-OHDA-induced neuronal cell apoptosis.

3.2. Materials and Methods

3.2.1. Materials

Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) and 6-OHDA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT, USA). Penicillin and streptomycin were purchased from Wako (Tokyo, Japan).

Apoptosis/Necrosis Detection Kit (blue, green, red) and DCFDA/H2DCFDA Cellular ROS Assay Kit were purchased from Abcam (Cambridge, UK). Antibodies such as Beclin, caspase 3, pERK, extracellular signal-regulated kinases (ERK), and LC3A/B XP and LumiGLO reagent were purchased from Cell Signaling Technology (Danvers, MA, USA). RNAiso Plus was purchased from Takara Bio (Shiga, Japan). The THUNDERBIRD SYBR qPCR Mix was purchased from Toyobo (Tokyo, Japan).

3.2.2. Amber extract

Baltic amber (Kaliningrad, Russia) was crushed, powdered, and extracted in 50% ethanol at 40 ℃ for 1 h with stirring and double filtration. The extracted solution was depressurized and freeze-dried to form a powder (Kohaku Bio Technology, Tsukuba, Japan). Amber extract powder was dissolved in dimethyl sufoxide (DMSO), and the mixture was stored at -80 ℃ until use.

3.2.3. Cell culture

The human neuroblastoma cell line SHSY5Y (Riken Cell Bank, Tsukuba, Japan) was used for the experiments. SHSY5Y cells were cultured in DMEM/F12 medium with 10% FBS. After 24 h, the cells were pre-treated with amber extract for 24 h, followed by treatment with 6-OHDA for another 24 h. The cells were maintained in a humidified atmosphere under 5% CO2 at 37 °C.

3.2.4. MTT assay

Cell viability was measured using the MTT assay. The medium was replaced with 90% DMEM/F12 and 10% MTT, and the cells were cultured at 37 °C for 4 h. Then, 10% sodium dodecyl sulfate (SDS) was added, and the mixture was kept at room temperature overnight. Absorbance was measured at 570 nm using a microplate reader (BioTek, Tokyo, Japan).

3.2.5. Apoptosis assay

SHSY5Y cells were cultured in DMEM/F12 medium with 10% FBS. After 24 h, the cells were pre-treated with amber extract for 24 h, followed by treatment with 6-OHDA for another 6 h. The apoptosis/necrosis detection kit (blue, green, red) was used to quantify apoptotic, necrotic, and live cells, the cells were washed by buffer, and then replaced into Apoptosis buffer, for 20 mins at r.t., then, washed in buffer. Fluorescence was measured using a fluorescence microplate reader (BioTek, Tokyo, Japan), with fluorescence intensities of Ex/Em = 490/525 nm, Ex/Em = 550/650 nm, and Ex/Em = 405/450 nm for apoptotic, necrotic, and live cells, respectively.

3.2.6. ROS assay

SHSY5Y cells were cultured in DMEM/F12 medium with 10% FBS. After 24 h, the cells were pre-treated with amber extract for 24 h, followed by treatment with 6-OHDA for another 6 h. DCFDA/H2DCFDA Cellular ROS Assay Kit was used to measure intracellular ROS concentration, the cells were washed by buffer, and then replaced into DCFDA, for 20 mins at 37°C. Then, washed in buffer. Fluorescence was measured using a fluorescence microplate reader (BioTek, Tokyo, Japan), at a fluorescence intensity of Ex/Em = 485/535 nm.

3.2.7. Western blotting

The cells were collected via centrifugation, washed twice with cold phosphate-buffered saline, and lysed using RIPA buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 10

mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 0.1% SDS, 0.5% Na-deoxycholate, and protein inhibitor). After electrophoresis and transfer, the membrane was incubated with primary antibodies including Beclin, caspase 3, pERK, ERK, and LC3 A/B XP. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h. LumiGLO reagent was used to detect HRP. The bands were detected using an AE-9300 Ez-capture MG system (Atto Corporation, Tokyo, Japan). Protein expression levels were quantified using the ImageJ software (NIH, USA).

3.2.8. Data analysis

All experiments were repeated at least thrice. The results are expressed as the mean ± SD. The one-way ANOVA post hoc tukey's test was performed to compare data among the groups. Statistical significance was set at a p-value of <0.05.

3.3. Results

3.3.1. Amber extract protects SHSY5Y cells against 6-OHDA-induced cytotoxicity

Cell viability was measured using the MTT assay. Low concentrations of amber extract (below 50 μg/mL) showed no cytotoxicity. However, cells treated with 100 μg/mL amber extract showed significantly decreased viability compared with the cells in the control group (Fig. 10A). Significant decrease were observed in the viability of cells treated with 50, 75μM 6-OHDA, then 75μM 6-OHDA have been choosed in the further experiment(Fig. 10B).

Compared with the control group, a significant decrease in viability was observed for cells treated with 75 μM 6-OHDA, whereas treatment with amber extract showed a protective effect against 6-OHDA -induced cytotoxicity (Fig. 10C).

3.3.2. Amber extract decreases 6-OHDA-induced ROS generation in SHSY5Y cells

Cells treated with 75 μM 6-OHDA showed a significant increase in ROS levels compared with the cells in the control group; however, the levels decreased with amber extract treatment (Fig. 11A).

I also measured pERK protein levels, which are related to ROS generation. The pERK/ERK ratio was increased in the 6-OHDA group compared with that in the control group cells and decreased in the amber extract treatment (Fig. 11B &C).

3.3.3. Amber extract decreases 6-OHDA-induced apoptosis in SHSY5Y cells

To evaluate the effect of amber extract on apoptosis, the apoptosis assay was performed and the protein expression levels of caspase 3 were measured. Cells treated with 75 μM 6-OHDA showed a significant increase in cell death and cleaved caspase 3 protein level when compared with cells in the control group. In contrast, apoptosis and cleaved caspase-3 protein levels were lower in the amber extract-treated group than in the 6-OHDA group (Figures 12A, B, and C).

3.3.4. Effect of amber extract on autophagy-related gene expression in SHSY5Y cells

To confirm the effect of amber extract on autophagy, the protein expression levels of autophagy-related genes, Beclin1, and LC3 were measured. I observed that 6-OHDA decreased the protein levels of Beclin-1, but had no significant effect on LC3-II levels. However, the protein levels of Beclin-1 and LC3-II were higher in the amber extract-treated group than those in the 6-OHDA group (Fig. 13A, B &14 A, B). Furthermore, I measured the LC3 II/LC3 I ratio as another marker for autophagy. Cells treated with 6-OHDA showed no significant differences in the LC3 II/LC3 I ratio compared with cells in the control groups. However, the LC3 II/LC3 I ratio was increased in the amber extract group (Fig. 14C). These results suggested that amber extract promotes autophagy in SHSY5Y cells.

3.4. Discussion

6-OHDA can take up by the dopamine transporter (DAT) into cell and generate active oxygen, which causes the death of neuron (Blum et al. 2000; Schober 2004). In our previous study, amber extract can upregulate the mRNA level of SOD1 and SOD2 to downregulate ROS generation (Luo et al. 2021). Therefore, the purpose of this study was to focus on the effects of amber on 6-OHDA induced generate active oxygen and neuron death.

Cell viability was not different in the amber extract group (up to 50 μg/mL); however, it was significantly increased in the amber pre-treatment group compared with that in the 6-OHDA group. Thus, I concluded that amber extract protected the cells from 6-OHDA-induced cytotoxicity. Moreover, this result considered that amber may has preventive effect under 6-OHDA cytotoxicity.

Apoptosis assay was performed to confirm the effects of amber extract on 6-OHDA induced cell death. The fluorescence intensity of apoptotic cells to healthy cells was increased in the 6-OHDA group, which is consistent with a previous study (Woodgate et al. 1999), and decreased in the amber pre-treatment group. Moreover, the protein levels of caspase 3 and cleaved caspase 3 were measured. The results of the amber group showed that cleaved caspase 3 showed a concentration-dependent decrease compared with that in the 6-OHDA group. This result suggested that amber has a protective effect against 6-OHDA-induced cell death.

In recent years, many studies have suggested that mitochondrial dysfunction (Gu et al. 1998) and oxidative stress (Dias et al. 2013; Annunziato et al. 2003) play important roles in PD. 6-OHDA can enter neuronal cells and generate intracellular ROS, causing neuronal cell death (Blum et al. 2000; Schober 2004). In my study, the increase in ROS in the 6-OHDA group weas clearly observed, this observation was consistent with that in previous studies (Bernstein et al. 2011). And increased ROS were decreased by the amber

treatment. Therefore, amber reduced cell apoptosis because of the possible effect of ROS scavenging.

In a previous study, oxidative stress induced by ROS generation led to apoptosis via caspase 3 activation (Annunziato et al. 2003). However, unlike cleaved caspase 3, ROS generation was not significantly different between the cells treated with various concentrations of amber extract. This suggested that in addition to its ROS scavenging effect, amber may exert its effect via another signaling pathway to protect cells from apoptosis.

ERK1/2 have been reported as important regulators of neuronal responses (Chu et al. 2004; Subramaniam et al. 2010). Activation of the ERK signaling pathway may be related to cell death (Chu et al. 2004). In vitro and postmortem studies have shown that ERK1/2 activation plays an important role in 6-OHDA-induced cell death (Kulich et al. 2001; Zhu et al. 2002). Therefore, I measured the protein levels of pERK and ERK. The results showed that the phosphorylation of ERK in the 6-OHDA group was significantly increased, consistent with a previous study (Chu et al. 2004), whereas amber inhibited the phosphorylation of ERK. This also suggested that amber extract decreases apoptosis via the ROS-mediated ERK signaling pathway.

In a previous study, α-synuclein aggregates were found to be a neuropathological hallmark of PD (Poewe et al. 2017). One of the important degradation pathways of α-synuclein is autophagic proteolysis (autophagy) (Webb et al. 2003; Dehay et al. 2010). Autophagy is an intracellular degradative process. It is usually occurring in stress conditions, such as the presence of abnormal proteins and nutrient deficiency, etc.(Yun et al. 2018). Rapamycin, a inducer of autophagy, has been shown to have a protective effect on PD-related dopaminergic neurodegeneration (Dehay et al. 2010). These studies showed that autophagy may represent a potential neuroprotective strategy in PD. In our previous study, amber was shown to promote autophagy (Luo et al. 2021). Therefore, I detected the protein levels of Beclin1 and LC3, which are autophagy-related markers. The results showed that Beclin1 expression in the 6-OHDA group decreased significantly, ,

whereas no significant change was observed in LC3II expression, which was consistent with that in previous study (He et al. 2018). However, the amber group showed a significant increase in Beclin1 and LC3II protein levels.

These results suggested that amber extract can protect neuronal cells against 6-OHDA-induced cell apoptosis by upregulating autophagy. Interestingly, an amber concentration of 50 μg/mL did not show the highest values of results in autophagy, whereas a concentration of 25 μg/mL induced higher protein levels of both Beclin1 and LC3II (Figs.13B & 14C). However, in LC3 II/LC3 I ratio, a marker of autophagosome formation, showed the same effect on 25 μg/mL and 50 μg/mL group (Fig.14C). This indicated that a low concentration of amber extract may have a better therapeutic effect.

As Fig.15 showed, 6-OHDA also can induce neuroinflammation, upregulation of the iNOS, and NO production (Zhang et al. 2011), which mean may have the relative with inflammatory, in this experiment, the NO production and iNOS protein level are not measured, but in our previous study, amber has the ability on anti-inflammatory (Tian et al. 2021). This is suggested that amber may also protect SHSY5Y cells from iNOS/NO signaling pathway.

In summary, amber extract protected SHSY5Y cells from 6-OHDA-induced apoptosis. Additionally, it decreased intracellular ROS and phosphorylation of ERK, and promoted autophagy. These results indicated that amber can be potentially used as a novel therapeutic and prophylactic candidate for PD.

General Discussion

4.1 Active compound in amber

Amber is a type of fossilized resin obtained from plants. Nowadays, the largest known deposit of amber is in the Baltic region. Baltic amber is the most popular and commonly used in folk medicine in Russia (Mills et al. 1984). In previous studies, many effects of Amber, such as the anti-allergic effect (Maruyama et al. 2018), promotion of collagen production, and suppression of melanin production (Suzuki et al. 2020) have been investigated. In our previous studies, we found that amber extract also has anti-inflammatory effect (Tian et al. 2021), the ability to reduce fat accumulation (Sogo et al. 2021).

Amber contains many bioactive compounds, such as monoterpenes succinic, monoterpenoids, sesquiterpenoids, and other compounds (Mills et al. 1984; Yamamoto et al. 2006). Monoterpenes and Monoterpenoids are a series of chemicals that are widely diffused in plants, the basic structure is composed of two bound isoprene units (Kabir et al. 2020). Monoterpenes have been reported to have antioxidant, anti-inflammatory, anti-diabetic, hepatoprotective, anti-tumor activities and can modulate autophagy (Ashrafizadeh et al. 2020). Monoterpenoids also have been reported to have anti-inflammatory effects (Bi et al. 2017) and one kind of treatment for chronic pain (Perri et al. 2020). Moreover, Sesquiterpenoids also have been reported to have anti-inflammatory effect (Hoang et al. 2016) and neuroprotective Effects (Xu et al. 2018). Some compounds have shown the protect effect on inflammatory, oxidant and neurol. However, there are still have many other unknown compounds in amber. Further study will focus on identifying all compound of the amber extract and elucidating the compound that have the potential effect on neuroprotective effects.

4.2 Neuroinflammatory

Neuroinflammatory also became an important part of neurodegenerative diseases, such as PD and AD (Hirsch et al. 2009; McKenzie et al. 2017; Gelders et al. 2018). In a previous study, neuroinflammatory has become an important role in AD (Leng et al. 2021), and the number of neuronal cells that died of neuroinflammation was far more than that of amyloid-β. In previous studies, kujigamberol, which is isolated from kuji amber, has anti-allergic effects (Kimura et al. 2012; Maruyama et al. 2018). Moreover, other bioactive compounds, such as monoterpenes succinic, monoterpenoids and sesquiterpenoids also been found to have anti-inflammatory effect (Ashrafizadeh et al. 2020; Bi et al. 2017; Hoang et al. 2016). According to these knowledge, it is suggested that the protective effect of amber extract may possible not only by one single component but also by the combined action of many different types of compounds.

In cell, also have one kind of apoptosis, called contagious apoptosis. This is one kind of process that one cell death can affect another cell nearby and lead to another cell death (Li et al. 2000; Friedlander 2003). And contagious apoptosis also happened in the neuron system. In previous study, during the neuronal cell death process, the level of inflammatory cytokines, such as IL-1β, TNF-α were increased (Yuan et al. 2000). In our study group, amber has anti-inflammatory effect on LPS induced inflammatory cell model (Tian et al. 2021). This is suggested that amber may have the ability on anti-neuroinflammatory. Furthermore, may not only stop the apoptosis on one cell, but also inhibitor the inflammatory cytokines, etc., to release. And finally decreased contagious apoptosis. Further study will focus on inflammatory cytokines, such as IL-1β, TNF-α, and iNOS, NO production which relative to inflammatory.

4.3 ROS

Reactive oxygen species (ROS) play an important role in our life. A low concentration of ROS is relative to some physiological functions such as cell cycle progression and proliferation, etc. but the high concentration of ROS can damage the protein, membranes,

and finally activation of the cell death processes. In mammals' cells have several different antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and peroxiredoxins (Redza-Dutordoir et al. 2016). In some preview studies, ROS was increased in some neurodegenerative diseases, such as AD and PD (Kehrer et al. 2015; Brieger et al. 2012; Ghezzi et al. 2017). In the results of ROS level, amyloid-β and 6-OHDA upregulated the ROS level which was decreased by amber extract. However, the results of the DPPH assay, show that a low concentration of amber (concentration used in experiments) has no chemical scavenging activity, this is suggested that amber decreased the ROS level are not by amber itself. The treatment of amber showed the upregulation of the mRNA level of SOD1 and SOD2 which are two kinds of important enzymes on antioxidants. This is suggested that amber may have the ability to suppress the abnormal ROS level by upregulation of the antioxidant enzymes. Because 90% of intracellular ROS are generation from mitochondria (Zhang et al. 2019). In Capture II the Bcl2 and Bax mRNA levels and mitochondrial membrane potential were measured, and results show that amber protects the cell from apoptosis through the mitochondria pathway. Further study will focus on the activation of Nrf2, which is relative to the ROS level and inflammatory, and the effect of amber on other antioxidant species in the cell.

4.4 Autophagy

Autophagy is an intracellular degradative process. It is usually occurring in stress conditions, such as the presence of abnormal proteins and nutrient deficiency, etc. In many neurodegenerative diseases, such as AD, PD, and Amyotrophic lateral sclerosis (ALS), etc., are found the abnormal protein aggregation (Guo et al. 2018). Dysregulated autophagy has a strong relative with many neurodegenerative diseases, therefore the regulation of autophagy become one of the potential therapeutic targets of neurodegenerative diseases (Menzies et al. 2015; Menzies et al. 2011; Sarkar 2013). Autophagy occurs through two different pathways: mTOR dependent and independent pathway (Sarkar 2013). The central core of mTOR has two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 promotes protein synthesis and is a

major negative regulator of autophagy, mTORC2 also plays a negative regulator of autophagy (Ballesteros-Álvarez et al. 2021). mTOR independent pathway is regulated other signaling pathway and finally activation autophagy. In this experiment, I measured Beclin1 and LC3, which are involved in autophagosome formation. As the results showed in amyloid-β model, amyloid-β, as one kind of protein that is easy to aggregation, increased autophagy, which was consistent with previous studies (Singh et al. 2017; Lv et al. 2009). And in 6-OHDA model, 6-OHDA, one kind of drug can take up by dopamine transporter into the cell and generate active oxygen, impair the function of autophagy, which was consistent with the previous study (He et al. 2018). And in both experiments, amber extract shows the ability to upregulation the protein level of Beclin1 and LC3II, which means promoted the autophagosome formation. Further study will focus on upstream signaling pathways, such as mTOR, AMPK, PI3K/AKT, and MAPK/Erk signaling pathways.

4.5 Chinese traditional medicine

In nowadays, many medicines for neurodegenerative diseases are being developed. For the best effect, sometimes require the combination of several different medicines. However because of the side effect, recently, the development of multifunctional drugs is gathering attention. These medicines should have at least 2 different effects. Such as cholinesterase inhibitor, anti-inflammatory, antioxidant, anti-apoptotic, activation of mitochondrial-dependent cell-survival genes and proteins, etc. (Youdim et al. 2005). Also, some medicine like levodopa, for the treatment of PD, and Donepezil, for the treatment of AD, may cause many side effects. However, Traditional Chinese medicine shows the potential ability on neurodegenerative diseases due to the multifunctional effect and few side effects (Hu et al. 2019). Some natural compounds isolated from Chinese traditional medicines, such as curcumin, epigallocatechin gallate, ginsenosides, etc. were reported to have the multifunctional effect, such as anti-inflammatory, antioxidant, autophagy regulation, etc. both in vitro and in vivo (Luo et al. 2019; Basnet et al. 2011; Jakubczyk et al. 2020; Choi et al. 2016). In this experiment, I used another traditional Chinese

medicine, Amber. Similar to other natural compounds, the extract of amber shows the effect on anti-apoptosis, antioxidant, autophagy regulation, and in amyloid-β induced cell death model show the effect on BACE1 production (Yang et al. 2005; Choi et al. 2016; Rezai-Zadeh et al. 2005). This suggests that amber may have multifunctional effect, however, still need more evidence to prove this theory.

Future prospects

In this experiment, I found and determined that amber extract can protect SHSY5Y cell from amyloid-β and 6-OHDA cytotoxicity by decreased the ROS level and promoted autophagy. However still have a lot of things that have not been clarified. I am looking forward to further experiments to perfect this theory.

The first is that identifying all compound of the amber extract. The amber solution used in the experiment are mixture. And which component is most effect is still unknow, because several different compounds show the potential ability on antioxidant and inflammatory, I expect to find all the active component, and find the best ratio of each component.

Secondly, I will focus on neuroinflammatory, which have relative with neurodegenerative diseases. I expect to measure some inflammatory cytokines, such as IL-1β, TNF-α, and iNOS, NO production.

Thirdly, I will focus on signaling pathway are involve in autophagy. Because Beclin1 and LC3 were clarified. So, I expect to measure upstream signaling pathway protein, such as mTOR, AMPK, etc. Also will to measured the protein level of aggregation protein, such as Tau protein and α-Synuclein.

Finally, in this experiment, I only used one kind of medicine to induce cell apoptosis. I plan to different kind of medicine to induce cell apoptosis, and finally use a mouse model to verify the efficacy of amber extract/component in vivo. This proves whether amber extract/component can use as a effective medicine for AD and PD.

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References

Annunziato, L., Amoroso, S., Pannaccione, A., et al. Apoptosis induced in neuronal cells by oxidative stress: Role played by caspases and intracellular calcium ions. In *Toxicology Letters*, 2003.139(2-3):125-33.

Anon. 2021 Alzheimer's disease facts and figures. *Alzheimer's and Dementia* 2021. 17(3).

Ashrafizadeh, M., Ahmadi, Z., Mohammadinejad, R., et al. Monoterpenes modulating autophagy: A review study. *Basic and Clinical Pharmacology and Toxicology* 2020. 126(1): p.9–20.

Awasthi, A., Matsunaga, Y., Yamada, T. Amyloid-beta causes apoptosis of neuronal cells via caspase cascade, which can be prevented by amyloid-beta-derived short peptides. *Experimental Neurology* 2005. 196(2):282-9.

Ballesteros-Álvarez, J., Andersen, J.K. mTORC2: The other mTOR in autophagy regulation. *Aging Cell* 2021. 20(8).

Basnet, P., Skalko-Basnet, N. Curcumin: An anti-inflammatory molecule from a curry spice on the path to cancer treatment. *Molecules* 2011. 16(6).

Behl, C., Davis, J.B., Lesley, R., et al. Hydrogen peroxide mediates amyloid β protein toxicity. *Cell* 1994. 77(6):817-27.

Bernstein, A.I., Garrison, S.P., Zambetti, G.P., et al. 6-OHDA generated ROS induces DNA damage and p53- and PUMA-dependent cell death. *Molecular Neurodegeneration* 2011. 6(1).

Bi, C., Bi, S., Li, B. Processing of mutant β-amyloid precursor protein and the clinicopathological features of familial alzheimer's disease. *Aging and Disease* 2019. 10(2):383-403.

Bi, X., Han, L., Qu, T., et al. Anti-inflammatory effects, SAR, and action mechanism of monoterpenoids from radix paeoniae alba on LPS-stimulated RAW264.7 cells. *Molecules* 2017. 22(5).

Blum, D., Torch, S., Nissou, M.F., et al. Extracellular toxicity of 6-hydroxydopamine on PC12 cells. *Neuroscience Letters* 2000. 283(3).

Bové, J., Prou, D., Perier, C., et al. Toxin-induced models of Parkinson's disease. *NeuroRx* 2005. 2(3).

Brieger, K., Schiavone, S., Miller, F.J., et al. Reactive oxygen species: From health to disease. *Swiss Medical Weekly* 2012. 142.

Brookmeyer, R., Johnson, E., Ziegler-Graham, K., et al. Forecasting the global burden of Alzheimer's disease. *Alzheimer's and Dementia* 2007. 186-91.

Cao, Q., Jiang, Y., Cui, S.Y., et al. Tenuifolin, a saponin derived from Radix Polygalae, exhibits sleep-enhancing effects in mice. *Phytomedicine* 2016. 23(14):1797-1805.

Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., et al. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. *Redox Biology* 2018. 14:450-464.

Cho, M.H., Cho, K., Kang, H.J., et al. Autophagy in microglia degrades extracellular β-amyloid fibrils and regulates the NLRP3 inflammasome. *Autophagy* 2014. 10(10):1761-75.

Choi, R.J., Roy, A., Jung, H.J., et al. BACE1 molecular docking and anti-Alzheimer's disease activities of ginsenosides. *Journal of Ethnopharmacology* 2016. 190.

Chu, C.T., Levinthal, D.J., Kulich, S.M., et al. Oxidative neuronal injury: The dark side of ERK1/2. *European Journal of Biochemistry* 2004. 271(11).

Dauer, W., Przedborski, S. Parkinson's disease: Mechanisms and models. *Neuron* 2003. 39(6).

Dehay, B., Bové, J., Rodríguez-Muela, N., et al. Pathogenic lysosomal depletion in Parkinson's disease. *Journal of Neuroscience* 2010. 30(37).

Dias, V., Junn, E., Mouradian, M.M. The role of oxidative stress in parkinson's disease. *Journal of Parkinson's Disease* 2013. 3(4).

Dong, L., May, B.H., Feng, M., et al. Chinese Herbal Medicine for Mild Cognitive Impairment: A Systematic Review and Meta-Analysis of Cognitive Outcomes. *Phytotherapy Research* 2016. 30(10):1592-1604.

Exner, N., Lutz, A.K., Haass, C., et al. Mitochondrial dysfunction in Parkinson′s disease: Molecular mechanisms and pathophysiological consequences. *EMBO Journal* 2012. 31(14).

Friedlander, R.M. Apoptosis and Caspases in Neurodegenerative Diseases. *New England Journal of Medicine* 2003. 348(14).

Gelders, G., Baekelandt, V., Van der Perren, A. Linking neuroinflammation and neurodegeneration in parkinson's disease. *Journal of Immunology Research* 2018. 2018:4784268.

Ghezzi, P., Jaquet, V., Marcucci, F., et al. The oxidative stress theory of disease: levels of evidence and epistemological aspects. *British Journal of Pharmacology* 2017. 174(12).

Ghobrial, I.M., Witzig, T.E., Adjei, A.A. Targeting Apoptosis Pathways in Cancer Therapy. *CA: A Cancer Journal for Clinicians* 2005. 55(3):178-94.

Goedert, M. Filamentous nerve cell inclusions in neurodegenerative diseases: Tauopathies and α-synucleinopathies. *Philosophical Transactions of the Royal Society B: Biological Sciences* 1999. 354(1386).

Gu, M., Cooper, J.M., Taanman, J.W., et al. Mitochondrial DNA transmission of the mitochondrial defect in Parkinson's disease. *Annals of Neurology* 1998. 44(2).

Guo, F., Liu, X., Cai, H., et al. Autophagy in neurodegenerative diseases: pathogenesis and therapy. *Brain Pathology* 2018. 28(1).

Harada, J., Sugimoto, M. Activation of caspase-3 in β-amyloid-induced apoptosis of cultured rat cortical neurons. *Brain Research* 1999. 842(2):311-23.

Hardy, J., Selkoe, D.J. The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science* 2002. 297(5580):353-6.

He, X., Yuan, W., Li, Z., et al. 6-Hydroxydopamine induces autophagic flux dysfunction by impairing transcription factor EB activation and lysosomal function in dopaminergic neurons and SH-SY5Y cells. *Toxicology Letters* 2018. 283:58-68.

Hirsch, E.C., Hunot, S. Neuroinflammation in Parkinson's disease: a target for neuroprotection? *The Lancet Neurology* 2009. 8(4).

Hoang, L.S., Tran, M.H., Lee, J.S., et al. Inflammatory inhibitory activity of sesquiterpenoids from Atractylodes macrocephala rhizomes. *Chemical and Pharmaceutical Bulletin* 2016. 64(5).

Hoehn, M.M., Yahr, M.D. Parkinsonism: Onset, progression, and mortality. *Neurology* 1967. 17(5).

Hu, Y., Wang, J. Interactions between clopidogrel and traditional Chinese medicine. *Journal of Thrombosis and Thrombolysis* 2019. 48(3).

Jakubczyk, K., Drużga, A., Katarzyna, J., et al. Antioxidant potential of curcumin—a meta-analysis of randomized clinical trials. *Antioxidants* 2020. 9(11).

Jouanne, M., Rault, S., Voisin-Chiret, A.S. Tau protein aggregation in Alzheimer's disease: An attractive target for the development of novel therapeutic agents. *European Journal of Medicinal Chemistry* 2017. 139.

Kabir, A., Cacciagrano, F., Tartaglia, A., et al. Analysis of monoterpenes and monoterpenoids. In *Recent Advances in Natural Products Analysis*, 2020, 274-286

Kehrer, J.P., Klotz, L.O. Free radicals and related reactive species as mediators of tissue injury and disease: Implications for Health. *Critical Reviews in Toxicology* 2015. 45(9).

Kimura, K.I., Minamikawa, Y., Ogasawara, Y., et al. Kujigamberol, a new dinorlabdane diterpenoid isolated from 85 million years old Kuji amber using a biotechnological assay. *Fitoterapia* 2012. 83(5).

Kulich, S.M., Chu, C.T. Sustained extracellular signal-regulated kinase activation by 6-hydroxydopamine: Implications for Parkinson's disease. *Journal of Neurochemistry* 2001. 77(4).

Leng, F., Edison, P. Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? *Nature Reviews Neurology* 2021. 17(3).

Li, M., Ona, V.O., Guégan, C., et al. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science* 2000. 288(5464).

Luo, H., Vong, C.T., Chen, H., et al. Naturally occurring anti-cancer compounds: Shining from Chinese herbal medicine. *Chinese Medicine (United Kingdom)* 2019. 14(1).

Luo, Y., Zhou, S., Haeiwa, H., et al. Role of amber extract in protecting SHSY5Y cells against amyloid β1-42-induced neurotoxicity. *Biomedicine and Pharmacotherapy* 2021. 141.

Lv, J., Jia, H., Jiang, Y., et al. Tenuifolin, an extract derived from tenuigenin, inhibits amyloid-β secretion in vitro. *Acta Physiologica* 2009. 196(4):419-25.

Maruyama, M., Kobayashi, M., Uchida, T., et al. Anti-allergy activities of Kuji amber extract and kujigamberol. *Fitoterapia* 2018. 127.

McKenzie, J.A., Spielman, L.J., Pointer, C.B., et al. Neuroinflammation as a Common Mechanism Associated with the Modifiable Risk Factors for Alzheimer's and Parkinson's Diseases. *Current Aging Science* 2017. 10(3).

Menzies, F.M., Fleming, A., Rubinsztein, D.C. Compromised autophagy and neurodegenerative diseases. *Nature Reviews Neuroscience* 2015. 16(6).

Menzies, F.M., Moreau, K., Rubinsztein, D.C. Protein misfolding disorders and macroautophagy. *Current Opinion in Cell Biology* 2011. 23(2).

Mills, J.S., White, R., Gough, L.J. The chemical composition of Baltic amber. *Chemical Geology* 1984. 47;1–2; 15-39.

Mizushima, N., Komatsu, M. Autophagy: Renovation of cells and tissues. *Cell* 2011. 147(4).

Mount, C., Downton, C. Alzheimer disease: Progress or profit? *Nature Medicine* 2006. 12(7).

Moussa, C.E.H. Beta-secretase inhibitors in phase I and phase II clinical trials for Alzheimer's disease. *Expert Opinion on Investigational Drugs* 2017. 1131-1136.

Muramatsu, N., Akiyama, H. Japan: Super-aging society preparing for the future. *Gerontologist* 2011. 51(4).

ONU. *World Population Prospects: The 2017 Revision*. 2017.

ONU. *World population prospects 2019*. 2019.

Pattingre, S., Tassa, A., Qu, X., et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005. 122(6):927-39.

Pei, H., Ma, L., Cao, Y., et al. Traditional Chinese Medicine for Alzheimer's Disease and Other Cognitive Impairment: A Review. *American Journal of Chinese Medicine* 2020. 48(3):487-511.

Perri, F., Coricello, A., Adams, J.D. Monoterpenoids: The Next Frontier in the Treatment of Chronic Pain? *J — Multidisciplinary Scientific Journal* 2020. 3(2): 195–214.

Poewe, W., Seppi, K., Tanner, C.M., et al. Parkinson disease. *Nature Reviews Disease Primers* 2017. 3.

Pringsheim, T., Jette, N., Frolkis, A., et al. The prevalence of Parkinson's disease: A systematic review and meta-analysis. *Movement Disorders* 2014. 29(13).

Przedborski, S., Ischiropoulos, H. Reactive oxygen and nitrogen species: Weapons of neuronal destruction in models of Parkinson's disease. *Antioxidants and Redox Signaling* 2005. 7(5–6).

Redza-Dutordoir, M., Averill-Bates, D.A. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochimica et Biophysica Acta - Molecular Cell Research* 2016. 1863(12).

Rezai-Zadeh, K., Shytle, D., Sun, N., et al. Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. *Journal of Neuroscience* 2005. 25(38).

Roychaudhuri, R., Yang, M., Hoshi, M.M., et al. Amyloid β-protein assembly and Alzheimer disease. *Journal of Biological Chemistry* 2009. 284(8):4749-53.

Sarkar, S. Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: Autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. *Biochemical Society Transactions* 2013. 41(5).

Schober, A. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell and Tissue Research* 2004. 318(1).

Shi, C., Zhao, L., Zhu, B., et al. Protective effects of Ginkgo biloba extract (EGb761) and its constituents quercetin and ginkgolide B against β-amyloid peptide-induced toxicity in SH-SY5Y cells. *Chemico-Biological Interactions* 2009. 181(1):115-23.

Singh, A.K., Bissoyi, A., Kashyap, M.P., et al. Autophagy Activation Alleviates Amyloid-β-Induced Oxidative Stress, Apoptosis and Neurotoxicity in Human Neuroblastoma SH-SY5Y Cells. *Neurotoxicity Research* 2017. 32(3):351-361.

Sogo, E., Zhou, S., Haeiwa, H., et al. Amber extract reduces lipid content in mature 3t3-l1 adipocytes by activating the lipolysis pathway. *Molecules* 2021. 26(15).

Son, S.M., Shin, H.J., Byun, J., et al. Metformin facilitates amyloid-β generation by β- And γ -secretases via autophagy activation. *Journal of Alzheimer's Disease* 2016. 51(4):1197-208.

Subramaniam, S., Unsicker, K. ERK and cell death: ERK1/2 in neuronal death. *FEBS Journal* 2010. 277(1).

Suzuki, S., Abe, J., Kudo, Y., et al. Inhibition of melanin production and promotion of collagen production by the extract of Kuji amber. *Bioscience, Biotechnology and Biochemistry* 2020. 84(3).

Swaminathan, G., Zhu, W., Plowey, E.D. BECN1/Beclin 1 sorts cell-surface APP/amyloid β precursor protein for lysosomal degradation. *Autophagy* 2016. 12(12):2404-2419.

Tian, Y., Zhou, S., Takeda, R., et al. Anti-inflammatory activities of amber extract in lipopolysaccharide-induced RAW 264.7 macrophages. *Biomedicine and Pharmacotherapy* 2021. 141.

Toh, W.H., Gleeson, P.A. Dysregulation of intracellular trafficking and endosomal sorting in Alzheimer's disease: Controversies and unanswered questions. *Biochemical Journal* 2016. 473(14):1977-93.

Toral-Rios, D., Pichardo-Rojas, P.S., Alonso-Vanegas, M., et al. GSK3β and Tau Protein in Alzheimer's Disease and Epilepsy. *Frontiers in Cellular Neuroscience* 2020. 14.

Tysnes, O.B., Storstein, A. Epidemiology of Parkinson's disease. *Journal of Neural Transmission* 2017. 124(8).

Uddin, M.S., Stachowiak, A., Al Mamun, A., et al. Autophagy and Alzheimer's disease: From molecular mechanisms to therapeutic implications. *Frontiers in Aging Neuroscience* 2018. 10(JAN):1–18.

Vassar, R. BACE1 inhibitor drugs in clinical trials for Alzheimer's disease. *Alzheimer's Research and Therapy* 2014. 6(9):89.

Walsh, D.M., Klyubin, I., Fadeeva, J. V., et al. Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 2002. 416(6880):535-9.

Wang, H., Xu, Y., Yan, J., et al. Acteoside protects human neuroblastoma SH-SY5Y cells against β-amyloid-induced cell injury. *Brain Research* 2009. 1283:139-47.

Wang, L., Jin, G., Yu, H., et al. Protective effect of Tenuifolin against Alzheimer's disease. *Neuroscience Letters* 2019. 705:195-201.

Watson, D., Castaño, E., Kokjohn, T.A., et al. Physicochemical characteristics of soluble oligomeric Aβ and their pathologic role in Alzheimer's disease. *Neurological Research* 2005. 27(8):869-81.

Webb, J.L., Ravikumar, B., Atkins, J., et al. α-synuclein Is Degraded by Both Autophagy and the Proteasome. *Journal of Biological Chemistry* 2003. 278(27).

Woodgate, A., MacGibbon, G., Walton, M., et al. The toxicity of 6-hydroxydopamine on PC12 and P19 cells. *Molecular Brain Research* 1999. 69(1).

Xu, J., Zhu, H.L., Zhang, J., et al. Sesquiterpenoids from Chloranthus anhuiensis with Neuroprotective Effects in PC12 Cells. *Journal of Natural Products* 2018. 81(6): 1391–1398.

Yamamoto, S., Otto, A., Krumbiegel, G., et al. The natural product biomarkers in succinite, glessite and stantienite ambers from Bitterfeld, Germany. *Review of Palaeobotany and Palynology* 2006. 140(1–2).

Yan, H., Li, L., Tang, X.C. Treating senile dementia with traditional Chinese medicine. *Clinical interventions in aging* 2007. 2(2):201–208.

Yang, F., Lim, G.P., Begum, A.N., et al. Curcumin inhibits formation of amyloid β oligomers and fibrils, binds plaques, and reduces amyloid in vivo. *Journal of Biological Chemistry* 2005. 280(7).

Yin, R., Xue, J., Tan, Y., et al. The Positive Role and Mechanism of Herbal Medicine in Parkinson's Disease. *Oxidative Medicine and Cellular Longevity* 2021. 2021:9923331.

Youdim, M.B.H., Buccafusco, J.J. Multi-functional drugs for various CNS targets in the treatment of neurodegenerative disorders. *Trends in Pharmacological Sciences* 2005. 26(1).

Yu, L. Chinese herbal medicine for patients with mild to moderate Alzheimer disease based on syndrome differentiation: a randomized controlled trial. *Journal of Chinese Integrative Medicine* 2012. 10(7):766–776.

Yuan, J., Yankner, B.A. Apoptosis in the nervous system. *Nature* 2000. 407(6805).

Yun, C.W., Lee, S.H. The roles of autophagy in cancer. *International Journal of Molecular Sciences* 2018. 19(11).

Zelko, I.N., Mariani, T.J., Folz, R.J. Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology and Medicine* 2002. 33(3):337-49.

Zeng, Q., Siu, W., Li, L., et al. Autophagy in Alzheimer's disease and promising modulatory effects of herbal medicine. *Experimental Gerontology* 2019. 119(January):100–110.

Zhang, L., Yu, H., Zhao, X., et al. Neuroprotective effects of salidroside against beta-amyloid-induced oxidative stress in SH-SY5Y human neuroblastoma cells. *Neurochemistry International* 2010. 57(5):547-55.

Zhang, L., Dong, Y., Xu, X., et al. The role of autophagy in Parkinson's disease. *Neural Regeneration Research* 2012. 7(2):141–145.

Zhang, W., Hu, X., Shen, Q., et al. Mitochondria-specific drug release and reactive oxygen species burst induced by polyprodrug nanoreactors can enhance chemotherapy. *Nature Communications* 2019. 10(1).

Zhang, Z.J., Cheang, L.C.V., Wang, M.W., et al. Quercetin exerts a neuroprotective effect through inhibition of the iNOS/NO system and pro-inflammation gene expression in PC12 cells and in zebrafish. *International Journal of Molecular Medicine* 2011. 27(2).

Zhu, J.H., Kulich, S.M., Oury, T.D., et al. Cytoplasmic aggregates of phosphorylated extracellular signal-regulated protein kinases in lewy body diseases. *American Journal of Pathology* 2002. 161(6).

Figures and legends

Fig.1

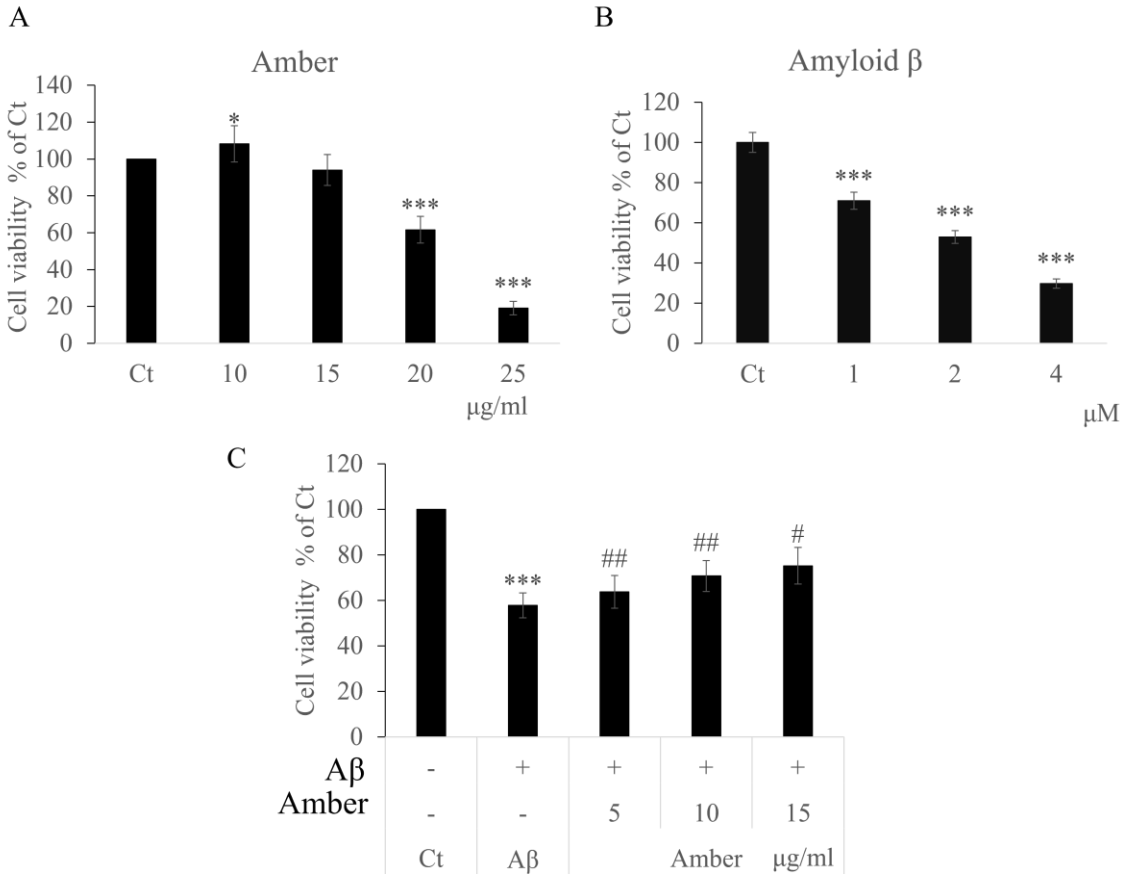
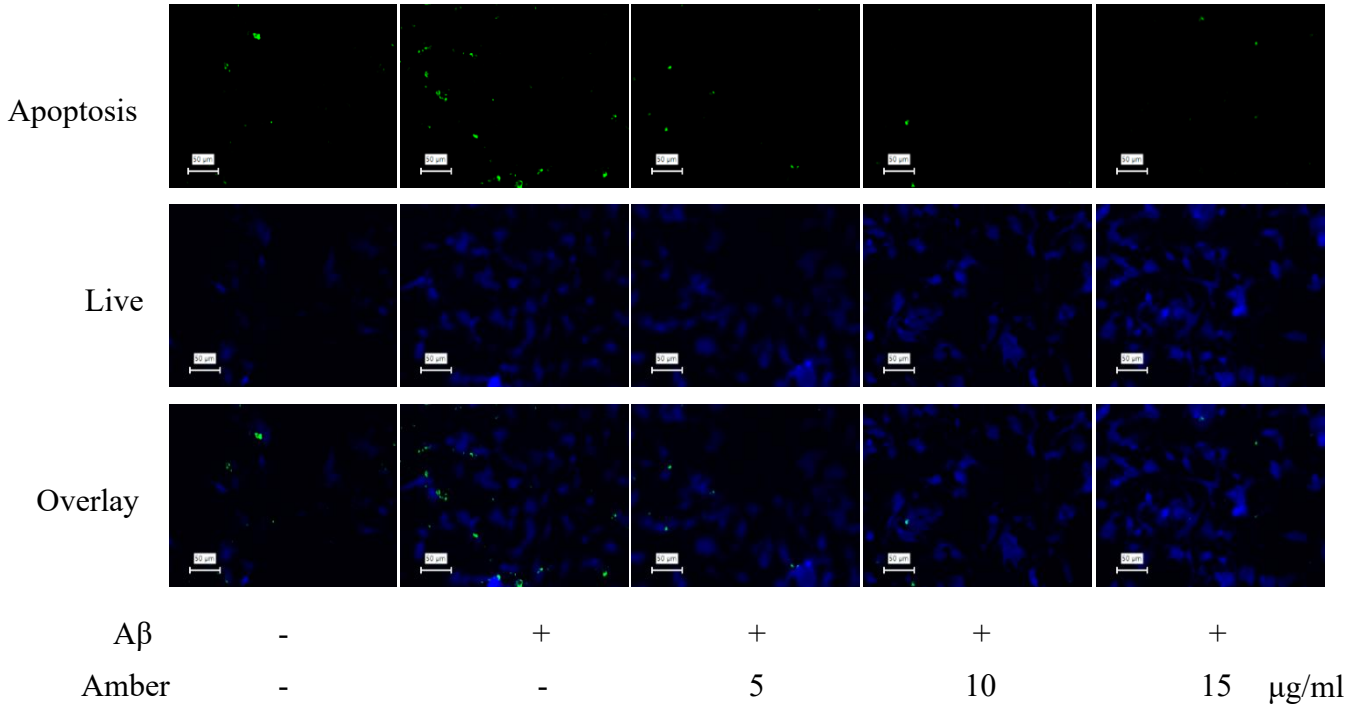


Fig.1. Effect of amber extract on amyloid-β (Aβ) (1-42)-induced cytotoxicity. Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) Cytotoxicity of amber extract. (B) Cytotoxicity of Aβ (1-42). (C) Effect of amber extract on Aβ (1-42)-induced cell death. Cell viability was determined using MTT assay. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, *** P<0.001 vs. Control, #P<0.05 vs. Aβ, ##P<0.01 vs. Aβ.

Fig.2

A



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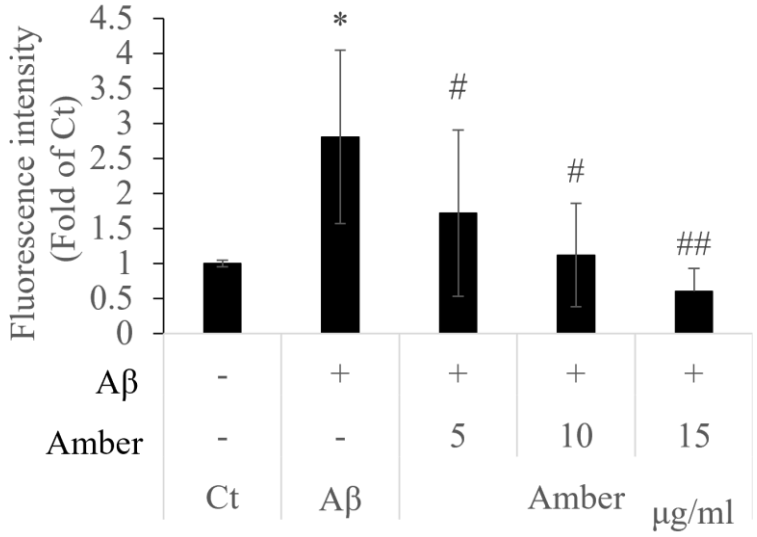


Fig. 2 Effect of amber extract on Aβ (1-42)-induced cell apoptosis. Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) Apoptosis analyzed using a fluorescence microscope. The

images were captured using fluorescence microscopy. Green: apoptotic cells, blue: live cells. (B) The fluorescence intensity of apoptosis was quantified using ImageJ. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, ** P<0.01 vs. Control, #P<0.05 vs. Aβ, ##P<0.01 vs. Aβ.

Fig.3

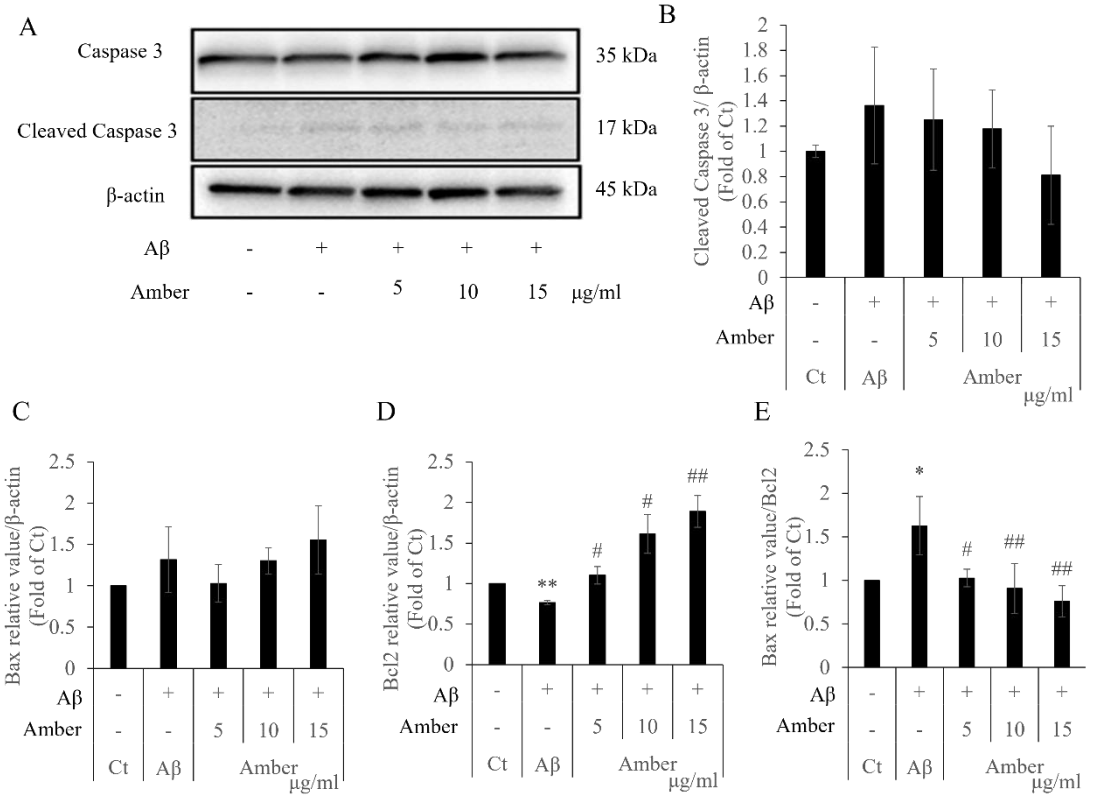


Fig. 3 Effect of amber extract on Aβ (1-42)-induced cell apoptosis. Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) Representative western blot image of caspase 3 and cleaved caspase 3. (B) The protein bands of cleaved caspase 3 were quantified using ImageJ. (C) mRNA levels of Bax, (D) mRNA levels of Bcl-2, (E) mRNA levels of Bax/Bcl-2 ratio, and they were normalized to the control. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, ** P<0.01 vs. Control, #P<0.05 vs. Aβ, ##P<0.01 vs. Aβ.

Fig.4

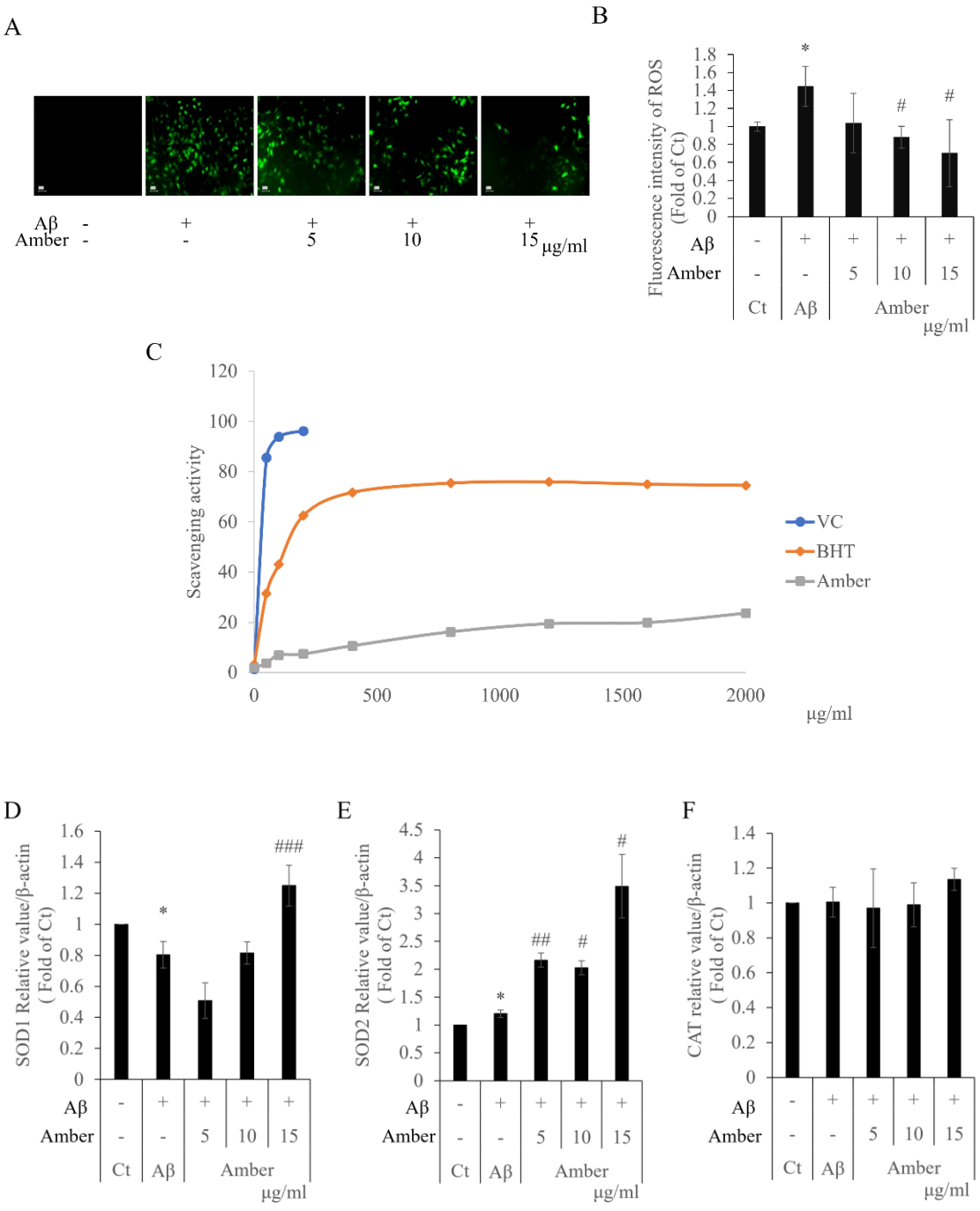
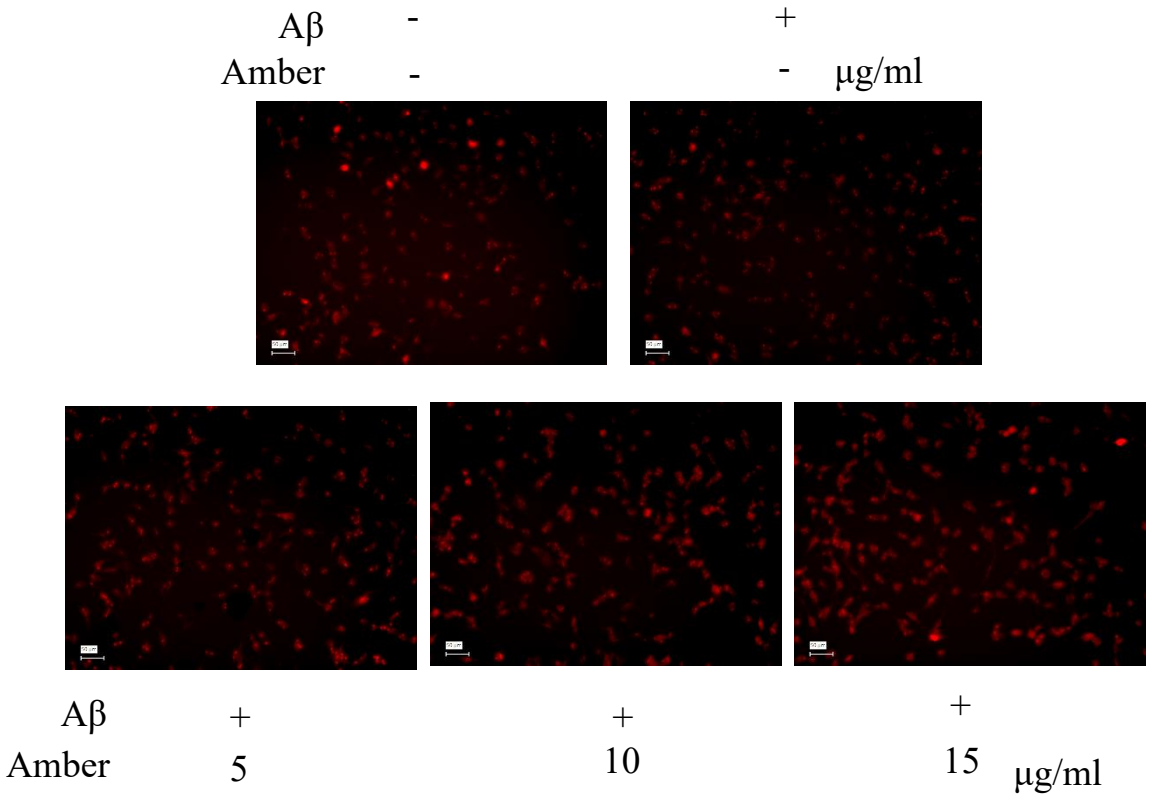


Fig.4 Effect of amber extract on Aβ (1-42)-induced intracellular reactive oxygen species (ROS) generation. Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) ROS analyzed using a fluorescence microscope. The images were captured using fluorescence microscopy. (B) The fluorescence intensity of intracellular ROS generation was quantified using

ImageJ. (C) Scavenging activity of Vitamin C, BHT and Amber extract on free radical procedure. (D) mRNA levels of SOD1, (E) mRNA levels of SOD2, (F) mRNA levels of CAT, and normalized to the control. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. Aβ, ##P<0.01 vs. Aβ, ###P<0.001 vs. Aβ.

Fig.5

A



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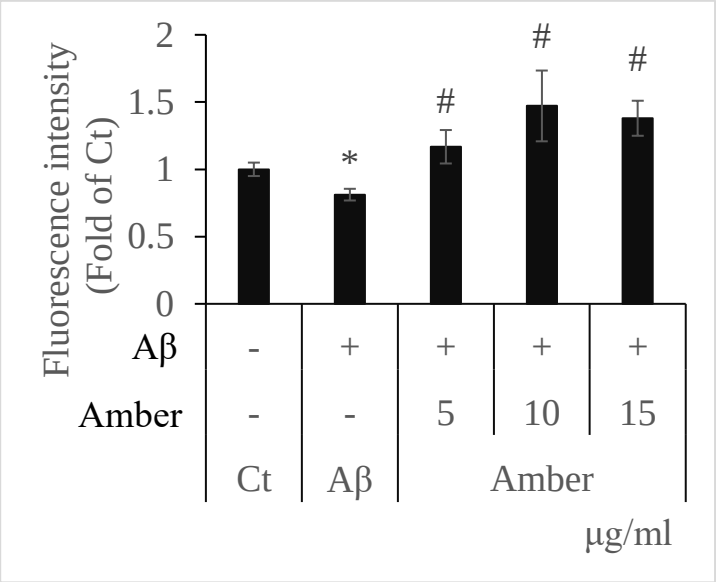

Fig. 5. Effect of amber extract on Mitochondrial membrane potential (MMP). Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) Mitochondrial membrane potential (MMP) analyzed using a fluorescence microscope. The images were captured using fluorescence microscopy. (B) The fluorescence intensity of MMP was quantified using ImageJ. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. Aβ.

Fig.6

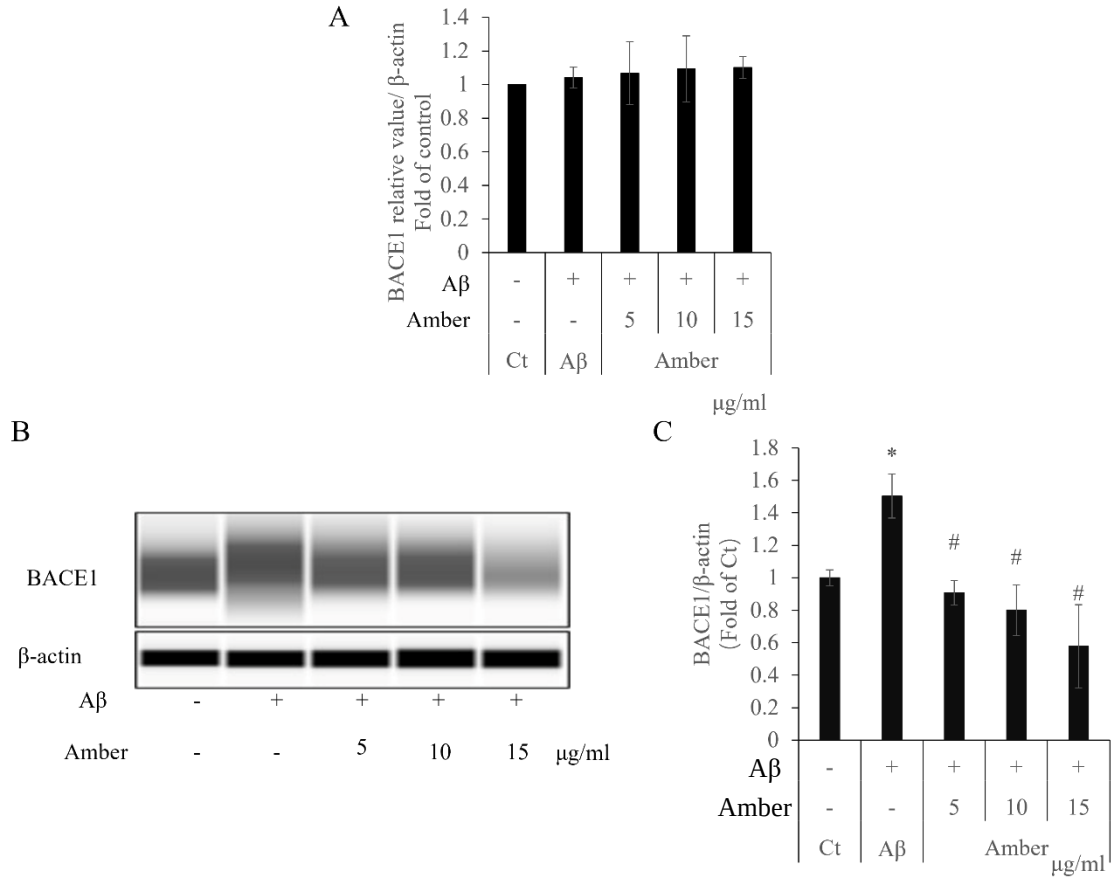

Fig.6 Effect of amber extract on BACE1 expression. Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) mRNA levels of BACE1, normalized to the control. (B) Representative western blot image of BACE1. (C) The protein bands were quantified using ImageJ. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. Aβ, ##P<0.01 vs. Aβ, ###P<0.001 vs. Aβ.

Fig.7

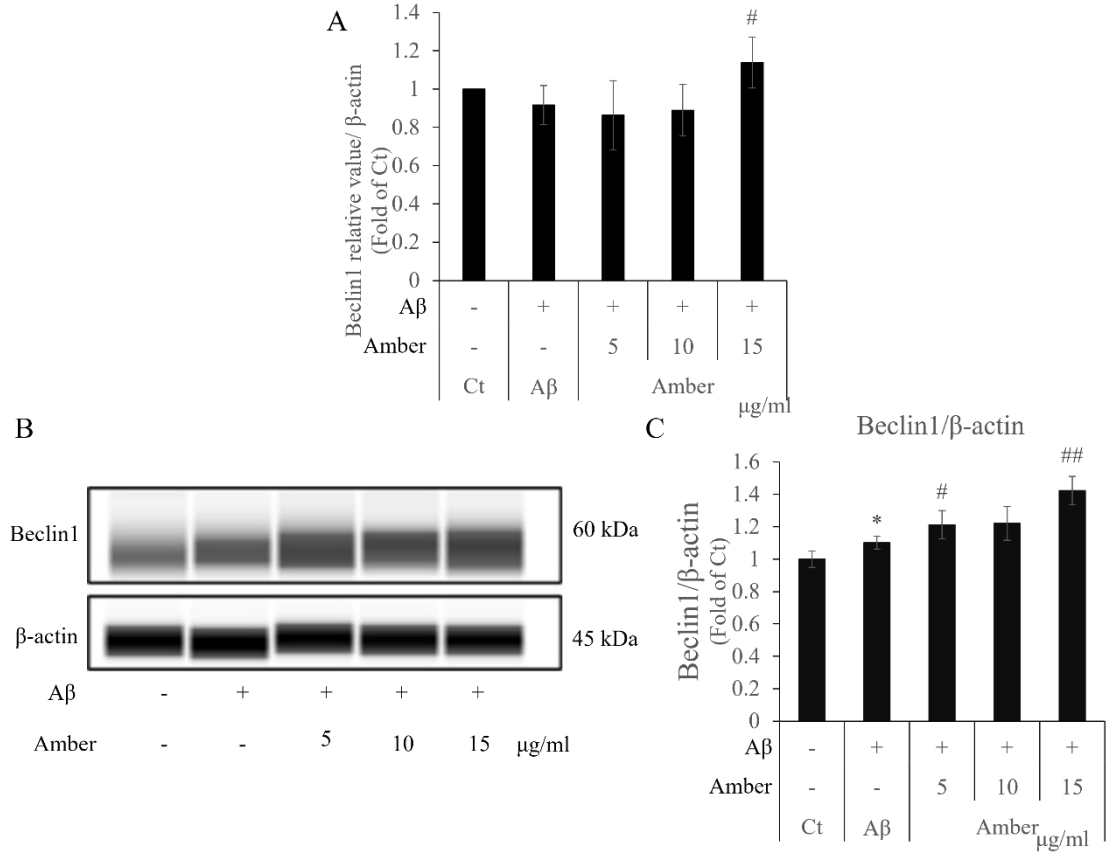

Fig.7 Effect of amber extract on autophagy gene expression. Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) mRNA levels of beclin1, normalized to the control. (B) Representative western blot image of Beclin1. (C) The protein bands were quantified using ImageJ. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. Aβ, ##P<0.01 vs. Aβ.

Fig.8

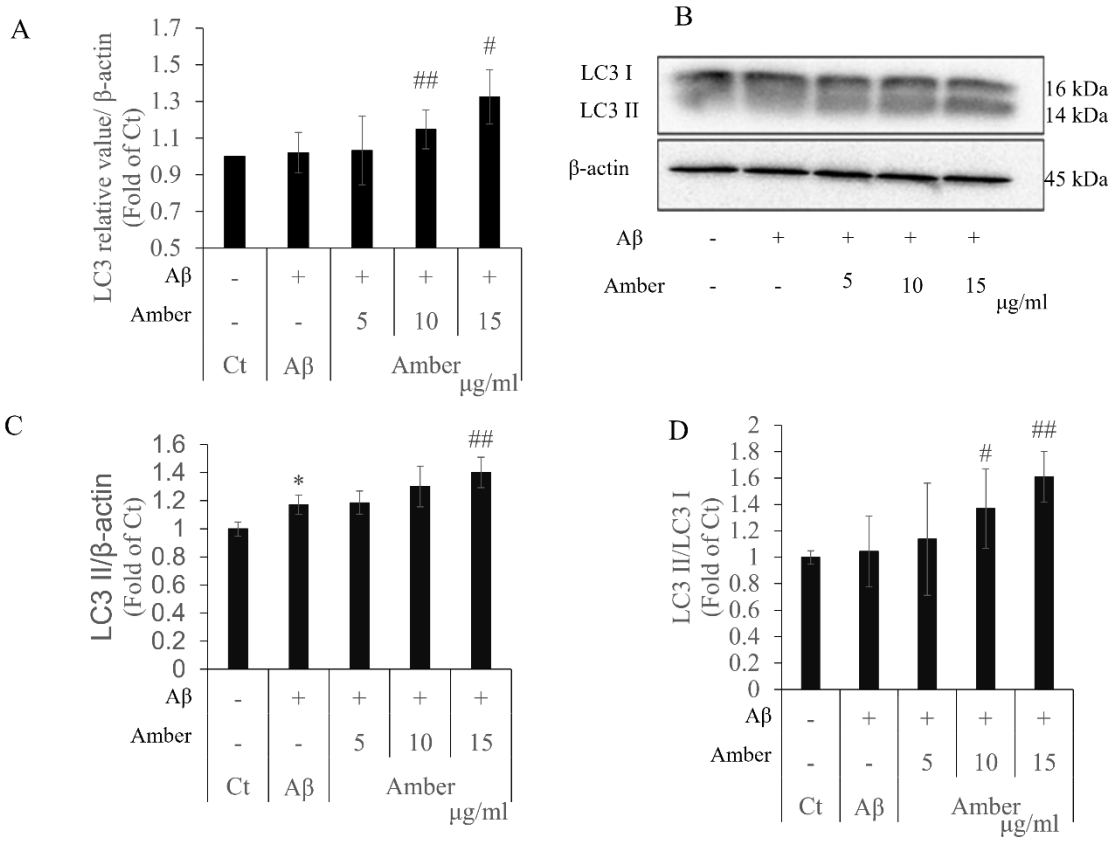

Fig.8 Effect of amber extract on autophagy gene expression. Control: (Aβ amber) - -, Aβ: (Aβ amber) + - (A) mRNA levels of LC3, normalized to the control. (B) Representative western blot image of LC3. (C) The protein bands were quantified using ImageJ. (D) The relative ratios of LC3 II/LC3 I bands density. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. Aβ, ##P<0.01 vs. Aβ.

Fig.9

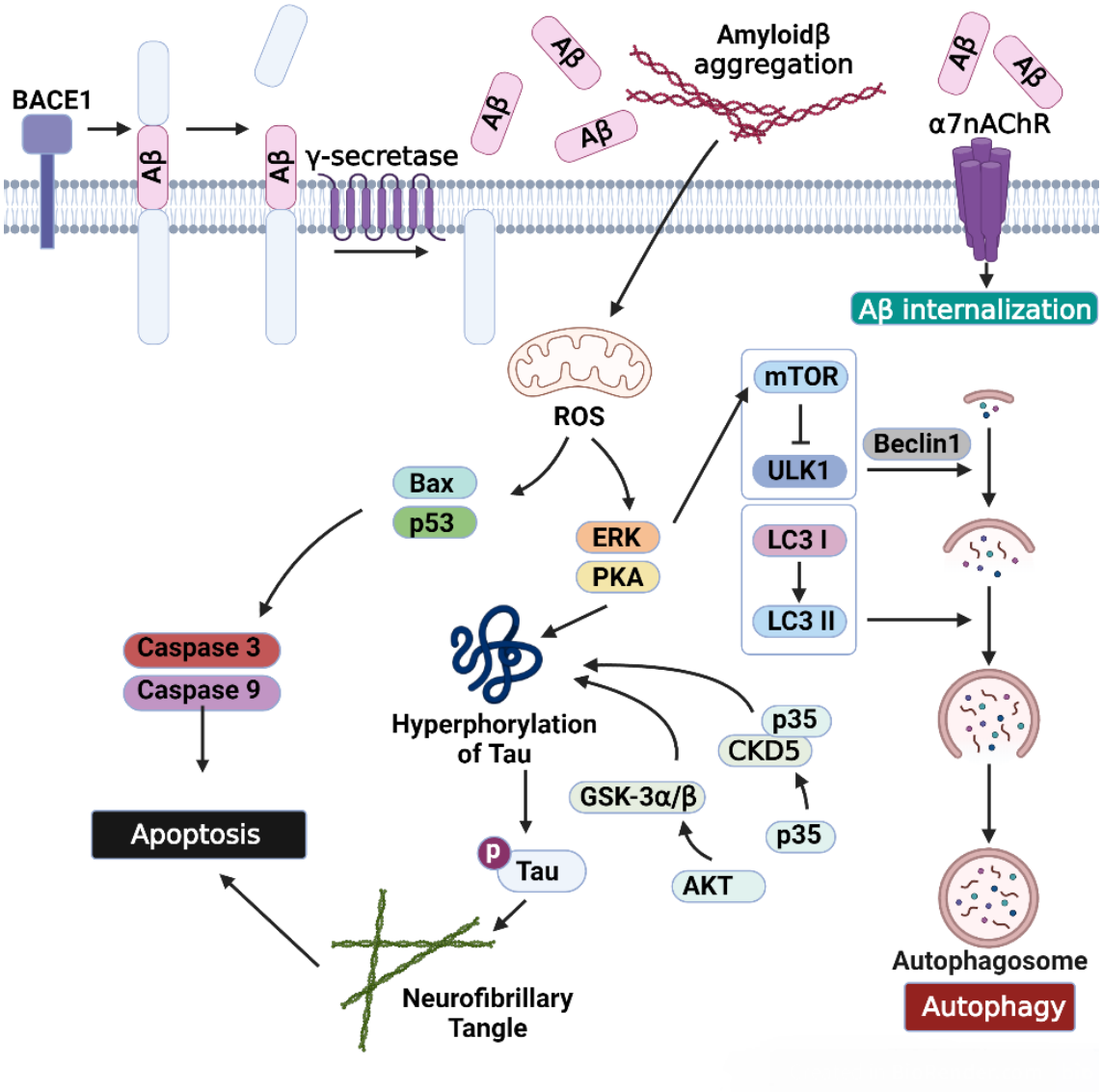

Fig.9 Signaling pathway of amyloid-β. BACE1 is one of the enzymes that cleaves Amyloid-β precursor protein (APP) and finally produced amyloid-β. Amyloid-β is easy to aggregation, and ROS formation, finally cause cell apoptosis.

Fig.10

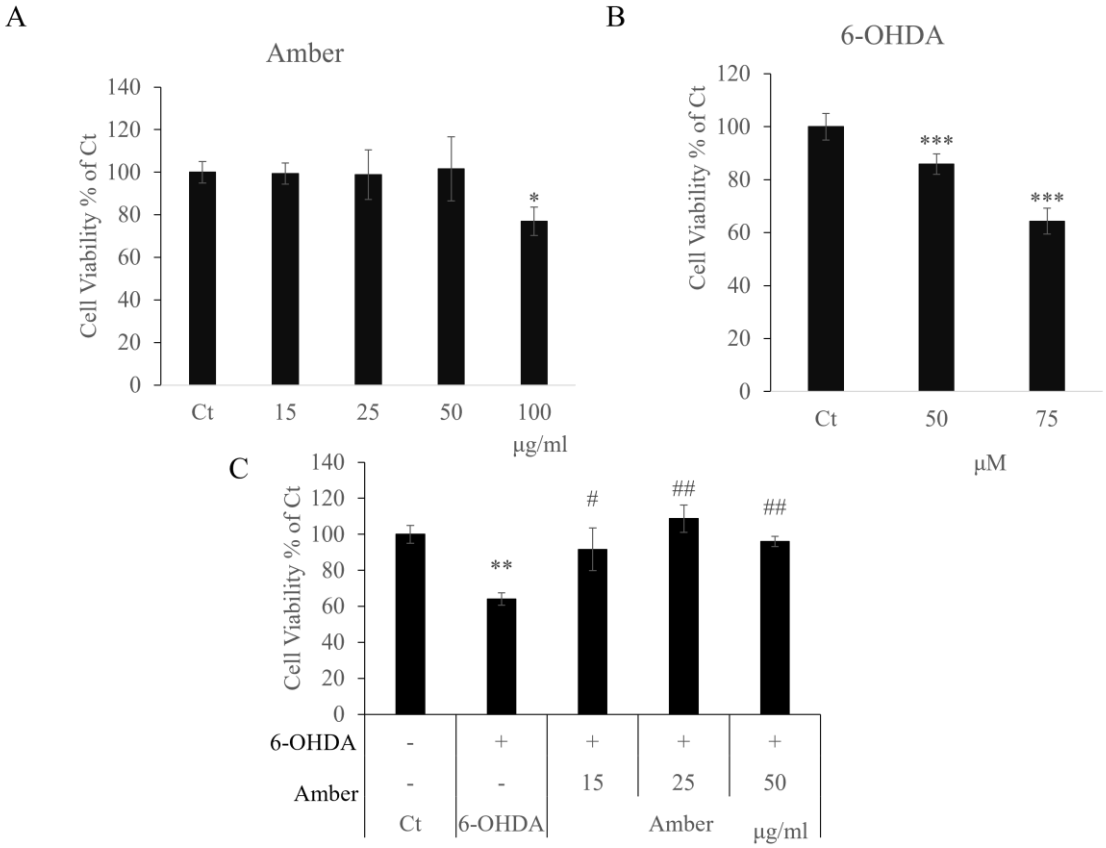

Fig.10 Effect of amber extract on 6-hydroxydopamine-induced cytotoxicity. Control: (6-OHDA amber) - -, 6-OHDA: (6-OHDA amber) + - (A) Cytotoxicity of amber extract. (B) Cytotoxicity of 6-OHDA (C) Effect of amber extract on 6-OHDA -induced cell death. Cell viability was determined using MTT assay. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, ** P<0.01 vs. Control, #P<0.05 vs. 6-OHDA, ##P<0.01 vs. 6-OHDA.

Fig.11

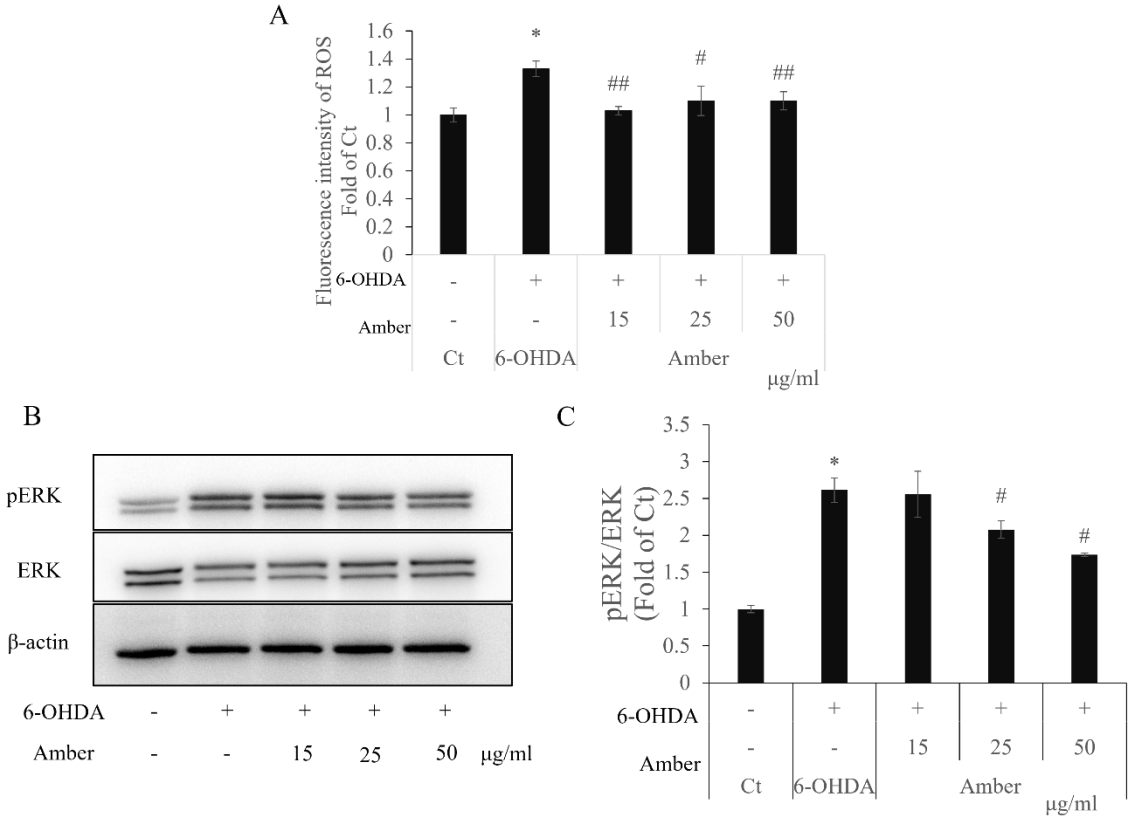

Fig.11 Effect of amber extract on 6-OHDA -induced intracellular reactive oxygen species (ROS) generation. Control: (6-OHDA amber) - -, 6-OHDA: (6-OHDA amber) + - (A) ROS analyzed using a fluorescence microplate reader. (B) Representative western blot image of pERK and ERK. (C) The protein bands were quantified using ImageJ. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. 6-OHDA, ##P<0.01 vs. 6-OHDA.

Fig.12

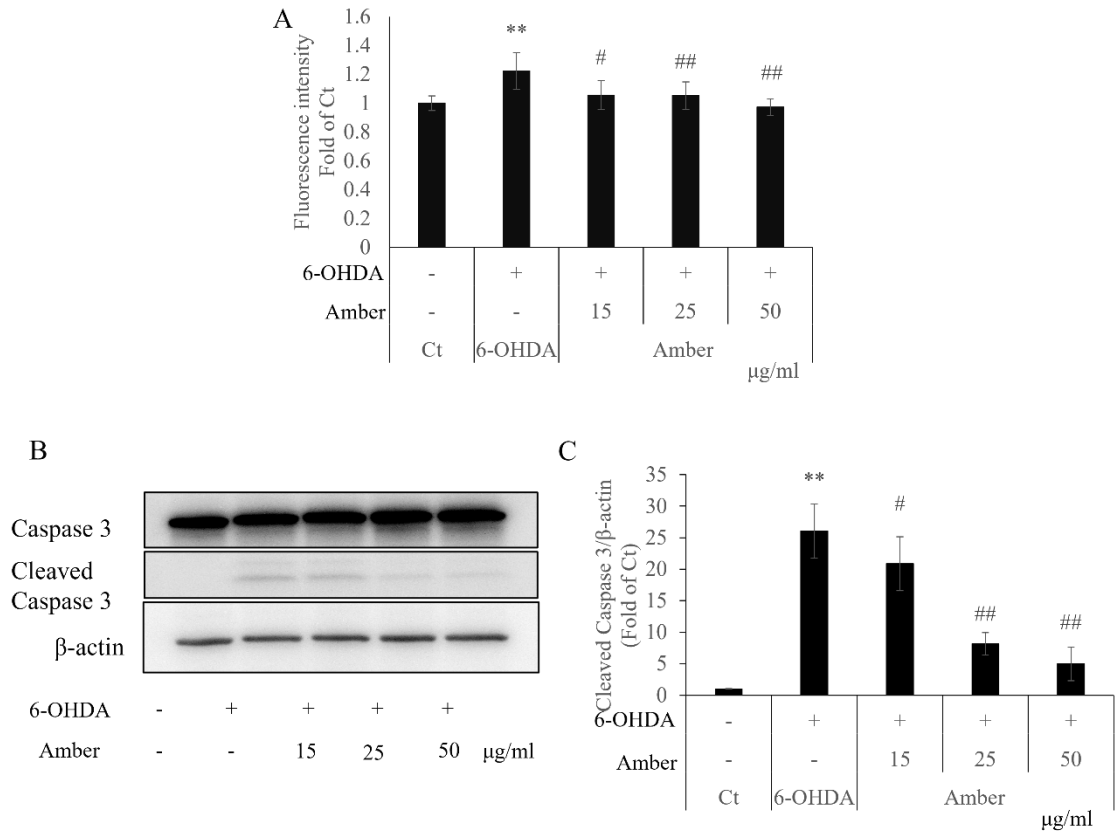

Fig. 12 Effect of amber extract on 6-OHDA -induced cell apoptosis. Control: (6-OHDA amber) - -, 6-OHDA: (6-OHDA amber) + - (A) Apoptosis analyzed using a fluorescence microplate reader. (B) Representative western blot image of caspase 3 and cleaved caspase 3. (C) The protein bands of cleaved caspase 3 were quantified using ImageJ. The results are expressed as mean ± SD. N≥3, ** P<0.01 vs. Control, #P<0.05 vs. 6-OHDA, ##P<0.01 vs. 6-OHDA.

Fig.13

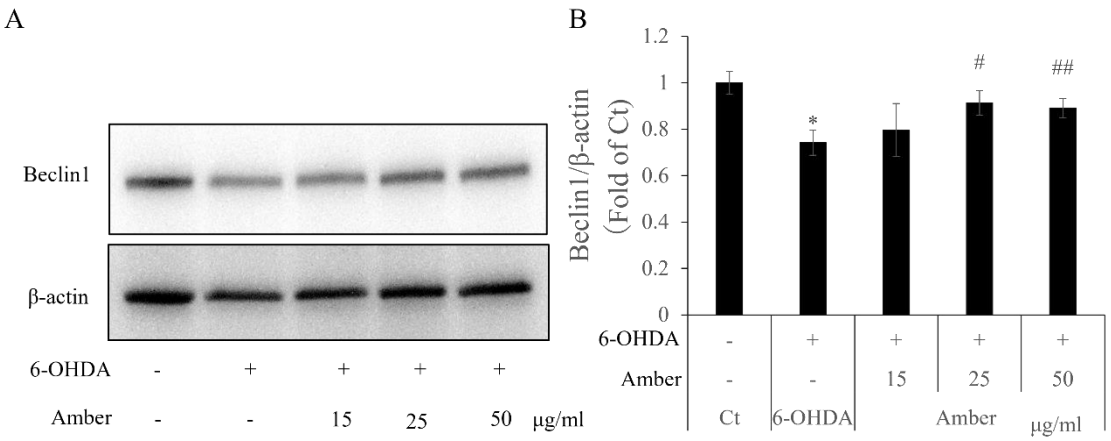

Fig. 13 Effect of amber extract on autophagy gene expression. Control: (6-OHDA amber) - -, 6-OHDA: (6-OHDA amber) + - (A) Representative western blot image of Beclin1. (B)The protein bands of Beclin1 were quantified using ImageJ. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. 6-OHDA, ##P<0.01 vs. 6-OHDA.

Fig.14

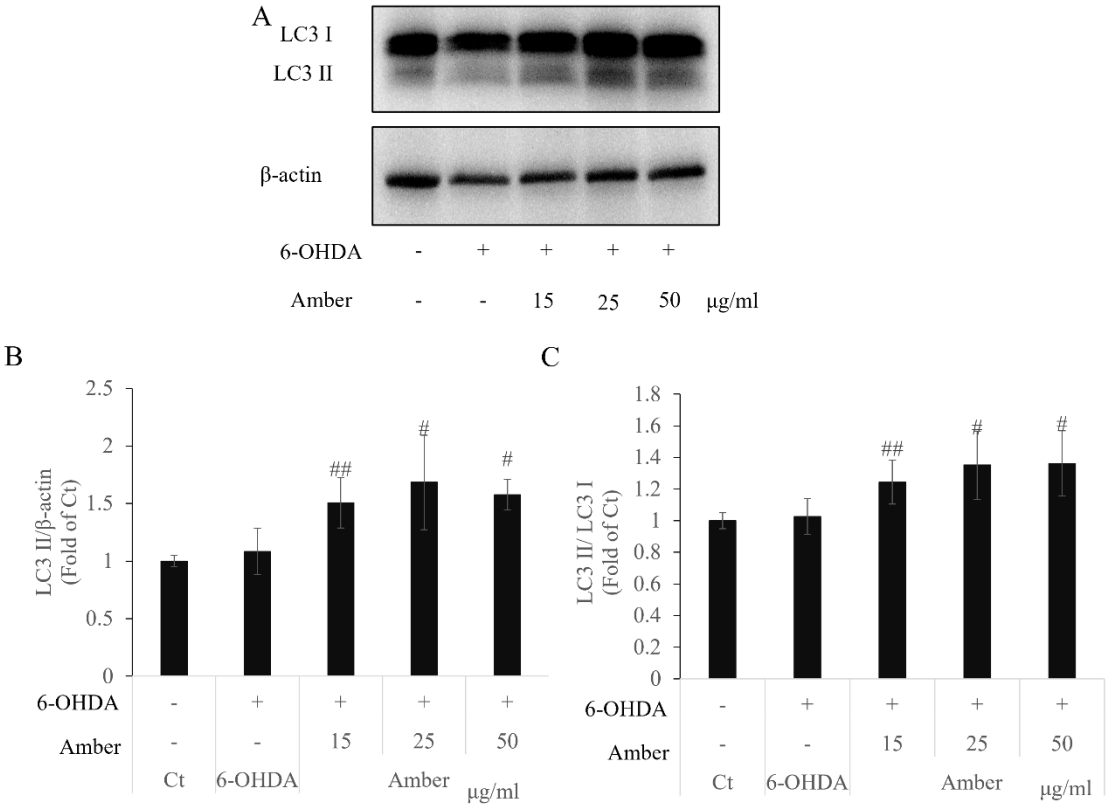

Fig.14 Effect of amber extract on autophagy gene expression. Control: (6-OHDA amber) - -, 6-OHDA: (6-OHDA amber) + - (A) Representative western blot image of LC3. (B) The protein bands of LC3II were quantified using ImageJ. (C) The relative ratios of LC3 II/LC3 I bands density. The results are expressed as mean ± SD. N≥3, * P<0.05 vs. Control, #P<0.05 vs. 6-OHDA, ##P<0.01 vs. 6-OHDA.

Fig.15

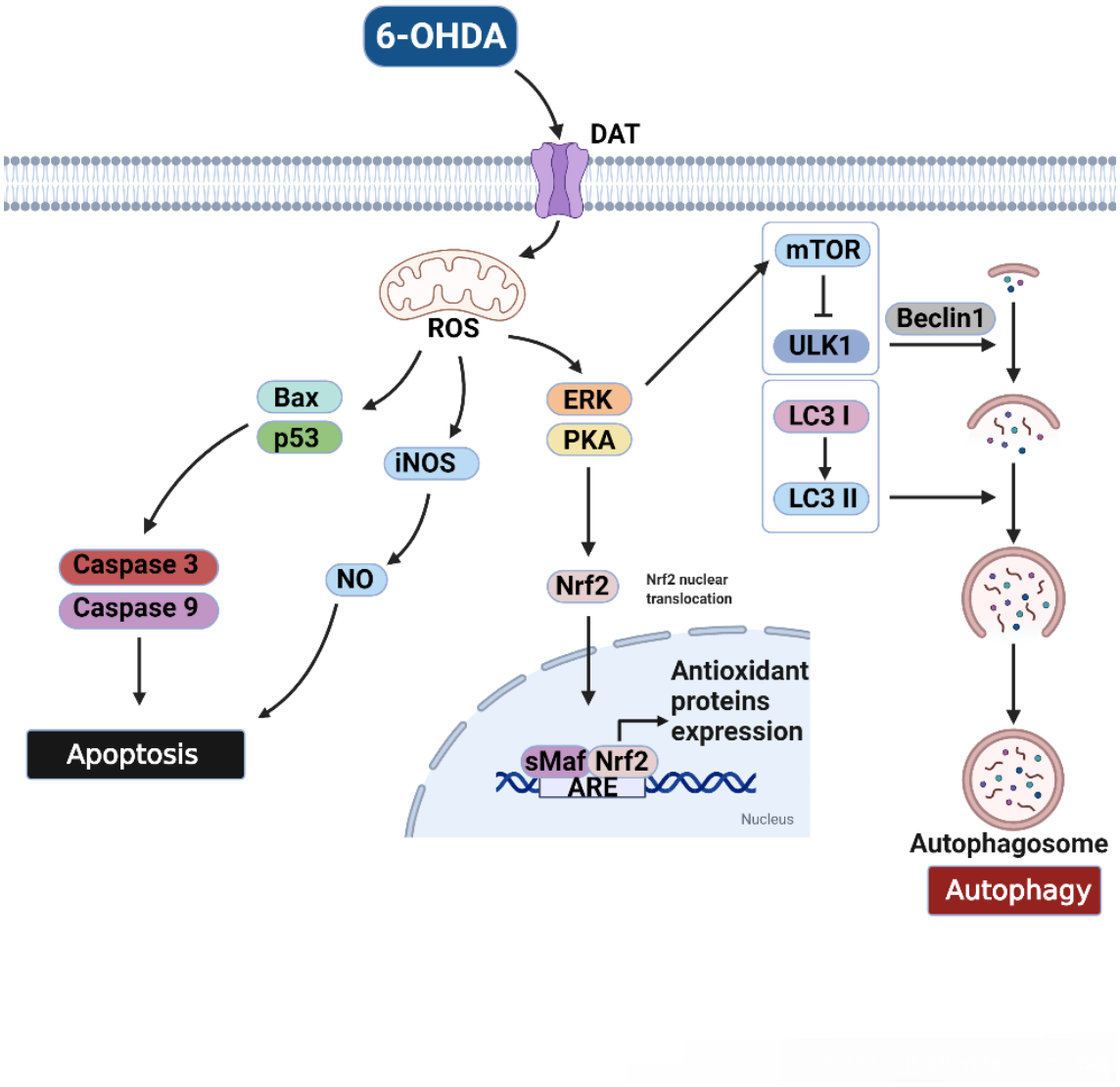

Fig.15 signaling pathway of 6-OHDA. 6-OHDA can take up by the dopamine (DA) transporter into cell and generate active oxygen, and finally cause cell apoptosis.