

Article

Protective Effects of *Allium senescens* Extract against 6-Hydroxydopamine in Neurons

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Abstract: Oxidative neurodegeneration causes various neuronal diseases, including Alzheimer's, Parkinson's, and Huntington's diseases. This study aimed to demonstrate the protective effect of *Allium senescens* leaf extracts on 6-hydroxydopamine (6-OHDA)-stressed SH-SY5Y cells, which are known to be optimal for neurotoxic research. The levels of apoptotic markers were evaluated using quantitative polymerase chain reaction (qPCR) and flow cytometry. The localization of apoptotic cells in vivo was analyzed using whole-mount immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Additionally, the production of reactive oxygen species (ROS) was estimated using flow cytometry. 6-OHDA induced ROS production in neuroblasts and in vivo, but treatment with the extract protected against the 6-OHDA-induced increase in ROS levels. Under oxidative stress, the extract performs three protective functions: decreasing ROS production, preventing mitochondrial apoptosis, and protecting the central and ventral nervous systems. These results also suggest that the extract can be useful in the development of functional foods for the prevention of neural damage caused by oxidative stress.

Keywords: *Allium senescens*; neurodegeneration; apoptosis; 6-OHDA; oxidative stress



Citation: Park, Y.; Yun, M.; Lee, S.; Kim, B. Protective Effects of *Allium senescens* Extract against 6-Hydroxydopamine in Neurons. *Appl. Sci.* **2024**, *14*, 5579. <https://doi.org/10.3390/app14135579>

Academic Editors: Monica Gallo and Dino Musmarra

Received: 1 May 2024

Revised: 7 June 2024

Accepted: 24 June 2024

Published: 26 June 2024



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1. Introduction

Neurodegenerative disorders are caused by oxidative stresses, the dysfunction of mitochondria, and misfolded proteins [1,2]. These disorders are enhanced by exposure to specific chemicals including 6-OHDA, 1-methyl-4-phenyl tetrahydropyridine (MPTP), annonacin, and antagonistic compounds to β 2-adrenoreceptor [3–5]. Oxidative inflammation is a common feature of chronic neurodegenerative pathologies in the central nervous system (CNS) [6]. In neurodegenerative disorders, various antioxidants in phytoextracts are effective in preventing the disorders without side effects [7]. Additionally, the activation of various compounds, including levodopa, dopamine agonists, safinamide, and monoamine oxidase B (MAO-B) inhibitors has been used to treat the disorders [8,9]. SH-SY5Y cells and neuroblastoma cells are used widely in various neuronal studies for metabolism, neurotoxicity, neuroprotection, and differentiation. Among these, this cell line is the most applied for neurodegenerative disorders [7,10]. In neurodegenerative studies, cell lines exposed to 6-hydroxydopamine (6-OHDA) are commonly utilized [11]. 6-OHDA, a neurotoxin, is known to induce neurodegenerative disorders besides PD [12]. Although 6-OHDA does not induce all neurodegenerative disorders, 6-OHDA exposure models in neurons have presented various pathologic features, including neurodegeneration, inflammation, and apoptosis due to oxidative stress [12]. 6-OHDA enters dopaminergic and noradrenergic neurons and inhibits the reuptake of their neurotransmitters [13]. During neuronal destruction, reactive oxygen species (ROS), such as superoxide, accumulate in neurons [14]. Under

intrinsic cellular stresses, such as DNA damage, hypoxia, UV, and exposure to chemicals, mitochondria in neurons release cytochrome c through assembled Bax proteins, and the released cytochromes and activated caspase 9 enhance cellular apoptosis [15]. When 6-OHDA triggers the production of ROS, monoamine oxidase (MAO) A and B are expressed in the neurons, and MAOB produces ROS through the degradation of dopamine in neurons [16]. To protect against stress, cells express several proteins, including members of the B-cell leukemia/lymphoma 2 (Bcl-2) family [17], cytokine response modifier A (crmA) [18], and inhibitors of apoptosis proteins (IAPs) [19]. The Bcl-2 family of 20 proteins is involved in the regulation of cellular apoptosis. The Bcl-2 proteins (A1, Bcl-2, Bcl-w, Bcl-xL, Bcl-B, and Mcl-1; myeloid cell leukemia sequence 1) inhibit apoptotic proteins, including Bax, Bak, and Bok [20]. Caspase-3 (Cas3) interacts with caspase-8 and -9, which play a central role in apoptosis, and Cas3 activates the production of the A β peptide from the cleavage of the amyloid-beta 4A precursor protein associated with Alzheimer's disease [21].

Allium senescens is a perennial aromatic herb distributed in northern Europe and Asia, especially on Ulleung Island in Republic of Korea [22]. *A. senescens* is effective in refreshing blood and controlling cholesterol levels in the blood [23] to activate detoxification and restore functions in the liver [24]. Based on the reported chemical compositions [25], *A. senescens* extract contains various bioactive compounds, including allin, allicin, coumaric acid, ferulic acid, and isoquercitrin.

2. Materials and Methods

2.1. *A. senescens* Extract

The leaves of *A. senescens* (KSB, Ulleung, Republic of Korea) were dried and ground (35 mesh) prior to being extracted twice with hydration for 90 minutes at 60 °C. The filtered extract was concentrated (R-3000, Buchi Labortechnik, AG, Essen, Germany) at 60 °C using a Rotary Evaporator system (DAIHAN, Wonju, Republic of Korea) and lyophilized using a freeze dryer (FD8505, Ilshin Lab Co., Seoul, Republic of Korea). Before use, the extract was sterilized with a 0.2 μ m PVDF syringe filter (Sartorius, GO, Göttingen, Germany). The extract was supported by EVERBIO (CCB, Jincheon-gun, Republic of Korea).

2.2. Cell and Animal Plankton Cultures

SH-SY5Y cells (Korea Cell Bank, Seoul, Republic of Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μ g/mL penicillin, and 100 μ g/mL streptomycin at 37 °C and 5% CO₂. In animal plankton culture, the cysts (1 g) of brine shrimp (*Artemia franciscana*) were cultured in 3 L of artificial seawater (pH 8.2, 28 °C) with air supply for 2 days.

2.3. Exposure to Cell and Animal Plankton

To establish treatment dosages, SH-SY5Y cells were exposed to 0, 20, 40, 60, 80, and 100 μ M 6-OHDA (162957, SigmaAldrich, St. Louis, MI, USA) and 50, 100, 500, 1000, and 2000 μ g/mL of the extracts for 12 h. SH-SY5Y cells were exposed to 6-OHDA for 1 h after treatment with the extract (500 μ g/mL). After sorting early nauplii using a submarine assay [22], the treatment dosages of 6-OHDA (40, 80, 100 μ M) and the extract were established. For whole-mount immunohistochemistry and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, the nauplii were exposed to 6-OHDA for 30 min after treatment with the extract for 30 min.

2.4. Cell Viability Test

To evaluate viability, neuroblasts were stained with Annexin V-conjugated propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) and counted using a flow cytometer (FACScalibur, BD Biosciences, San Jose, CA, USA) and FlowJo 10.6.1 (BD Biosciences).

2.5. Quantitative PCR

Total RNA was extracted from cells using the RiboEx reagent (GeneAll, Seoul, Republic of Korea). The RNA was then reverse transcribed into cDNA using a Maxime RT PreMix (iNtRON, Seongnam, Republic of Korea), and quantitative PCR was performed with primers (Table 1) with the following cycling parameters: 1 min at 95 °C, followed by 35 cycles of 35 s at 59 °C and 35 cycles of 1 min at 72 °C. The expression levels of the target genes in the samples were normalized to those of the housekeeping gene *GAPDH*, and the relative quantities of the target genes were determined with respect to those of the control.

Table 1. The list of primers for qRT-PCR.

Gene	F/R *	Seq (5'→3')
<i>AKT</i>	F R	GGCTGCCAAGTGTCAAATCC AGTGCTCCCCCACTTACTTG
<i>NFκB-P50</i>	F R	CGGAGCCCTCTTTCACAGTT TTCAGCTTAGGAGCGAAGGC
<i>NFκB-P52</i>	F R	AGGTGCTGTAGCGGGATTTC AGAGGCACTGTATAGGGCAG
<i>Bcl2</i>	F R	CTGCTGACATGCTTGGAAAA ATTGGGCTACCCCAGCAATG
<i>Bax</i>	F R	AGCGCTCCCCCACTTACTTG GACAGGGACATCAGTCGCTT
<i>Cyt</i>	F R	ATGAATGACCACTCTAGCCA ATAGAAACAGCCAGGACCGC
<i>GAPDH</i>	F R	GTGGTCTCCTCTGACTTCAACA CTCTTCCTCTGTGCTCTTGCT

* means Forward/Reverse.

2.6. Evaluation of Modulation for Mitochondrial Apoptotic Markers

All cellular samples were fixed with 2% paraformaldehyde for 4 h and treated with 0.02% Tween 20 for 5 min. After blocking using an Fc blocking solution (BD Bioscience), the samples were incubated with fluorescein isothiocyanate (FITC)-anti-MAOB (Biocompare, Inc., South San Francisco, CA, USA) and Alexa Fluor 680-anti-Cas3 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 days at 37 °C. All samples were washed using phosphate-buffered saline and analyzed using a flow cytometer (BD FACScalibur) and FlowJo 10.7.0 (BD Bioscience).

2.7. Mitochondrial Apoptosis

After exposure to the various concentrations of extracts and 6-OHDA in Figure 1 for 3 days, SH-SY5Y cells were stained with JC-1 Mitochondrial Membrane Potential Assay Kit (Invitrogen, MA, USA), and mitochondrial activity was estimated using a flow cytometer (BD FACScalibur) and FlowJo 10.6.1 (BD Biosciences).

2.8. Cellular ROS Detection

All cultured cells exposed to various conditions were stained with DCFDA- Cellular ROS Assay Kit (Abcam, Cambridge, UK) for 30 min and were measured using a flow cytometer (BD FACScalibur) and FlowJo 10.7.0 (BD Bioscience).

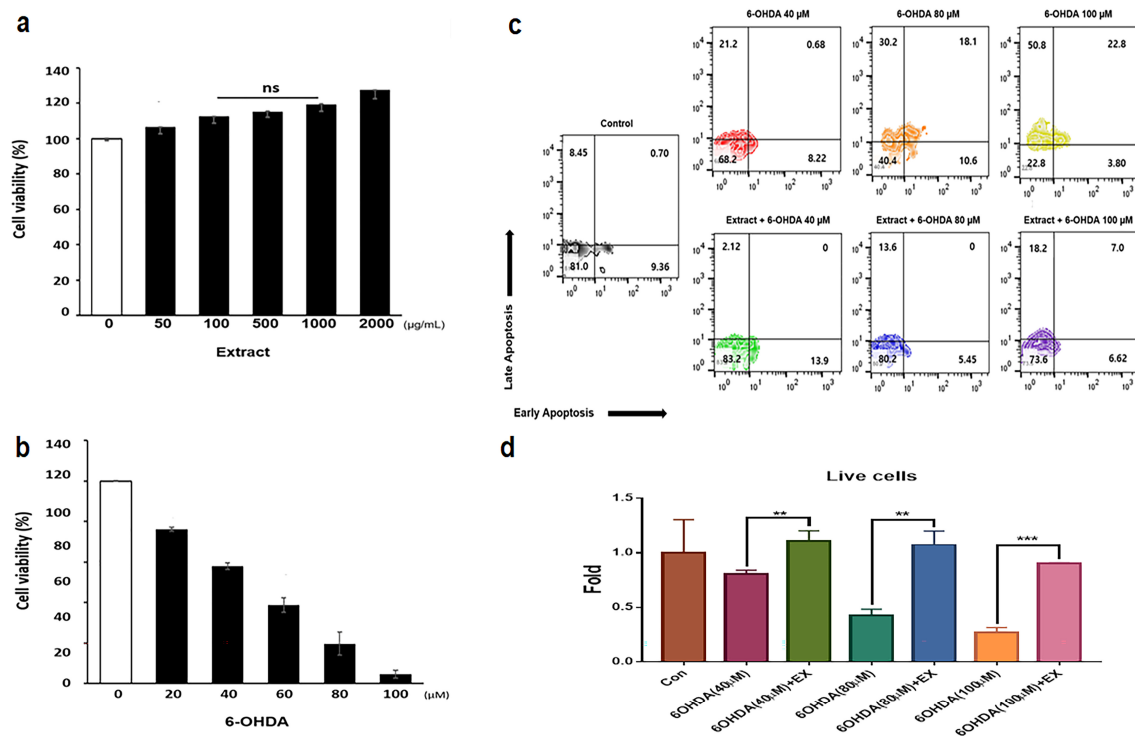


Figure 1. Cellular viability and apoptosis of *Allium senescens* extract and 6-OHDA in neuroblasts. (a,b) Cellular viability of neuroblasts treated with extract and 6-OHDA ($p < 0.05$). (c) Apoptosis of neuroblasts treated with extract (500); 500 $\mu\text{g/mL}$ and indicated concentrations of 6-OHDA. (d) Histograms showing relative fold changes in number of live cells under treatment with extract and 6-OHDA based on the panel (c). ns: not significant, EX: *Allium senescens* extract 500 $\mu\text{g/mL}$, 6-OHDA: 6-hydroxydopamine (** $p < 0.01$, *** $p < 0.001$).

2.9. Evaluation of Modulation for Apoptosis In Vivo

All nauplius samples were stained with the TUNEL assay kit (Thermo Fisher Scientific, Waltham, MA, USA), and the fluorescence intensity and count of stained colonies were estimated using a fluorescence microscope (Eclipse Ts-2, Nikon, Shinagawa, Japan) and the imaging software NIS-elements V5.11 (Nikon).

2.10. Whole-Mount Immunohistochemistry

All nauplius samples were treated with 4% paraformaldehyde for 4 h and treated with 0.02% Tween 20 for 15 min. The treated samples were stained with Alexa Fluor 680-anti-Cas3 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 4 days [21,22]. The fluorescence intensity of the stained nauplii was evaluated and imaged using a fluorescence microscope (Eclipse Ts-2, Nikon, Shinagawa, Japan) and imaging software NIS-elements V5.11 (Nikon). All cultured cells were pretreated with 10% BSA (Sigma, Kawasaki, Japan) for 4 h to the specificity of the antibodies.

2.11. Statistical Analysis

All experiments were analyzed by one-way analysis of variance (ANOVA) with a post hoc test (Scheffe's method) using Prism 7 (GraphPad, San Diego, CA, USA) software.

3. Results

This study explored the protective functions of *A. senescens* extract against 6-OHDA stress in neuroblasts. Moreover, the goal of this research was to explore the potentiality of the preventive function of the hydrolytic extract of *A. senescens* against oxidative neurodegeneration and to prove the usefulness of the extract as a functional food.

3.1. Prevention for Oxidative Apoptosis by *A. senescens* Extract

The extract was not cytotoxic to the neuroblasts (Figure 1a). Although at concentrations above 60 μ M 6-OHDA, the viability of neuroblasts was decreased dramatically (Figure 1b), the viability was maintained at 500 μ g/mL of the extract even though it was exposed to more than 80 μ M 6-OHDA (Figure 1c,d).

In contrast to 6-OHDA, which enhances neuroblast apoptosis without the extract, the extract attenuated neuroblast apoptosis in the presence of 6-OHDA (Figure 1c). Notably, although apoptosis was approximately 0.26 times under the 100 μ M 6-OHDA treatment, the extract enhanced the viability by approximately 0.91 times (Figure 1d). Without the extract, the mitochondrial activity was attenuated by approximately 30% under the 100 μ M 6-OHDA treatment (Figure 2a,c,d), and the activity was maintained in neuroblasts treated with both the extract (500 μ g/mL) and 6-OHDA (40, 80, 100 μ M) (Figure 2b–d).

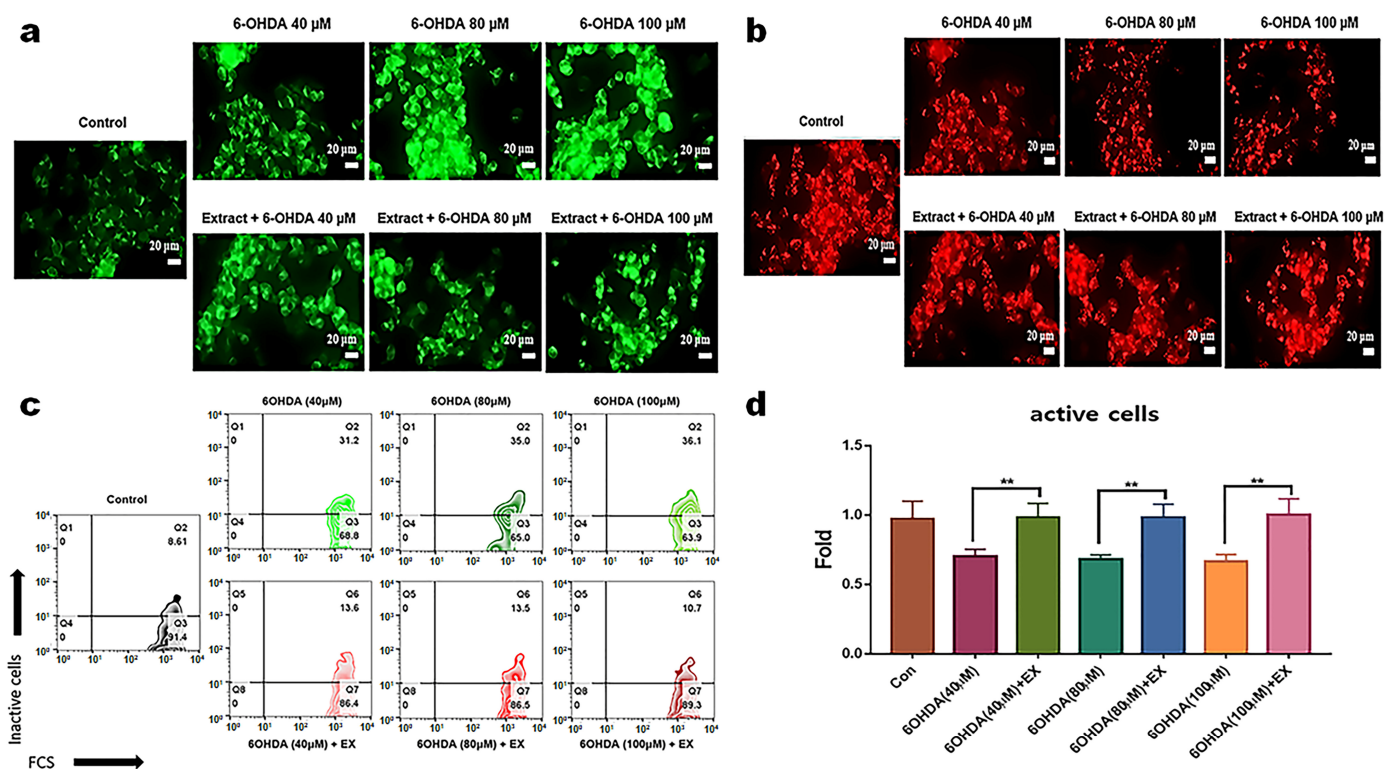


Figure 2. Protective effect of extract for mitochondrial apoptosis of neuroblasts. (a,b) Estimation of mitochondrial membrane potential using fluorescence microscopy. Red and green colors indicate live and dead cells, respectively. (c,d) Flow cytometric counts indicate active and inactive cells in histograms; populations of Q2 regions in the quadrant plots are dead cells, (d) showing the counts of inactivated cells based on the panel (c). EX: *Allium senescens* extract 500 μ g/mL, 6-OHDA: 6-hydroxydopamine (** $p < 0.01$) (white bars = 20 μ m).

3.2. Protection of Mitochondrial Activity by *A. senescens* Extract

Although 100 μ M 6-OHDA enhanced ROS production in neuroblasts, treatment with the extract attenuated this enhancement by a factor of approximately 2 under all concentrations of 100 μ M 6-OHDA (Figure 3). An increase in ROS derived from 100 μ M 6-OHDA enhanced the upregulation of MOB in neuroblasts (Figure 4a). Despite the activation of MAOB expression by 6-OHDA in neuroblasts, the extract effectively suppressed this upregulation in cells exposed to 80 and 100 μ M 6-OHDA (Figure 4a). The degree of suppression was approximately threefold lower compared to cells treated with 6-OHDA alone (Figure 4a). In agreement with these results, the levels of Cas3 were 1.8 times lower in cells treated with the extract (Figure 4b).

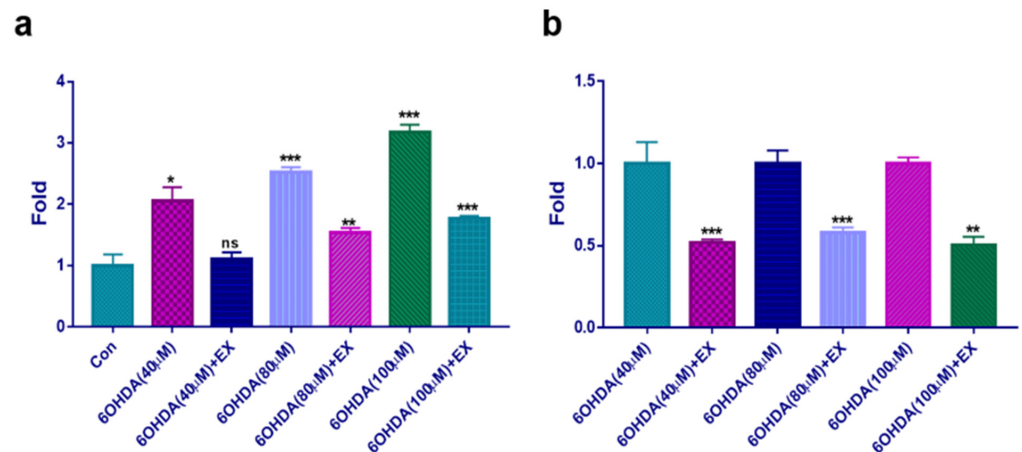


Figure 3. Evaluation of ROS production in neuroblasts treated with the extract. (a) ROS production under various conditions. (b) Relative fold changes derived from panel (a) comparing ROS production in cells exposed to various concentrations of 6-OHDA and treated with the extract. Con; control, EX: *Allium senescens* extract 500 µg/mL, 6-OHDA: 6-hydroxydopamine, ns: not significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

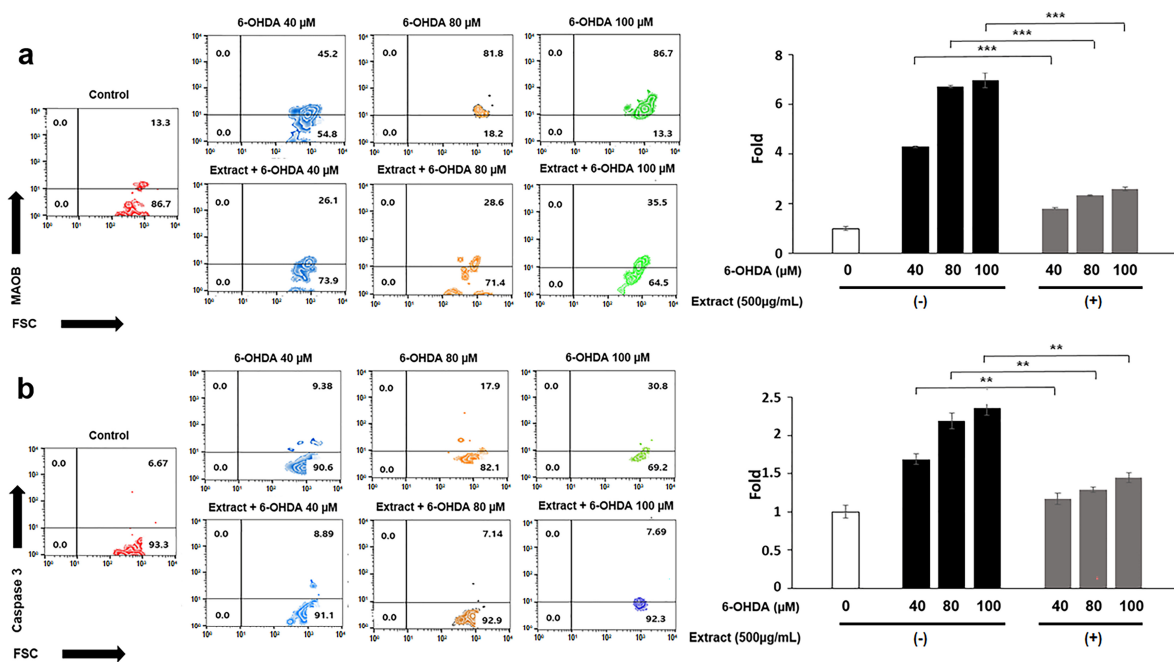


Figure 4. Expression of the markers for mitochondrial apoptosis in neuroblasts treated with the extract. (a,b) After gating for the population of live cells, the results for the markers of mitochondrial apoptosis were evaluated using flow cytometry. The Histograms showing relative fold changes in cell counts. EX: *Allium senescens* extract 500 µg/mL, 6-OHDA; 6-hydroxydopamine (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3. Modulation of Expression for Apoptotic Makers by *A. senescens* Extract

The extract inhibited the expression of apoptotic markers in neuroblasts. Although 6-OHDA induced the downregulation of anti-apoptotic markers, including serine/threonine kinase (AKT), nuclear factor kappa-light-chain-enhancer of activated B cells1 (P50), nuclear factor kappa-light-chain-enhancer of activated B cells 2 (P52), and BCL2, the extract effectively prevented the downregulation (Figure 5). Additionally, apoptotic markers such as Bcl-2-associated X-protein (Bax) and cytochrome C (CytC) were dramatically increased under 100 µM 6-OHDA treatment (Figure 5). Contrary to these results, the extract

upregulated all anti-apoptotic markers and suppressed the upregulation of apoptotic markers (Figure 5).

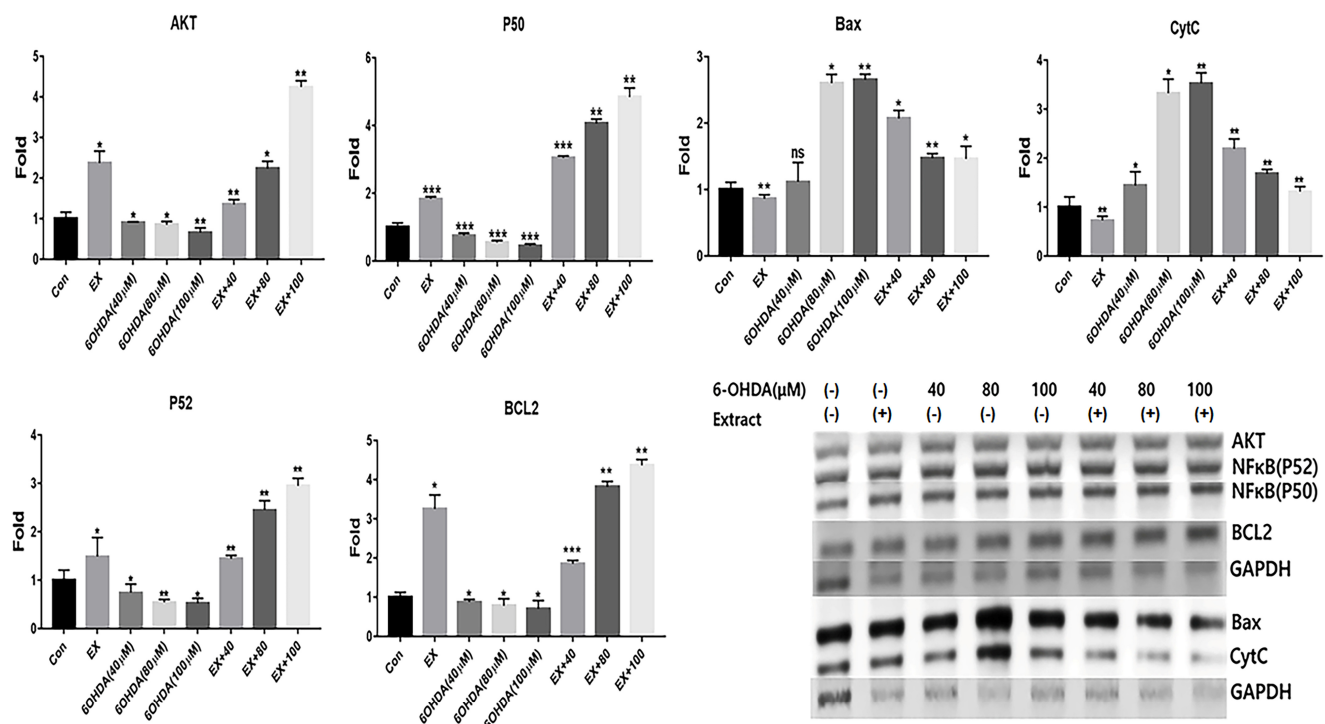


Figure 5. The mRNA levels for apoptotic markers in neuroblasts treated with the extract. Evaluation of the levels of the antiapoptotic markers (AKT, P50, P52, BCL-2) and apoptotic markers (Bax and CytC). Histograms showing relative fold changes in the signal intensity of the amplified DNA products. EX: *Allium senescens* extract 500 μg/mL, 6-OHDA: 6-hydroxydopamine, ns: not significant, EX+40, 80, 100: 6-OHDA treatment after exposure to the extract (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4. Protective Effects of *A. senescens* Extract against Oxidative Stress in Brine Shrimp

In vivo, 6-OHDA induced neuronal cell apoptosis during the development of brine shrimp (Figure 6). Following exposure to 6-OHDA, neurons of the CNS and segmental neurons in the VNS in nauplii were stained intensely with PI (red) and apo-BruU (green) (Figure 6). The fluorescence intensity was approximately 3.7 times higher than that of the control (Figure 6). Although all concentration were effective against 6-OHDA, 800 μg/mL of the extract strongly prevented neuronal apoptosis in the CNS and VNS, and notably, the extract was dramatically effective in the CNS of the nauplii (Figure 6). In segmental neurons in the thoracic region, apoptosis was attenuated in nauplii treated with 800 μg/mL of the extract (Figure 6). Whole-mount immunohistochemistry showed that 100 μM 6-OHDA induced mitochondrial apoptosis in the CNS and cellular colonies of the thoracic and abdominal regions (Figure 7). The apoptotic rate in the 6-OHDA-exposed cells was 2.3 times higher than that of the control, whereas the apoptotic rate in cells treated with 800 μg/mL extract was 11.5 times lower than that of the 6-OHDA-exposed CNS (Figure 7). Furthermore, the count and fluorescence intensity of ganglia in the thorax and abdomen were increased under the 6-OHDA treatment (Figure 6). Interestingly, despite exposure to 6-OHDA, the number of cell colonies were dramatically decreased in the groups treated with the extract (Figure 7).

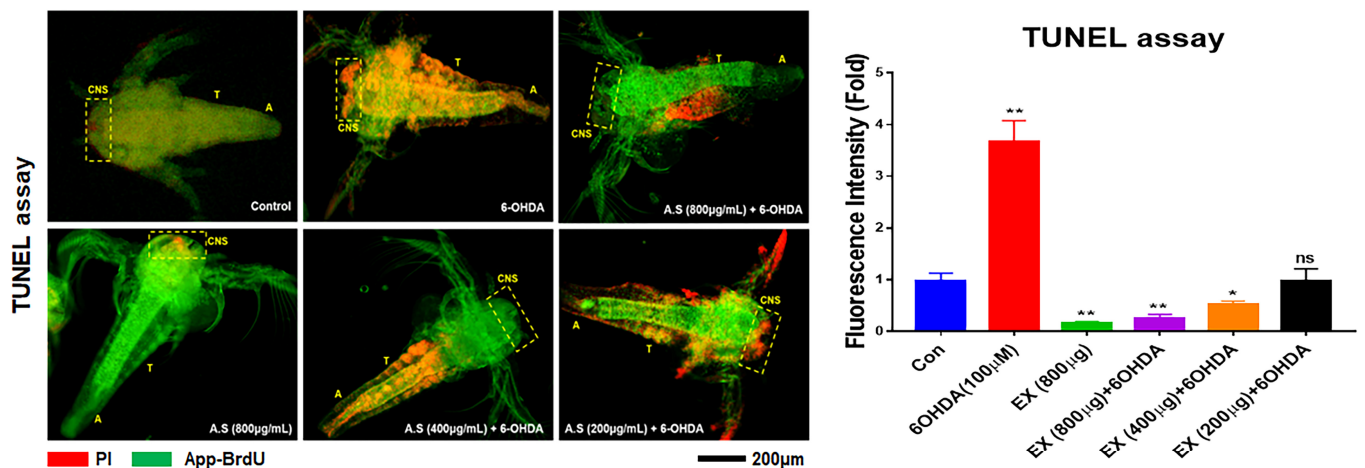


Figure 6. Effects of the extract on oxidative stress in the CNS and VNS of brine shrimp. Images for TUNEL assay in the CNS and VNS of brine shrimp exposed to 6-hydroxydopamine (6-OHDA). Histograms showing fluorescence intensity of cells in the CNS. CNS, central nervous system; T, thoracic ganglia region; A, abdominal ganglia region; ns, not significant; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VNS, ventral nervous system (* $p < 0.05$, ** $p < 0.01$).

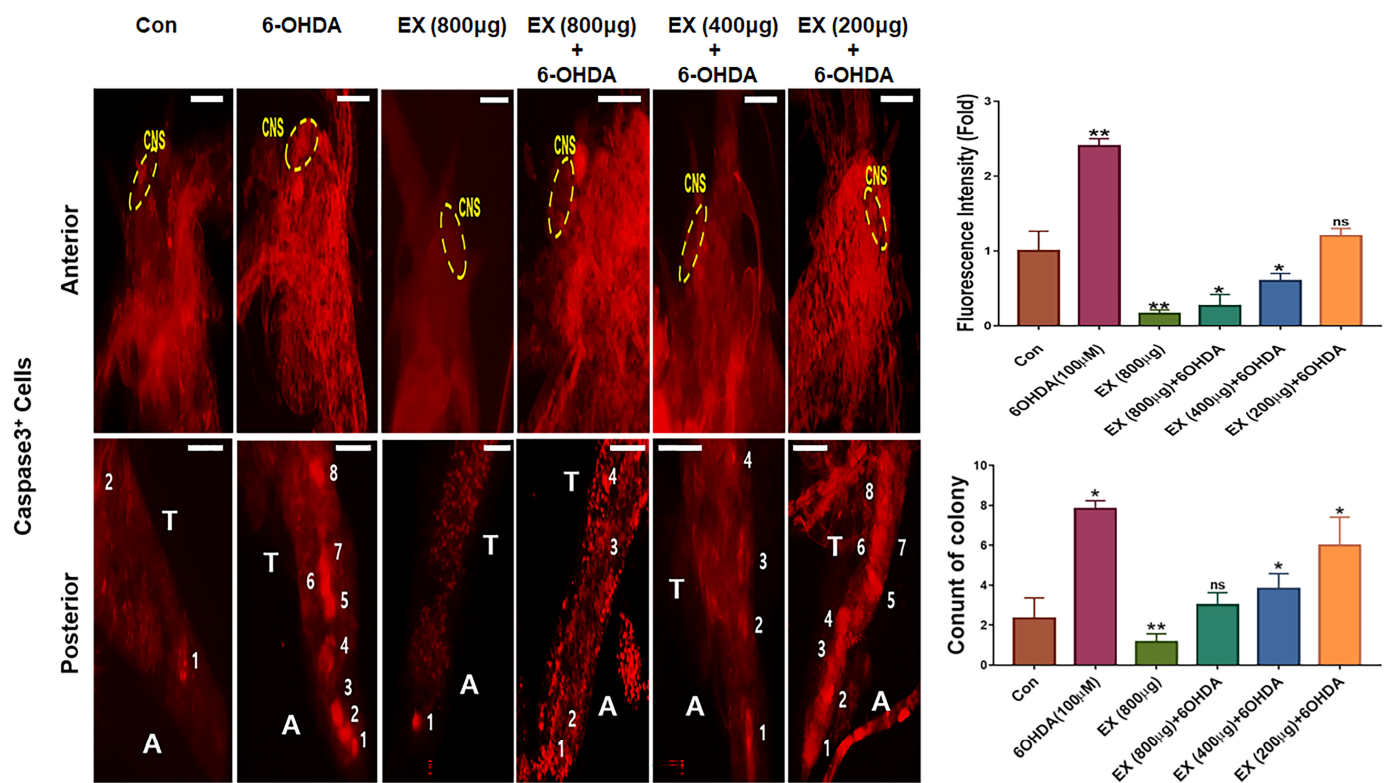


Figure 7. Effects of the extract on mitochondrial apoptosis in the CNS and VC of brine shrimp. Images for whole-mount immunohistochemistry with APC-labeled anti-caspase-3 in the CNS and VC of brine shrimp under 6-hydroxydopamine (6-OHDA) treatment. Histograms show the staining intensity of the cells. The numbers in the posterior regions represent cell colonies. CNS, central nervous system; T, thorax; A, abdomen; ns, not significant; VC, ventral cells, Ext; *Allium senescens* extract, 6-OHDA; 6-hydroxydopamine (* $p < 0.05$, ** $p < 0.01$).

4. Discussion

This study documented the protective function of *A. senescens* extract against modeled cells for oxidative neurodegeneration and an invertebrate animal: brine shrimp. Based

on the results of the cell viability analysis (Figure 1), the extract was not cytotoxic to neuronal cells. Non-cytotoxic phytoextracts are useful in the application of functional foods. Interestingly, this study demonstrated three protective functions against 6-OHDA-induced oxidative stress.

First, the extract prevented mitochondrial apoptosis by decreasing ROS levels in neuroblasts. Although 6-OHDA inhibits mitochondrial activity, the extract maintains its activity in addition to decreasing ROS under 6-OHDA treatment. Moreover, the extract downregulates MAOB and Cas3 in the neuroblasts. Under ROS accumulation, the CNS triggers neurodegeneration and accelerates aging [26]. ROS are toxic to various molecules, including proteins, lipids, carbohydrates, and DNA [27]. Furthermore, accumulated ROS causes neurodegenerative disorders [28]. These results document the potential functions of the extract in the prevention of oxidative neurodegeneration.

Second, the extract was found to drive the expression of apoptotic and anti-apoptotic genes. The extract activates the upregulation of anti-apoptotic genes but not of apoptotic genes. As shown in Figure 5, all concentrations of 6-OHDA decreased the apoptotic markers. However, the extract dramatically increased the levels of the apoptotic markers despite exposure to 6-OHDA. In particular, the protective effect of the extract for neurodegeneration increased dependently on the 6-OHDA concentrations (Figure 5). Increased ROS levels trigger the upregulation of apoptotic genes in the mitochondria [29]. In neurons, NF- κ B affects neuronal survival and apoptosis under mitochondrial dysfunction [30]. Furthermore, with increased ROS levels, the activation of NF- κ B without the upregulation of BCL-2 promote the activation of the apoptotic signaling pathway [30]. The activation of these two molecules indicates that the extract protects neuronal cells from apoptosis induced by 6-OHDA. Increased ROS levels inhibit the upregulation of *AKT* and *BCL-2* genes but not of *Bax*, which activates the upregulation of *CytC* and *Cas3* [31]. Although 100 μ M 6-OHDA significantly downregulates the *AKT* gene expression and upregulates *Bax* and *CytC* genes, the extract dramatically modulated *AKT*, *Bax*, and *CytC* gene expression to promote the survival of neuroblasts following exposure to 6-OHDA (Figure 5 and Supplementary Material). These results suggest that the extract is effective in preventing mitochondrial apoptosis in neuroblasts.

Third, the extract protected the CNS and VNS against oxidative apoptosis in vivo. In developing brine shrimp (Figure 6), the extract prevented the apoptosis of cells of the CNS and VNS and VC under the 6-OHDA treatment. Without the extract, the 6-OHDA treatment accelerated the apoptosis of the CNS and VNS and VC in brine shrimp. Interestingly, 6-OHDA intensely induced the apoptosis of protocerebrum, particularly mushroom bodies (MBs) in the CNS of nauplii, but the protective function of the extract against oxidative stress was dramatically effective in nauplii (Figures 6 and 7). The genus *Artemia* of anostracan crustaceans is also known as brine shrimp [32,33]. There are four distinct developmental stages of brine shrimp: cyst, emergence, nauplius, and adult [32,33]. This animal's plankton provides a useful model for neurological research because of its short developmental stages [32,33]. The central nervous system (CNS) and ventral system are actively developed during the nauplius stage of brine shrimp [32,33]. Brine shrimp is used in toxicological assays for chemicals, water pollution, and natural products [34]. Additionally, the neuronal development of brine shrimp is activated in the early nauplius [32]. Neuronal cells in the early nauplius show a sensitive response to toxic chemicals [32,34]. PD is caused by the degeneration of dopaminergic neurons in a specific area, called the substantia nigra. Corresponding with this specific area, in insects, the functions of the MBs include the reception and processing of sensory signals from olfactory, visual, mechanosensory systems; the establishment of memory for behavior; and motor control [35]. In brine shrimp, MBs are localized at the protocerebrum and are synthesized dopamine transporters [32]. At an extract concentration of 800 μ g/mL, although the protective effect was not intense in segmental neurons, the protection of the CNS was dramatically effective in early nauplii (Figure 6). These results suggest that the extract is more effective in the CNS than in the VNS, in vivo. Compared with cells treated with 6-OHDA alone, those treated with the

extract showed dramatically reduced mitochondrial apoptosis in the CNS and VC, in vivo (Figure 7). These protective effects of the extract in vivo provide conclusive evidence for its potential for the prevention of oxidative neurodegeneration, in addition to the results from the neuroblasts.

5. Conclusions

This study explored the effects of *A. senescens* extract on the prevention of oxidative neurodegeneration under 6-OHDA. Under 6-OHDA, the extract performs three protective functions: decrease ROS production, prevent mitochondrial apoptosis, and protect the CNS, VNS, and VC. *A. senescens* extract is an excellent candidate for a functional food material for the prevention of oxidative neurodegeneration diseases, in addition to maintaining brain health.

Supplementary Materials: The following is available online at: <https://www.mdpi.com/article/10.3390/app14135579/s1>, Figure S1: viability result, Figure S2: Full gels.

Author Contributions: Conceptualization and methodology, Y.P., M.Y. and S.L.; writing—original draft preparation, B.K.; writing—review and editing, B.K.; supervision, B.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by EVERBIO.

Institutional Review Board Statement: Not applicable. This study did not involve humans or vertebral animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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