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Neuroprotective effect of *Allium hookeri* against H_2O_2-induced PC12 cell cytotoxicity by reducing oxidative stress

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Abstract In this study, the anti-oxidative and neuro-protective effects of ethanolic extracts of the dried roots of *Allium hookeri* were investigated. Total phenolic contents and total flavonoid contents of *A. hookeri* extract depended on the ethanol concentrations used (50, 70 and 95%). In order to evaluate radical scavenging activity, DPPH and ABTS radical scavenging assays and ferric reducing powers were evaluated. The results showed the 95% ethanol extract of *A. hookeri* (95AH) had higher phenolic and flavonoid contents, and greater radical scavenging activities than 50 or 70% ethanol extracts of *A. hookeri*. The neuro-protective effects of 95AH were evaluated using H_2O_2-treated PC12 neuronal cells. Treatment of 95AH increased cell viability and superoxide dismutase and glutathione peroxidase activities, reduced lactate dehydrogenate release, reduced reactive oxygen species production, and increased Bcl-2/Bax ratio. HPLC revealed 95AH was rich in phenolics, especially catechin. These results demonstrate 95AH has substantial anti-oxidative and neuro-protective effects against H_2O_2-induced oxidative stress.

Keywords *Allium hookeri* · Antioxidant · Neuroprotection · Phenolics · Flavonoids

Introduction

Research interest in the development of treatments for neurodegenerative diseases like Parkinson's and Alzheimer's diseases continues to increase (Kim et al., 2017). Although the etiologies of brain neurodegenerative diseases have not been clearly elucidated, it has been shown the main cause is reactive oxygen species (ROS). Therefore, much attention has been focused on the development and research of antioxidants to control oxidative stress and free radical levels which are unstable, potent oxidants that easily react with many biomaterials, and in so doing cause oxidative stress (Park et al., 2016).

ROS are naturally occurring and are produced during normal metabolism (Kim et al., 2017), but when ROS components like superoxide anion ($O_2\bullet^-$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl) accumulate, they cause lipid peroxidation, protein oxidation, and DNA modification,which prevent cells functioning normally, and thus, cause cell death (Das and Roychoudhury, 2014; Park et al., 2016). The brain is an organ that utilizes much oxygen and contains high levels of lipid peroxides. However, antioxidant enzyme systems and molecular antioxidants are less active or present at lower levels in the brain than in other tissues (Cobley et al., 2018). Furthermore, the brain is

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Table 1 Total phenolic and flavonoid contents of the 50, 70, and 95% ethanol extracts of *A. hookeri*

Extract solvent	Yield (%)	Total phenolic contents (mg GAE[a)]/g)	Total flavonoids contents (mg NE[b)]/g)
50% EtOH	16.0	0.14 ± 0.01^{b}	0.07 ± 0.01^{b}
70% EtOH	11.3	0.11 ± 0.02^{c}	0.04 ± 0.01^{b}
95% EtOH	10.7	2.21 ± 0.02^{a}	0.27 ± 0.03^{a}

Results are presented as means ± SDs of three independent experiments

Values in the same column with different letters are significantly different from one another ($p < 0.05$)

[a]GAE, gallic acid equivalents on dry weight

[b]NE, naringin equivalents

highly susceptible to oxidative damage by ROS and free radicals, and resulting damage is believed to be closely related to neuronal death and neurodegenerative diseases. Awareness of the possibility of preventing age-related diseases is increasing. Also, health functional foods from plants resources have attracted attention, because it has less side effects and toxicity than artificial antioxidants (Kim et al., 2010; Song et al., 2000).

Allium hookeri is a member of the onion family (*Allium* sp.) and is widely grown in China, India, Bhutan, Myanmar, and East Asia (Bae and Bae, 2012; Lee et al., 2014). *A. hookeri* has an pungent odor like other members of this family and generates sweet, bitter, and spicy sensations when eaten. In China, *A. hookeri* has been used for medicinal purposes for more than 3000 years, and its leaves, sprouts, and roots are widely eaten (Ghahremani-majd et al., 2012; Rhyu and Park, 2013). It is also used as an alternative to onion (*Allium cepta*) and garlic (*Allium sativum*), but has higher levels of dietary sulfur than garlic and contains more phytosterol and total phenol than onions (Park et al., 2016). Recently, a Korean study (Park and Yoon, 2014) reported *A. hookeri* contains about six times the level of sulfur-containing compounds than garlic. In addition, *A. hookeri* has also been shown to have anti-inflammatory, anti-cancer, antioxidant, and anti-diabetic effects (Kim, 2017; Lee et al., 2014; Rhyu and Park, 2013).

The previous reports of bioactive compounds of *A. hookeri* were limited to sulfur-containing compounds (Rhyu and Park, 2013; Roh et al., 2016; Yang et al., 2017) and relatively few studies have been investigated the antioxidant and neuroprotective effects of *A. hookeri* and its phenolic compound. In the Jung et al. study (2016), essential oil from garlic or *A. hookeri* was administrated to mice and evaluated the neurogenesis effects on the mice brain. Results indicated *A. hookeri* essential oil reduced novel object recognition, neuroblast differentiation and neurogenesis proteins expression in mice brain, which was contrary to the garlic administration. In the Jeong et al. Study (2016), however, safe dosage was considered only for garlic. As it has been reported that high consuming of sulfur is toxic, thus purification for *A. hookeri* is necessary for diet (Lee et al., 2017).

Therefore, in the present study, we examined the antioxidant effects of the ethanolic extracts of the dried roots of domestic *A. hookeri* on hydrogen peroxide exposed PC12 cells. In addition, major phenolic compound, which is supposed to be attribute to antioxidative effect, was identified.

Materials and methods

Materials

Folin-Ciocalteu reagent, DPPH, ABTS, TPTZ, gallic acid, MTT, 2'7'-dichlorofluorescein diacetate (DCF-DA) and catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640, fetal bovine serum and phosphate buffered saline were purchased from Gibco (Waltham, MA, USA). Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) enzyme assay kits were purchased from Cayman Chemical (Ann Arbor, MI, USA). Primary antibodies Bax were from Cell signaling Technology (Danvers, MA, USA) and Bcl-2 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies were purchased from Bio-Rad Co. (Hercules, CA, USA). All other chemicals were analytical grade.

Sample preparation and extraction

Roots of fresh *A. hookeri* were purchased in Jincheon-gun, Chungcheongbuk-do province in March 2019, and then air dried at 20 ± 5 °C for 2 weeks. Dried *A. hookeri* (20 g) was extracted with 200 mL (w/v) of 50, 70 or 95% ethanol for 2 h at 50 °C twice and filtered through filter paper (No. 2, Whatman, Maidstone, UK). Filtrates were evaporated to dryness under vacuum at 50 °C using a rotary evaporator (EYELA N-1000, Riakikiai Co., Ltd., Tokyo, Japan) and freeze-dried (IlShin Co., Ltd., Yangju, Korea). Powdered samples of the 50, 70, and 95% *A. hookeri* extracts (50AH,

75AH, and 95AH, respectively) were stored at −20 °C until required.

Total phenolic and flavonoid contents

Total phenolic contents of the three extracts were determined using the Folin-Ciocalteu method (Park et al., 2016; Sapkota et al., 2010), with minor modifications. Samples and Folin-Ciocalteu reagent were mixed in 96-well plate, left for 3 min, and then 2% sodium carbonate was added and incubated at room temperature in the dark for 2 h. Absorbances were then measured using spectrophotometer (SpectraMax M3; Molecular Devices, LLC, Sunnyvale,CA, USA) at 760 nm. Gallic acid was used to produce the calibration curve, and results were expressed as mg gallic acid equivalents (GAE)/g of dry weight of *A. hookeri*.

Total flavonoid contents of extracts were determined using an aluminum chloride colorimetry-based method (Chang et al., 2002) with slight modification. Samples and 2% aluminium chloride methanolic solution were mixed in a 96-well plate and allowed to stand at room temperature for 15 min. Absorbances were measured using a spectrophotometer at 430 nm. Naringin was used to produce the calibration curve. Results are expressed as mg naringin equivalents (NE)/g of dry weight of *A. hookeri*.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured as previously described by Mensor et al. (2001). Three hundred microliters of DPPH solution was mixed with 50AH, 70AH, or 95AH samples in a 96-well plate and incubated in the dark for 30 min. Absorbances were measured at 515 nm using a spectrophotometer. Percentage scavenging activities of DPPH radicals were calculated using the following formula.

$$\text{DPPH radical scavenging activity } (\%) = \left(1 - \text{ABS}_{\text{sample}}/\text{ABS}_{\text{control}}\right) \times 100$$

where $\text{ABS}_{\text{control}}$ is the absorbance of DPPH solution with distilled water and $\text{ABS}_{\text{sample}}$ is extract absorbance in DPPH solution.

ABTS radical scavenging activity

ABTS radical scavenging activity was measured as described previously by Re et al. (1999). A stock solution of 7.4 mM ABTS and 2.45 mM potassium persulfate solution (1:1) was prepared and kept in 12–16 h at room temperature in the dark in produce ABTS radicals. This solution was then adjusted to an absorbance of 0.7 ± 0.02 at 732 nm using 50 mM phosphate buffer saline (pH 7.4) and mixed with 50AH, 70AH, or 95AH samples in a 96-well plate. The plate was then incubated in the dark for 30 min and absorbances were measured at 732 nm. Radical scavenging activities were expressed as percentage (%) of that of controls using distilled water.

$$\text{ABTS radical scavenging activity } (\%) = \left(1 - \text{ABS}_{\text{sample}}/\text{ABS}_{\text{control}}\right) \times 100$$

where $\text{ABS}_{\text{control}}$ is the absorbance of ABTS solution with distilled water and $\text{ABS}_{\text{sample}}$ is the absorbance of extract in ABTS solution.

Ferric reducing antioxidant power (FRAP)

FRAP was measured using the Benzie & Strain method (1996) with slight modification. FRAP reagent was prepared by dissolving 300 mM acetate buffer (pH 3.6), 10 mM TPTZ containing 40 mM HCl and 20 mM ferric chloride in distilled water and heating the mixture at 37 °C for 10–15 min. 50AH, 70AH, or 95AH and FRAP reagent were mixed thoroughly and kept at 37 °C for 4 min, and absorbances were measured at 593 nm. Reducing powers were expressed as μM ascorbic acid equivalents (AAE)/g of dry weight.

Cell culture

PC12 cells (a rat adrenal pheochromocytoma cell-line) were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI supplemented with 10% fetal bovine serum, 5% horse serum, 50 unit/mL of penicillin and 100 mg/mL of streptomycin and were incubated at 37 °C in a 5% CO_2 -95% air incubator.

MTT assay

PC12 viability after exposure to H_2O_2 (200 μM) was determined using an MTT assay. Cells were seeded at 3×10^4cells/well in 96-well plates and pretreated with 95AH (50–200 μg/mL) for 24 h. After exposure to H_2O_2 (200 μM) for 2 h, cells were stained with MTT solution (5 mg/mL in PBS) for 4 h, and after removing supernatants, formazan was dissolved using DMSO. Absorbances were measured using a spectrophotometer at 570 nm. Cell viabilities were expressed as percentages of the non-treated control.

Lactate dehydrogenase (LDH) release

LDH release was measured using an LDH detection kit (TaKaRa, Ohtsu, Japan). Briefly, cells were seeded at 3×10^4cells/well in 96-well plates and then treated in the same way as in MTT assays as described above. After cells

have been exposed to H_2O_2 (200 μM) for 2 h, culture supernatants were placed in a 96-well plate and LDH reagent was added and allowed to react for 20 min. The reaction was stopped by adding 1 N HCl and LDH release was determined by measuring absorbance at 450 nm using spectrophotometer. LDH release from cells was expressed as percentage of the non-treated control.

ROS measurement

In order to measure intracellular ROS contents, we used the oxidation sensitive fluorescence probe DCF-DA. Briefly, PC12 cells (3×10^4 cells/well) were incubated in 96-well plate and treated as MTT assay above. After being exposed to H_2O_2 (200 μM) for 2 h, cells were stained with 50 μM DCF-DA dye solution at 37 °C for 50 min. Cellular fluorescence was measured using a fluorescence microplate reader (BioTek, Winooski, VT, USA) at excitation and emission wavelengths of 485 and 528 nm, respectively.

Antioxidant enzymatic activity

In order to determine cellular antioxidant enzyme activities, PC12 cells were plated at 3×10^5 cells/mL in 6-well plates for 24 h, treated with 50, 100 or 200 μg/mL of 95AH for 24 h, and then exposed to 200 μM H_2O_2 for 2 h. Cells were then washed twice with ice-cold PBS and lysed in RIPA buffer (Sigma Aldrich). Lysates were centrifuged at 12,000 × *g* for 20 min at 4 °C, and protein concentrations in lysates were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). SOD activity was measured as Xu et al. (2011) described. GSH-Px activity was measured as Kang et al. (2012) described. Enzyme activities in 95AH treated samples were expressed as percentages of non-treated control values.

Western blot analysis

PC12 cells were plated at 3×10^5 cells/mL in 6-well plates for 24 h, treated with 50 or 100 μg/mL of 95AH for 24 h, and exposed to 200 μM H_2O_2 for 2 h. They were then washed twice with ice-cold PBS, and lysed in RIPA, and centrifuged at 12,000 × *g* for 20 min at 4 °C. Protein concentrations in lysates were determined using the Bradford assay. Western blotting was conducted by loading 15 μg/mL of total proteins into 10 and 12% SDS polyacrylamide gels. After separation, proteins were transferred to nitrocellulose membranes, which were blocked for 1 h at room temperature in Tween-Tris-buffered saline containing 5% skim milk, washed three times with PBS, incubated with primary antibodies (rabbit polyclonal to Bax and polyclonal to actin, 1:1000 dilution, and mouse monoclonal to Bcl-2, 1:1000 dilution) overnight at 4 °C with gentle agitation, washed three times with PBS, and blotted for 1 h at room temperature with secondary antibodies (1:4000 dilution). Finally, immunoblotted protein bands were detected using ECL solution (Amersham Corp., Tokyo, Japan).

HPLC analysis

Major phenols in 95AH were analyzed as previously described by Park et al. (2016) with minor modification using HPLC equipped with a diode array detector (HPLC–DAD, Dionex, Sunnylvale, CA, USA) and a C18 column (4.6 mm × 250 mm; GL Sciences Co., Ltd., Tokyo, Japan). The mobile phase consisted of (A): methanol and (B): 0.1% formic acid, and elution was conducted using linear gradients, as follows: 0–10% A (0–1 min), 10–50% A (1–30 min). The injection volume was 10 μL, flow rate was 0.34 mL/min, and UV detection was performed at 270 nm. Peaks were identified by comparing retention times and UV–Vis spectra with those of commercial standards. Quantification was determined using standard curve of the compound of a range of 0.25–1 mg/mL.

Statistical analysis

Intergroup comparisons were performed using analysis of variance followed by Duncan's multiple range test in PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). Results are presented as the means ± standard deviations (SDs) of at least three independent experiments. Statistical significance was accepted for p values < 0.05.

Results and discussion

Total phenolic and flavonoid contents

The yields of *A. hookeri* 50AH, 70AH, or 95AH extracts were 16.0, 11.3 and 10.7%, respectively (data not shown)

Phenolics are secondary metabolites with various structures and are widely distributed in the plant kingdom. It has been reported plant-derived phenolics have numerous bioactivities such as antidiabetic, anticholesterol, anticancer, and antioxidant effects (Jeong et al., 2007), and in particular, polyphenols are known to protect against free radical attack in vivo. Free radicals are molecules containing unpair electrons. They remove electrons from neighboring molecules to stabilize themselves in the initial stage of oxidation molecules (Sakihama et al., 2002). This molecular oxidation by electron transfer accelerates body aging and many diseases (Valko et al., 2007). The hydroxyl groups of phenolics act as hydrogen donors, and thus, stabilize the unpaired electrons and scavenge ROS (Kwon

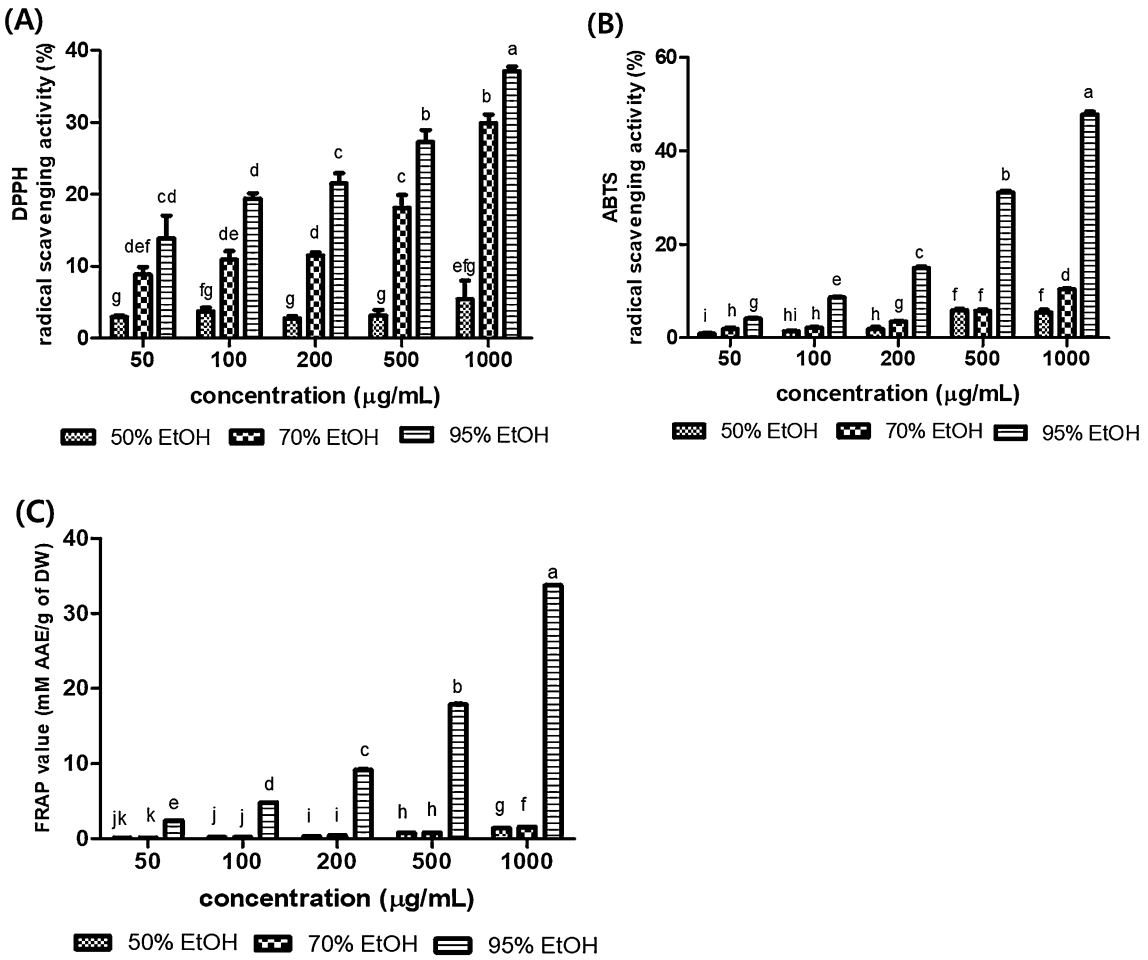

Fig. 1 Effects of ethanol concentration during extraction on the antioxidative activities of *A. hookeri* extracts. (**A**) DPPH radical scavenging activities. (**B**) ATBS radical scavenging activities. (**C**) Ferric reducing antioxidant powers. Results are presented as means ± SDs of three independent experiments. Data with different letters represent statistical differences from one another ($p<0.05$). [1)]AAE, ascorbic acid equivalent

et al., 2011; Sakamoto and Imai, 2017). Total phenolic and flavonoid contents of *A. hookeri* depended on the ethanol concentration used for extraction (Table 1). Total phenolic contents of 50AH, 70AH, and 95AH were 0.14, 0.11, and 2.21 mg GAE/g of dry weight, respectively, and total flavonoid contents were 0.07, 0.04 and 0.27 mg NE/g of dry weight, respectively. 95AH had higher total phenolic and flavonoids than the other two extracts.

In a study by Lee et al. (2014) total phenolic contents of an 80% ethanol extract were greater than in a water extract of *A. hookeri* root part. In the present study, 95AH contains the highest amount of flavonoid contents among three extracts, which also agrees with the findings of Lee et al. (2014) who reported *A. hookeri* is polyphenol rich and flavonoid poor.

Radical scavenging activity

Plant phenols and flavonoids have strong antioxidative activities, and their bioactivities are largely dependent on their phenol and flavonoid contents (You et al., 2018). The various compounds in plants show different antioxidative effects (Park et al., 2016), and in the present study, we used DPPH and ABTS radical scavenging assays to evaluate these effects and we also measured ferric reducing powers. The DPPH radical scavenging assay is widely used to evaluate the free radical scavenging activities of antioxidants from natural sources. DPPH accept electrons or hydrogen from bioactive substances such as ascorbic acid, tocopherol, and polyhydroxy and aromatic compounds, and forms stable species and is decolorized when reduced (Kwon et al., 2011). Figure 1A shows 50AH, 70AH, or 95AH dose-dependently increased radical scavenging activities. At a concentration of 1000 μg/mL the radical

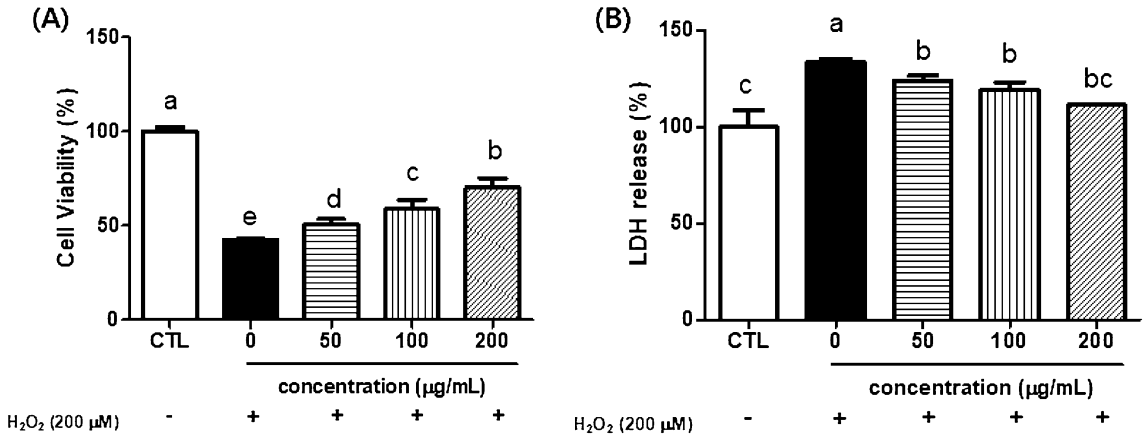

Fig. 2 Protective effects of 95AH on PC12 cells against H2O2 oxidative stress. (**A**) Cell viabilities. (**B**) LDH release. Cells were pretreated 95AH for 24 h and then incubated with or without 200 mM of H2O2 for 2 h. CTL, control; 95AH, 95% ethanol extract of *A. hookeri*. Results are presented as the means ± SDs of three independent experiments Data with different letters represent statistical differences from one another ($p<0.05$)

scavenging effects of 50AH, 70AH, and 95AH were 5.38, 29.89, and 37.14%, respectively.

ABTS radicals are generated by ABTS salt and potassium persulfate (a strong oxidizing agent). When the ABTS radicals encounter the antioxidant scavengers, its dark green color fade rapidly (Hwang et al., 2016). As shown in Fig. 1B, 50AH, 70AH, or 95AH dose-dependently scavenged radicals, and at 1000 μg/mL, their radical scavenging activities of 50AH, 70AH and 95AH were 5.47, 10.43, and 47.89%, respectively.

The ferric reducing power assay evaluates the reduction of the ferric to ferrous ion by antioxidants (Benzie and Strain, 1996). As shown in Fig. 1C, 50AH, 70AH, and 95AH at 1000 μg/mL had FRAP values of 1.41, 1.57, and 33.76 μM AAE, respectively. These results showed 95AH had the highest antioxidative activity, which matched phenolic and flavonoid contents. There are reports that the phenolic contents of plants are positively related to antioxidative effects, and the phenolic contents of *Allium* sp. plants were well correlated with DPPH and ABTS radical scavenging activity and FRAP results (Ghahremanimajd et al., 2012; Hong et al., 2011; Jeong et al., 2007). Jeong et al. (2007) also demonstrated that the bioactivities of phenolics are due to reactions between phenolic hydroxyls and large molecules such as proteins and enzymes (Kim et al., 2007).

Protective effect of *A. Hookeri* extract on H_2O_2 treated PC12 cells

H_2O_2 is an ROS produced under stressful conditions, and high concentrations of intracellular H_2O_2 are believed to mediate oxidative stress-induced cytotoxicity (Benzie and Strain, 1996). In PC12 cells, oxidative stress was produced by treating them with H_2O_2 (200 μM) for 2 h. This exposure to H_2O_2 reduced cells viability to 42.46% versus non-treated controls (Fig. 2A). However, 95AH pretreatment at 50, 100, and 200 μg/mL increased cells viability to 50.65, 58.64, and 70.27%, respectively ($p < 0.05$).

Neuronal cells contain much more unsaturated fatty acid rather than other cells. Therefore, when cells are damaged and cell membranes are compromised, LDH (a cytoplasmic enzyme) is released to media (You et al., 2018). As Fig. 2B shows, 200 μM H_2O_2 increased LDH leakage by 33.60% versus non-treated controls, and pretreatment with 50, 100, and 200 μg/mL of 95AH reduced LDH leakage by respective 7.76, 11.01, and 16.70% versus non-pretreated controls ($p < 0.05$).

At low concentrations, H_2O_2 regulates physiological processes like senescence, photosynthesis, cell cycle, growth, and development. In addition, H_2O_2 can easy to penetrate cell membranes and readily reacts with intracellular metal ions (Das and Roychoudhury, 2014). Furthermore, the over production of H_2O_2 can damage cellular molecules such as proteins, RNA, and DNA (Ren et al., 2010). It has been reported that *Allium* sp. possess antioxidant activity because it contains vitamins and flavonoids with antioxidant effects (Yin and Cheng, 1998). In a study by Kim et al. (2017), the neuronal protective cell viability was found to be greater for ethanol than water extracts of *A. hookeri* when cells were challenged with glutamate. We believe the protective effect of 95AH on H_2O_2 treated PC12 cells observed in the present study was due to the antioxidative effects of phenolics and flavonoids in 95AH.

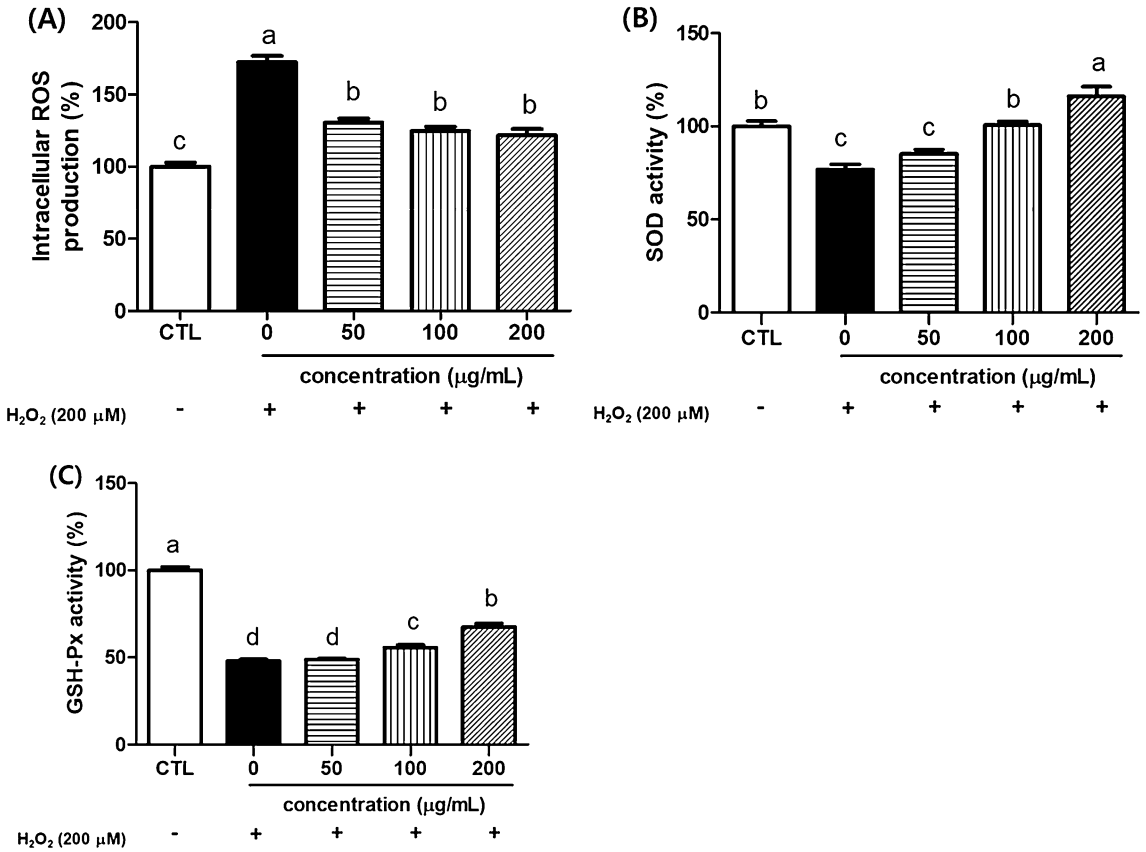

Fig. 3 Effect of 95AH on ROS inhibition and antioxidant enzyme activity in PC12 cells against H2O2 oxidative stress. (**A**) Intracellular ROS production. (**B**) SOD levels. (**C**) GSH-Px levels. Cells were pretreated 95AH for 24 h and then incubated with or without 200 mM of H2O2 for 2 h. CTL, control; 95AH, 95% ethanol extract of *A. hookeri*. Results are presented as the means ± SDs of three independent experiments Data with different letters represent statistical differences from one another ($p<0.05$)

Antioxidant defense system of *A. hookeri* extract on H_2O_2-treated PC12 cells

Elevated intracellular ROS levels are related to various chronic diseases and many studies have shown reductions in elevated ROS levels have cytoprotective effects (Park et al., 2016). DCF-DA is a non-polar compound that diffuses into cells and is then converted into a non-fluorescent polar derivative (DCFH) by cellular esterases. In the present of excessive intracellular ROS, cell membrane-impermeable DCFH is oxidized to the highly fluorescent DCF-DA (Hwang et al., 2016). In the present study (Fig. 3A), treatment of 200 μM H_2O_2 increased ROS levels by 72.50% versus non-treated controls. However, pretreatment with 50, 100, and 200 μg/mL of 95AH reduced ROS levels by respective 24.40, 27.76, and 27.44% versus non-pretreated controls ($p < 0.05$).

It has been reported that *Allium* sp. has notable antioxidant effects (Yin and Cheng, 1998). In a study by Kim et al. (2017), an *A. hookeri* extract effectively inhibited ROS production in glutamate treated neuronal cells. Rho et al. (2016) also demonstrated that the administration of *A. hookeri* extract inhibited ROS levels in serum and the pancreatic tissues of type 1 diabetic mice. These results suggest the inhibitory effect of 95AH on ROS production in PC12 cells was probably due to its antioxidant activity.

The body possesses various defense systems against oxidative stress. Under normal conditions, cells maintain ROS balance using SOD and GSH-Px. Cellular oxidative stress can be caused by free radicals or by impaired antioxidant enzyme activities, and the resulting oxidative imbalance has been shown to be a cause of degenerative diseases such as cancer, cardiovascular diseases, renal diseases, neurological disorders, and diabetes (Hwang et al., 2016; Jaiswal and Rizvi, 2014).

While oxygen is metabolized in aerobic organisms, $O_2^{\bullet-}$, a precursor of ROS, is generated and initiate lipid and protein oxidation (Sakamoto and Imai, 2017). SOD, however, is a pivotal antioxidative enzyme that converts the $O_2^{\bullet-}$ to relatively non-toxic H_2O_2 because H_2O_2 can oxidized when catalyzed by metal ions (Collin, 2019). Then, GSH-Px converts H_2O_2 to oxygen and water (Das and Roychoudhury, 2014).

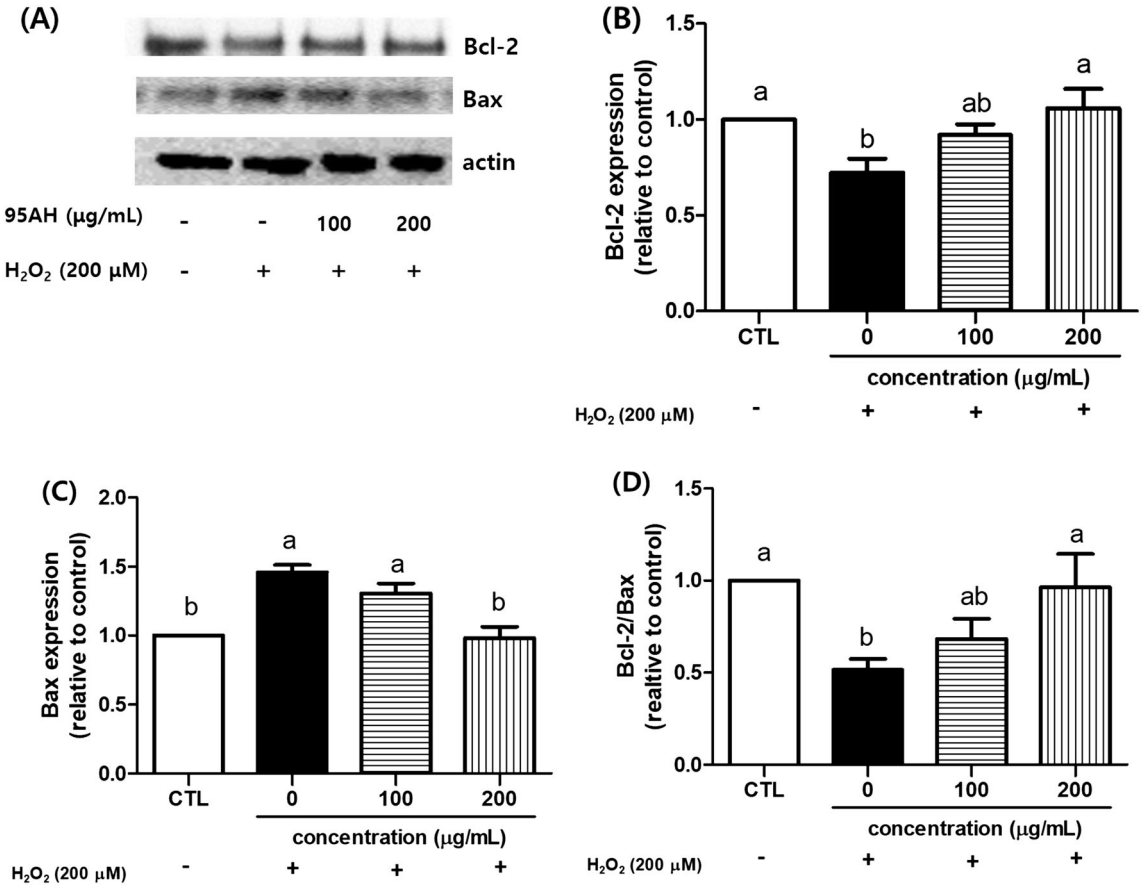

Fig. 4 Effects of 95AH on the expressions of Bcl-2 and Bax in H_2O_2 treated PC12 cells. Cells were pretreated with the indicated concentrations of 95AH for 24 h and then treated with H_2O_2 for 2 h. Protein levels of Bax and Bcl-2 were analyzed by western blotting using actin as the internal control. (**A**) Representative protein expressions of Bcl-2 and Bax. (**B**) Expression level of Bcl-2. (**C**) Expression level of Bax. (**D**) Bcl-2/Bax ratio. CTL, control; 95AH, 95% ethanol extract of *A. hookeri*. The results shown are representative of three independent experiments. Data with different letters represent statistical differences from one another ($p<0.05$)

To determine the effect of 95AH on the antioxidant potentials of neuronal cells exposed to H_2O_2 oxidative stress, we measured antioxidant enzyme activities (Fig. 3B, C). Treatment with 200 μM H_2O_2 reduced SOD activity to 76.78% compared to non-treated controls ($p < 0.05$). However, pretreatment with 95AH at 50, 100 and 200 μg/mL resulted in SOD activities of 85.25, 100.6, and 116.1%, respectively ($p < 0.05$). Regarding GSH-Px activities, 200 μM H_2O_2 H_2O_2 treatment reduced GSH-Px activity to 48.04% versus non-treated controls. However, pretreatment with 50, 100, and 200 μg/mL of 95AH increased this to 48.77, 55.68 and 67.42%, respectively ($p < 0.05$).

It was demonstrated that endogenouse or exogenous (diet or dietary supplement) antioxidants protect cellular antioxidant systems against oxidative stress. Because they can balance or increase the levels of antioxidant enzymes by neutralizing free radicals or activating nuclear factor erythroid-derived 2-related factor 2, a redox homeostasis regulator (Kurutas, 2015). Previously, there are similar results that pretreatment of *A. hookeri* extract restored antioxidant enzyme activities. In cultured hepatocyte, 80% ethanol *A. hookeri* extract upregulated antioxidant enzyme activities such as SOD, GSH-Px and catalase against H_2O_2 (Park et al., 2016). Also, it was shown in an animal study that administering *A. hookeri* extract for 4 weeks increased serum contents of SOD in diabetic mice (Kim, 2017). These results indicate that 95AH helps maintain the enzymatic activities of SOD and GSH-Px.

Effect of *A. hookeri* extract on the expressions of apoptotic proteins

Mitochondria importantly produce ROS and are particularly vulnerable to oxidative stress (Hwang et al., 2016; You et al., 2018). In particular, when mitochondria membrane potentials are impaired by oxidative stress, mitochondrial energy production is suppressed and apoptosis follows (Yang et al., 2013). Bcl-2 family proteins (e.g., Bax and Bcl-2) regulate cell death and apoptosis. Bcl-2 plays an important anti-apoptotic role, and critically

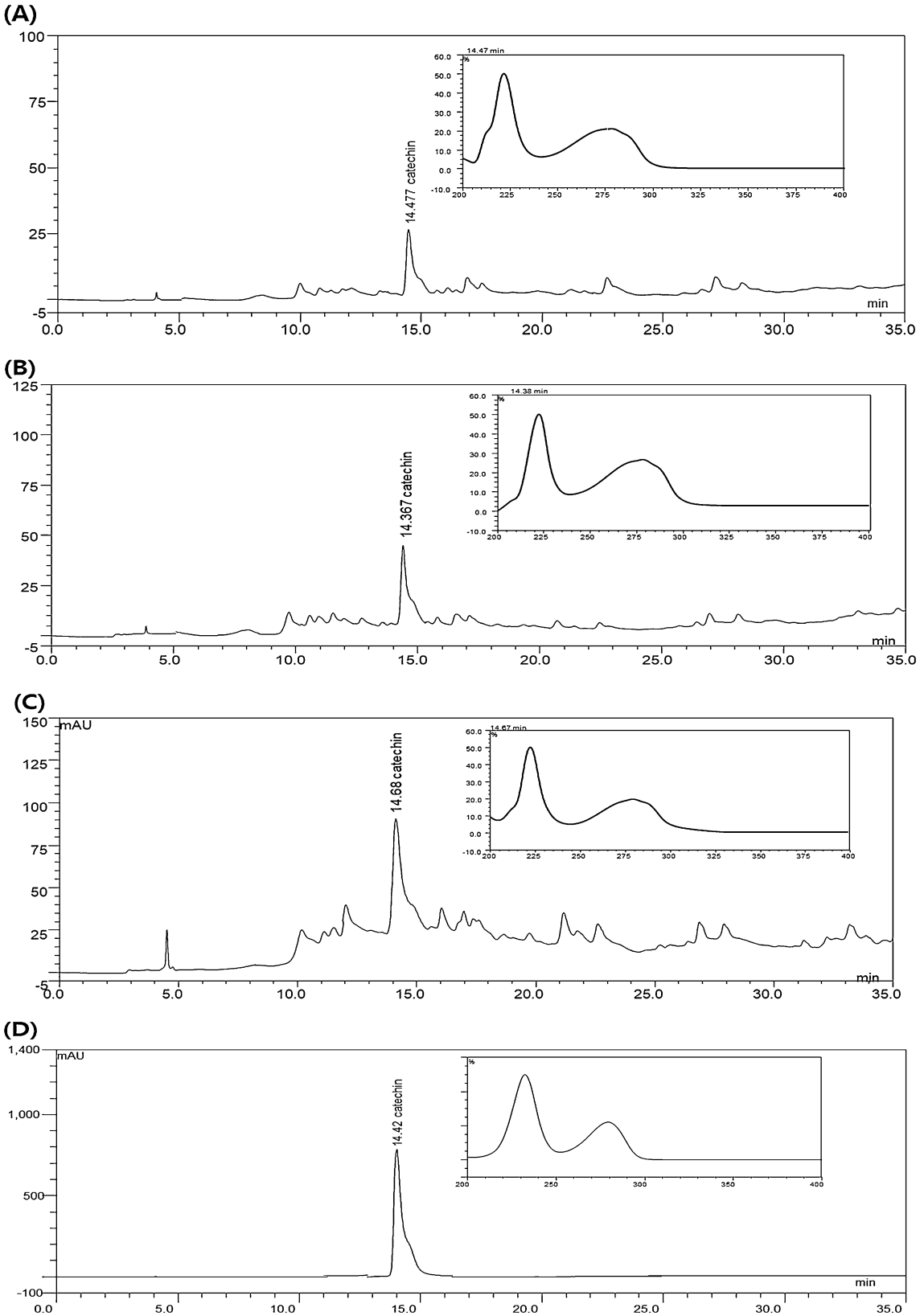

Fig. 5 HPLC Chromatograms of 50%, 70%, and 95% ethanol A. *hookeri* extract and catechin at 270 nm. Phenolic compounds were identified by comparing retention times and spectrum (RTs) and UV–Vis spectrum with commercial standards. (**A**): 50% ethanol *A. hookeri* extract. (**B**): 70% ethanol *A. hookeri* extract. (**C**): 95% ethanol *A. hookeri* extract. (**D**): Catechin. All samples were run in triplicate

inhibits apoptosis when cells are exposed to different stressors. On the other hand, Bax is pro-apoptotic protein, and does so by forming heterodimers with and thus deactivating Bcl-2 (Kim et al., 2010).

To investigated the inhibitory effect of 95AH on neuronal cell death, we examined the expressions of proteins related to apoptosis in H_2O_2 treated PC12 cells (Fig. 4). Treatment with 200 μM H_2O_2 for 2 h significantly decreased Bcl-2 expression by 28.06%, whereas pretreatment with 100 and 200 μg/mL of 95AH increased Bcl-2 expression by 41.84 and 53.29%, respectively compared with H_2O_2 treated PC12 cells ($p < 0.05$). On the other hand, treatment with H_2O_2 increased Bax expression by 45.90% whereas pretreatment with 100 and 200 μg/mL of 95AH decreased Bax expression by 10.55 and 32.73% as compared with H_2O_2 treated PC12 cells ($p < 0.05$). The Bcl-2/Bax protein ratio in 200 μM H_2O_2 treated PC12 cells was reduced to 48.38%, but pretreatment with 95AH at 100 and 200 μg/mL increased Bcl-2/Bax ratios by 32.11 and 86.78%, respectively, compared to H_2O_2 treated cells (both $p < 0.05$).

Previous studies have reported plants used as spices, such as onions and garlic, contain phenolics with excellent antioxidant properties, and the antioxidant properties of the plants effectively improve neuronal diseases such as Alzheimer's disease (Bahaeddin et al., 2016; Lee et al., 2014). Yang et al. (2013) evaluated the inhibitory effect of onion ethanol extract in glutamate treated neuronal cells, and observed it suppressed Bax but increased Bcl-2 protein expressions, which agrees with our findings.

Identification of major phenolic compound in *A. hookeri* extract

A. hookeri contains a variety of bioactive compounds including proteins, sugars, ascorbic acid, phytochemicals, and phenolics. In particular, the phenolics in *A. hookeri* are known to have considerable antioxidant effects by scavenging ROS and free radicals. In addition, many reports have concluded the redox activities of phenolics help maintain homeostasis when cells are exposed to various stresses (Das and Roychoudhury, 2014; Park et al., 2016; Roh et al., 2016). In a previous study, it was demonstrated that an ethanol extract of *A. hookeri* contained several phenolics, such as gallic acid, protocatechuic acid, catechin, and coumaric acid (Park et al., 2016).

In the present study, we used an HPLC spectroscophy approach to identify major phenol in 50AH, 70AH and 95AH (Fig. 5). The largest HPLC peak was identified at an RT of 14.4, 14.3 and 14.6 min in 50AH, 70AH and 95AH, respectively. The maximum absorption was at 278.5, 279.5 and 278.6 nm in 50AH, 70AH and 95AH UV–Vis spectrum, which was similar to the catechin standard (14.4 min at 278.8 nm). The amounts of catechin in 50AH, 70AH and 95AH were 2.2, 3.1, and 5.2 mg/g of dry weight, respectively (data not shown). Park et al. (2016) demonstrated that the amount of phenolic was catechin in 80% ethanol *A. hookeri* extract and was higher than water *A. hookeri* extract, which was similar pattern to the present study. On the other hand, very small or no peak of other phenolic compound was detected, showing different results from Park et al. (2016).

Catechin is main component of green tea and red wine, and has been reported to have great antioxidative effect and prevent diseases caused by oxidative stress (Monajjemi et al., 2011). A previous study reported that catechin from the onion (*Allium cepta* L.) effectively scavenged free radicals and inhibited serum levels of malondialdehyde and LDL (Jaiswal and Rizvi, 2014). In addition, a strong relation was observed between the antioxidative effects of various *Allium* sp. plants and its catechin content (Beretta et al., 2017). In this study, it was identified that 95AH has the highest amount of catechin contents, which was similar to the effects of radical scavenging and antioxidant enzymes. Therefore, we speculate that catechin may affect the antioxidant and protective effects of 95AH on H_2O_2-induced oxidative stress.

In the present study, we examined the antioxidant activities and neuro-protective effects of *A. hookeri* extracts obtained using different ethanol concentrations (50, 70, and 95%). The 95AH was found to have the highest total phenolic and flavonoid contents, and DPPH, ABTS, and FRAP assays showed it had greater radical scavenging activities than the other two extracts. 95AH was also found to have neuro-protective effects, as it inhibited oxidative damage and intracellular ROS levels and increased the activities of SOD and GSH-Px and increased Bcl-2/Bax ratio. Furthermore, HPLC showed that catechin was the most abundant phenolic expecially in 95AH. Based on these results, we believe *A. hookeri* extract offers a potential treatment for neuronal diseases.

Author contributions Experiment and wrote paper: SHY and SHR, Designed experiments and supervised: HJP and GHK. All authors reviewed and approved the final manuscript.

Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

References

Bae GC, Bae DY. The anti-inflammatory effects of ethanol extract of *Allium hookeri* cultivated in South Korea. Korean. J. Herbol.27: 55-61 (2012)

Bahaeddin Z, Yans A, Khodagholi F, Sahranavard S. Neuroprotection and anxiety like behavior reduction of *Allium hirtifolium* and *Astragalus hamosus* in the Aβ-injected rat. Res. J. Pharmacogn. 3: 39-49 (2016)

Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239: 70-76 (1996)

Beretta HV, Bannoud F, Insani M, Berli F, Hirschegger P, Galmarini CR, Cavagnaro PF. Relationships between bioactive compound content and the antiplatelet and antioxidant activities of six *Allium* vegetables species. Food Technol. Biotechnol. 55: 266 (2017)

Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. 10: 178-182 (2002)

Cobley JN, Fiorello ML, Bailey DM. 13 reasons why the brain is susceptible to oxidative stress. Redox Biol. 15: 490-503 (2018)

Collin F. Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases. Int. J. Mol. Sci. 20: 2407 (2019)

Das K, Roychoudhury A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. 2: 53 (2014)

Ghahremani-majd H, Dashti F, Dastan D, Mumivand H, Hadian J, Esna-Ashari M. Antioxidant and antimicrobial activities of Iranian mooseer (*Allium hirtifolium* Boiss) populations. Hortic. Environ. Biote. 53: 116-122 (2012)

Hong E, Park KH, Kim GH. Phenolic-enriched fractions from *perilla frutescens var*. acuta: Determinating rosmarinic acid and antioxidant activity. J. Food Biochem. 35: 1637-1645 (2011)

Hwang KA, Hwang YJ, Song J. Antioxidant activities and oxidative stress inhibitory effects of ethanol extracts from *Cornus officinalis* on raw 264.7 cells. BMC Complement Altern. Med. 16: 196 (2016)

Jaiswal N, Rizvi SI. Onion extract (*Allium cepa* L.), quercetin and catechin up-regulate paraoxonase 1 activity with concomitant protection against low-density lipoprotein oxidation in male Wistar rats subjected to oxidative stress. J. Sci. Food Agric. 94: 2752-2757 (2014)

Jeong HJ, Park SB, Kim SA, Kim HK. Total polyphenol content and antioxidative activity of wild grape (*Vitis coignetiae*) extracts depending on ethanol concentrations. J. Korean Soc. Food Sci. Nutr. 36: 1491-1496 (2007)

Jung HY, Lee KY, Yoo DY, Kim JW, Yoo M, Lee S, Yoo K-Y, Yoon YS, Choi JH, Hwang IK. Essential oils from two *Allium* species exert effects on cell proliferation and neuroblast differentiation in the mouse dentate gyrus by modulating brain-derived neurotrophic factor and acetylcholinesterase. BMC Complement Altern. Med. 16: 431 (2016)

Kang W, Yang H, Hong HJ, Han CH, Lee YJ. Anti-oxidant activities of kiwi fruit extract on carbon tetrachloride-induced liver injury in mice. Korean J. Vet. Res. 52: 270-280 (2012)

Kim MW. Effects of Dietary Supplementation with *Allium hookeri* root on hepatic enzyme contents in streptozotocin-induced diabetic rats. J. East Asian Soc. Diet. Life 27: 399-407 (2017)

Kim JY, Lee JA, Kim KN, Song GP, Park SY. Antioxidative and antimicrobial activities of *Euphobia helioscopia* extracts. J. Korean Soc. Food Sci. Nutr. 36: 1106-1112 (2007)

Kim JH, Yang HK, Hong HJ, Kang WY, Kim DG, Kim SC, Song KJ, King D, Han CH, Lee YJ. Neuroprotective effects of Korean kiwifruit against t-BHP-induced cell damage in PC12 cells. Korean J. Plant Res. 23: 165-171 (2010)

Kim JY, Ko W, Kim AJ. Effects of *Allium hookeri* extracts on glutamate-induced neurotoxicity in HT22 cells. Korean J. Pharmacon. 48: 31-37 (2017)

Kurutas EB. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutr. J. 15: 71 (2015)

Kwon JW, Lee HK, Park HJ, Kwon TO, Choi HR, Song JY. Screening of biological activities to different ethanol extracts of *Rubus coreanus Miq*. Korean J. Med. Crop Sci. 19: 325-333 (2011)

Lee KW, Kim YS, Park PJ, Jeong JH. Comparison of effect of water and ethanolic extract from roots and leaves of *Allium hookeri*. J. Korean Soc. Food Sci. Nutr. 43: 1808-1816 (2014)

Lee NY, Cho WY, Jang HJ, Lee CH. Effect of Dietary supplementation of *Allium hookeri* root and processed sulfur on the growth performance of guinea pigs. Int. J. Anim. Vet. Sci. 11: 614-617 (2017)

Mensor LL, Menezes FS, Leitão GG, Reis AS, Santos TCD, Coube CS, Leitão SG. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 15: 127-130 (2001)

Monajjemi M, Khosravi M, Honarparvar B, Mollaamin F. Substituent and solvent effects on the structural bioactivity and anticancer characteristic of catechin as a bioactive constituent of green tea. Int. J. Quantum Chem. 111: 2771-2777 (2011)

Park JY, Yoon KY. Comparison of the nutrient composition and quality of the root of *Allium hookeri* grown in Korea and Myanmar. Korean J. Food Sci. Technol. 46: 544-548 (2014)

Park SY, Je JY, Ahn CB. Phenolic Composition and Hepatoprotective Activities of *Allium hookeri* against hydrogen-peroxide-induced oxidative stress in cultured hepatocytes. J. Food Biochem. 40: 284-293 (2016)

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26: 1231-1237 (1999)

Ren G, Qiao HX, Yang J, Zhou CX. Protective effects of steroids from *Allium chinense* against H_2O_2-induced oxidative stress in rat cardiac H9C2 cells. Phytother. Res.24: 404-409 (2010)

Rhyu DY, Park SH. Characterization of alkyl thiosulfinate in *Allium hookeri* root using HPLC-ESI-MS. J. Korean Soc. Appl. Biol. Chem. 56: 457-459 (2013)

Roh SS, Kwon OJ, Yang JH, Kim YS, Lee SH, Jin JS, Jeon YD, Yokozawa T, Kim HJ. *Allium hookeri* root protects oxidative stress-induced inflammatory responses and β-cell damage in pancreas of streptozotocin-induced diabetic rats. BMC Complement. Altern. Med. 16: 63 (2016)

Sakamoto T, Imai H. Hydrogen peroxide produced by superoxide dismutase SOD-2 activates sperm in *Caenorhabditis elegans*. J. Biol. Chem. 292: 14804-14813 (2017)

Sakihama Y, Cohen MF, Grace SC, Yamasaki H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. Toxicology 177: 67-80 (2002)

Sapkota K, Park SE, Kim JE, Kim S, Choi HS, Chun HS, Kim SJ. Antioxidant and antimelanogenic properties of chestnut flower extract. Biosci. Biotechnol. Biochem. 74: 1527-1533 (2010)

Song JC, Park NK, Hur HS, Bang MH, Baek NI. Examination and isolation of natural antioxidants from Korean medicinal plants. Korean J. Med. Crop Sci. 8: 94-101 (2000)

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39: 44-84 (2007)

Xu Y, Xin Y, Diao Y, Lu C, Fu J, Luo L, Yin Z. Synergistic effects of apigenin and paclitaxel on apoptosis of cancer cells. PloS ONE 6: e29169 (2011)

Yang EJ, Kim GS, Kim JA, Song KS. Protective effects of onion-derived quercetin on glutamate-mediated hippocampal neuronal cell death. Pharmacogn. Mag. 9: 302 (2013)

Yang MH, Kim NH, Heo JD, Rho JR, Ock KJ, Shin EC, Jeong EJ. Comparative evaluation of sulfur compounds contents and antiobesity properties of *Allium hookeri* prepared by different drying methods. Evid. Based Complementary Altern. Med. 10.1155/2017/2436927 (2017)

Yin MC, Cheng WS. Antioxidant activity of several *Allium* members. J. Agr. Food Chem. 46: 4097-4101 (1998)

You S, Jang M, Kim G. Antioxidant activity and neuroprotective effect of root bark of *Morus alba* L. extract against hydrogen peroxide-induced cytotoxicity in PC12 cells. J. Korean Soc. Food Sci. Nutr. 47: 519-627 (2018)