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Rhus verniciflua Stokes Extract and Its Flavonoids Protect PC-12 Cells against H_2O_2-Induced Cytotoxicity

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Rhus verniciflua Stokes (RVS), an herbal medicine found in East Asia, was extracted and further fractionated to investigate its antioxidant capacity and neuroprotective effects. The RVS ethyl acetate (EtOAc) fraction had the highest level of total phenolics and antioxidant capacity among all solvent fractions tested. Pretreatment of PC-12 cells with the EtOAc fraction effectively attenuated H_2O_2-induced oxidative damage. Furthermore, the EtOAc fraction significantly attenuated caspase-3 activity, resulting in inhibition of H_2O_2-induced apoptosis. We identified and quantified fustin, sulfuretin, and butein in the EtOAc fraction using accurate mass quadrupole time-of-flight mass spectrometry and reversed-phase high-performance liquid chromatography. The intracellular antioxidant capacity and superoxide dismutase (SOD) activity were significantly increased in PC-12 cells treated with the EtOAc fraction and with individual flavonoids. When cells were pretreated with the EtOAc fraction or individual flavonoids and then co-incubated with diethyldithiocarbamic acid (an inhibitor of SOD activity), cell viability against H_2O_2-induced oxidative stress was attenuated. These results suggest that the RVS EtOAc fraction and its flavonoid constituents protect PC-12 cells against H_2O_2-induced neurotoxicity through their antioxidant properties.

Keywords: *Rhus verniciflua* Stokes, neuroprotective effect, quadrupole time-of-flight mass spectrometry, caspase-3 activity, superoxide dismutase activity

Introduction

Reactive oxygen species (ROS) regulate cellular signaling pathways related to the cell cycle, proliferation, and apoptosis [1] and are associated with altered cellular oxidation and impaired cellular function. ROS include the superoxide radical anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). Intracellular ROS are generated endogenously as by-products of aerobic metabolism, such as mitochondrial oxidative phosphorylation, and they can be generated from exogenous sources such as cytokines and bacterial invasion [2]. ROS accumulation causes DNA cleavage, protein oxidation, and lipid peroxidation in neuronal cells, thus inducing age-related and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (AD). AD is ranked as the 7th leading cause of death in the United States and is characterized by the progressive deterioration of cognition and memory loss [3].

H_2O_2 is one of ROS that, in its electrically neutral form, diffuses through cellular membranes [1]. Exogenous H_2O_2 is considered to be a mediator of apoptosis that induces oxidative stress in neuronal cells. Hydroxyl radicals derived from H_2O_2 can lead to cytotoxicity in neuronal cells and downregulate antioxidant enzyme expression, inducing caspase-3 activation. Thus, H_2O_2 has been widely used to damage PC-12 cells derived from rat pheochromocytoma [4].

Various studies have suggested that some dietary flavonoids have protective effects against oxidative stress,

neuronal apoptosis, and neurodegenerative processes [5, 6]. Flavonoids can act as direct antioxidants to protect nerve cells against ROS. Some flavonoids are taken up into the cell and/or bind to the cell membrane to prevent intracellular dysfunction and oxidation of lipid membranes [7]. Flavonoids also increase antioxidant enzyme activity, which is the primary endogenous antioxidant defense system in living organisms [8]. Endogenous antioxidant enzymes reduce oxidative stress in cells and prevent apoptosis of neuronal cells. Superoxide-induced caspase-3 activation is inhibited by superoxide dismutase (SOD) and enhanced by the SOD inhibitor ammonium tetrathiomolybdate [9]. Antioxidant molecules such as *N*-acetyl cysteine prevent superoxide-induced neuronal cell death by inhibiting caspase-3 activity [10]. Furthermore, flavonoids inhibit 6-hydroxydopamine (6-OHDA)-induced neuronal cell death by enhancing SOD activity and the radical scavenging properties of the cells [11].

Rhus verniciflua Stokes (RVS), a deciduous tree of the Anacardiaceae family, has traditionally been used as a food ingredient and herbal medicine in East Asia. It is well known that the flavonoids fustin, sulfuretin, and butein are abundant in RVS [12]. In recent years, RVS extracts have been shown to have neuroprotective effects against glutamate-induced toxicity in rat cortical cells and rotenone-induced apoptosis in the SH-SY5Y human neuronal cell line [13, 14]. Furthermore, sulfuretin and fustin from RVS have protective in vitro effects against amyloid beta-associated and 6-OHDA-induced neuronal cell death, respectively [15, 16]. However, the neuroprotective effects of RVS on H_2O_2-induced neuronal PC-12 cell death have rarely been studied.

In this study, we determined the neuroprotective effects of an RVS extract and its flavonoids (butein, sulfuretin, and fustin) in PC-12 cells exposed to H_2O_2. An aqueous methanol extract of RVS was further fractionated to obtain organic solvents and water fractions. We determined the total phenolic contents and antioxidant capacity of the RVS solvent fractions. Three major flavonoids in the ethyl acetate (EtOAc) fraction were identified and quantified using accurate mass quadrupole time-of-flight mass spectrometry (Q-TOF MS) and reversed-phase high performance liquid chromatography (HPLC). Based on an in vitro cell model, we determined the neuroprotective effects of the EtOAc fraction and individual flavonoids against H_2O_2-induced cytotoxicity in PC-12 cells. Specifically, we evaluated the effects of the EtOAc fraction and its flavonoid constituents on cell viability, caspase-3 activity, and intracellular ROS formation. Furthermore, we investigated the effects of tested samples on SOD enzyme activity in PC-12 cells.

Materials and Methods

Materials

Folin-Ciocalteu's phenol reagent, gallic acid, 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), ascorbic acid, hydrogen peroxide, dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), butein, diethyldithiocarbamic acid (DETC), formic acid, and the caspase-3 assay kit were products of Sigma-Aldrich Co. LLC. (USA). 2,2′-Azo-*bis*(2-amidino-propane)dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (USA). Fustin and sulfuretin were obtained from Extrasynthése (France). RPMI 1640 medium and Hank's balanced salt solution was purchased from Welgene Inc. (Korea). Fetal bovine serum (FBS) and streptomycin were obtained from Gibco BRL (USA). The SOD assay kit-WST was obtained from Dojindo Molecular Technologies (Japan). All other chemicals were of analytical or HPLC grade.

Preparation of RVS Extract

RVS was purchased from a local market (Jecheon, Korea) and extracted with 80% (v/v) aqueous methanol using a homogenizer at 15,000 rpm for 15 min, followed by sonication for 30 min. The mixture was soaked and agitated frequently at room temperature for 24 h and then filtered through Whatman #2 filter paper (Whatman International Limited, UK). The residues were re-extracted by repeating the above steps. The filtrate was evaporated at 37°C and dissolved in deionized water. Extracts dissolved in deionized water were partitioned with organic solvents to yield *n*-hexane, EtOAc, *n*-butanol, chloroform, and water fractions. The fractions were then evaporated and stored at −20°C until analysis.

Total Phenolic Content

The total phenolic content of each RVS fraction was determined by using Folin-Ciocalteu's method [17]. Briefly, the tested samples were mixed with deionized water, and Folin-Ciocalteu's phenol reagent and 7% (w/v) Na_2CO_3 solution were added. After 90 min at room temperature, the absorbance was read against the prepared blank at 750 nm. Total phenolic content is expressed as mg gallic acid equivalents (GAE)/g of solid fraction.

Antioxidant Capacity

Antioxidant capacity was measured by the ABTS radical assay [18]. The resulting blue-green ABTS radical solution was adjusted to an absorbance of 734 nm at approximately 0.650 ± 0.020. The samples were then added to the ABTS radical solution. After the mixture was incubated in a water bath at 37°C for 10 min, the decline in absorbance was measured at 734 nm. Antioxidant capacity is expressed as mg vitamin C equivalents (VCE)/g of solid fraction.

Effects of RVS Solvent Fractions on PC-12 Cell Viability

PC-12 cells (ATCC, USA) derived from a transplantable rat pheochromocytoma were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in a humid 5% CO_2 and 95% air environment at 37°C. Cells (2×10^4 cells/well) were seeded in 96-well plates and incubated for 24 h before sample treatment. After incubation, the PC-12 cells were pretreated with various concentrations of solvent fractions for 24 h and then cultured for an additional 1 h with 200 μM H_2O_2. The control group was treated with the same medium without test samples and H_2O_2. Cell viability was measured using MTT reduction assay. Cells were incubated with 0.5 mg MTT/ml for 4 h at 37°C. MTT formazan crystals formed by viable cells were dissolved by the addition of DMSO and were quantified by measuring the absorbance using a microplate reader (Bio-Rad Laboratories, USA) at 570 and 630 nm as the test and reference wavelength, respectively.

Caspase-3 Activity

PC-12 cells (5×10^6 cells/well) were plated in 6-well plates and incubated for 24 h before sample treatment. After treatment with the EtOAc fraction for 24 h, cells were further incubated with 200 μM H_2O_2 for 24 h. Caspase-3 activity was determined using a colorimetric caspase assay kit according to the manufacturer's instructions. Absorbance was measured at 405 nm using an Infinite M200 (Tecan Austria GmbH, Austria). Caspase-3 activity is expressed as the percentage of the OD_{405} value to the control, which was set to 100%.

HPLC and Q-TOF MS Analyses

A reversed-phase HPLC system (LC-20; Shimadzu, Japan) equipped with a Symmetry C_{18} column (5 μm, 4.6 mm × 250 mm; Waters Corp., USA), an autosampler (SIL-20A; Shimadzu), a photodiode array detector (SPD-M20A; Shimadzu), a binary pump (LC-20AD; Shimadzu), and a vacuum degasser was used to quantify individual flavonoids from the RVS EtOAc fraction. A linear solvent gradient of binary mobile phase with solvent A (0.1% (v/v) formic acid in deionized water) and solvent B (0.1% (v/v) formic acid in acetonitrile) was applied as follows: 92% A/8% B at 0 min, 89% A/11% B at 4 min, 76% A/24% B at 25 min, 72.5% A/27.5% B at 27 min, 40% A/60% B at 50 min, 92% A/8% B at 52 min, and 92% A/8% B at 60 min. The detector was set at 280 nm for fustin and 370 nm for sulfuretin and butein. The flow rate was 1 ml/min and the injection volume was 20 μl. The concentration of each flavonoid was determined using authentic standard curves.

Flavonoids in the RVS EtOAc fraction were qualitatively analyzed by using an Agilent 6530 Q-TOF with an Agilent 1200 Series rapid resolution liquid chromatograph system (Agilent, USA). The Q-TOF MS was operated with an orthogonal electrospray source in negative ion mode. Separation of individual flavonoids was performed on a Symmetry C_{18} column (5 μm, 4.6 mm × 250 mm; Waters Corp.). The drying gas (nitrogen) temperature was set at 350°C with a flow of 10 l/min, the nebulizer pressure was 50 psi, the fragmentor voltage was 185 V, and the capillary voltage was 4,000 V. The mass range was set from m/z 100 to 1,000. The same linear solvent gradient as described under the HPLC conditions was applied.

Measurement of Intracellular Oxidative Stress

The concentration of intracellular ROS was measured using a fluorescent probe (DCFH-DA) assay. After the cells were cultured with the solvent fractions and the three flavonoids identified in the EtOAc fraction for 24 h, they were washed with fresh PBS and incubated with 50 μM DCFH-DA in Hank's balanced salt solution at 37°C for 30 min. Then, 50 μM H_2O_2 was added to each well for 1 h. Fluorescence was quantified at 530 and 485 nm (emission and excitation, respectively) using a microplate reader (Infinite M200; Tecan Austria GmbH).

Determination of Cellular Superoxide Dismutase Activity

Cells were seeded in 6-well plates at a density of 2×10^6 cells/well and incubated for 24 h. The cells were subsequently treated with the EtOAc fraction and its flavonoids for 24 h. After incubation, cells were harvested and centrifuged at 2,000 ×g for 10 min at 4°C. After discarding the supernatant, cells were frozen for 30 min at −20°C and sonicated in an ice bath for 15 min. This step was repeated twice, and the cells were centrifuged at 10,000 ×g for 10 min at 4°C. The supernatant was used to determine SOD activity using the SOD assay kit-WST, following the manufacturer's instructions. Absorbance was detected at 450 nm using a microplate reader (Infinite M200; Tecan Austria GmbH). To normalize SOD activity, the amount of protein in each lysate was measured.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD) of three replicate determinations. An analysis of variance and Duncan's multiple range test at the level of $p < 0.05$ were applied to determine differences among data. All statistical analyses were performed using SAS software (ver. 8.2; SAS Institute Inc., USA).

Results

Total Phenolic Content and Antioxidant Capacity

The total phenolic contents of five RVS solvent fractions are shown in Table 1. The EtOAc fraction exhibited the highest total phenolic content (911.1 mg GAE/g) followed by the *n*-butanol fraction (328.6 mg GAE/g), *n*-hexane fraction (246.7 mg GAE/g), chloroform fraction (235.2 mg GAE/g), and water fraction (66.9 mg GAE/g).

The ABTS radical scavenging assay is based on the spectrophotometric measurement of cation radical concentrations and is widely used to determine the antioxidant capacity of

Table 1. Total phenolic content and antioxidant capacity in various fractions of *Rhus verniciflua* Stokes.

Fractions	Total phenolic content (mg gallic acid equiv./g solid fraction)	Antioxidant capacity (mg vitamin C equiv./g solid fraction)
n-Hexane	246.7 ± 18.8[1]c[2]	243.2 ± 16.6d
Chloroform	235.2 ± 4.1c	339.3 ± 6.9c
Ethyl acetate	911.1 ± 10.6a	1,648.1 ± 76.8a
n-Butanol	328.6 ± 8.6b	792.4 ± 47.8b
Water	66.9 ± 2.7d	91.5 ± 4.6e

[1]Each value is presented as the mean ± standard deviation (n = 3).

[2]Means with different letters in a column are significantly different by Duncan's multiple range test ($p < 0.05$).

fruit, vegetables, grains, and their products; therefore, we used it to determine the antioxidant capacity of each RVS fraction (Table 1). The EtOAc fraction had the highest antioxidant capacity (1,648.1 mg VCE/g) among all RVS solvent fractions tested. The overall antioxidant capacity of solvent fractions against the ABTS radical was established in decreasing order of EtOAc fraction > *n*-butanol fraction (792.4 mg VCE/g) > *n*-hexane fraction (339.3 mg VCE/g) > chloroform fraction (243.2 mg VCE/g) > water fraction (91.5 mg VCE/g).

Effects of RVS Solvent Fractions on Cell Viability

The protective effects of the RVS solvent fractions against H_2O_2-induced cell injury were observed by MTT reduction. None of the solvent fractions were cytotoxic at the highest concentrations tested, as determined by the MTT assay (data not shown). As shown in Fig. 1, the mitochondrial metabolic activity of PC-12 cells treated with 200 μM H_2O_2 was approximately 30% of that of the control group (100%) in the MTT reduction assay. However, when cells were pretreated with 10 μg/ml of the EtOAc fraction, viability was significantly increased to approximately 70%. There were no significant differences among cells pretreated with 1 μg/ml of the EtOAc fraction, cells treated with the other solvent fractions (1 and 10 μg/ml), and cells exposed to H_2O_2 treatment only.

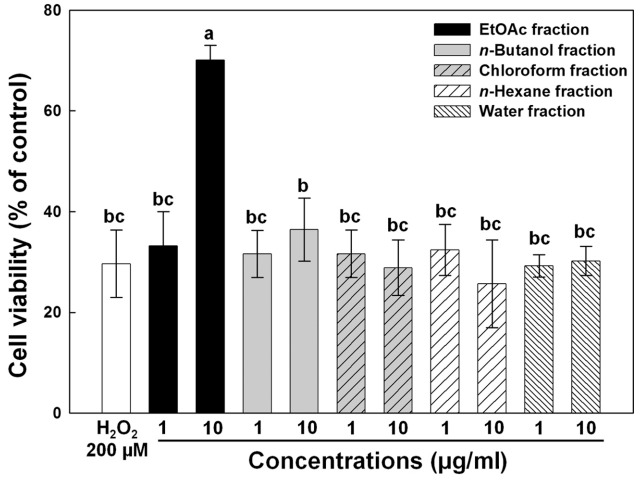

Fig. 1. Effects of five solvent fractions of *Rhus verniciflua* Stokes on the viability of PC-12 cells against H_2O_2-induced oxidative damage measured by MTT assay.

PC-12 cells were pretreated with solvent fractions for 24 h and exposed to 200 μM H_2O_2 for 1 h. Different letters on the bars indicate significant difference by Duncan's multiple range test ($p < 0.05$).

Effects of the EtOAc Fraction on Caspase-3 Activity in PC-12 Cells Exposed to H_2O_2

We investigated caspase-3 activity, a key inducer of apoptosis, in PC-12 cells exposed to H_2O_2. Caspase-3 activity was 319% higher in cells treated with H_2O_2 than in control cells (Fig. 2). However, in cells pretreated with the EtOAc fraction at concentrations of 1 and 10 μg/ml,

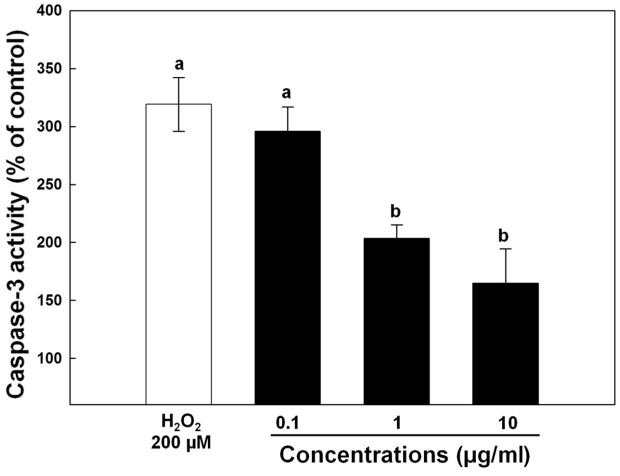

Fig. 2. Effects of the ethyl acetate fraction from *Rhus verniciflua* Stokes on H_2O_2-induced caspase-3 activity in PC-12 cells.

Different letters on the bars indicate significant difference by Duncan's multiple range test ($p < 0.05$).

caspase-3 activity was significantly decreased to approximately 203% and 165%, respectively.

HPLC and Q-TOF MS Analyses of Phenolics in the EtOAc Fraction

Among the solvent fractions, the EtOAc fraction had the highest level of total phenolics and antioxidant capacity (Table 1). Furthermore, the EtOAc fraction significantly increased cell viability and attenuated H_2O_2-induced caspase-3 activity (Figs. 1 and 2). We identified major flavonoids in the EtOAc fraction using Q-TOF MS by comparing full-scan data, fragment patterns, and retention time of authentic standards.

On the basis of the molecular ion and fragment patterns, we identified three major flavonoids from the EtOAc fraction as fustin, sulfuretin, and butein, with monoisotopic masses of 288.0634, 270.0528, and 272.0684, respectively. Full-scan data of the three flavonoids exhibited molecular ions at *m/z* 287.0570, 269.0449, and 271.0614 of $[M–H]^-$, respectively (Table 2). The flavonoids were further identified by retention time, UV-Vis spectra, and molar mass using authentic standards. The results tentatively indicated that all three flavonoids are present in the aglycone form. We did not observe fragment ions such as sugar moieties or methylated derivatives in the MS2 scan data.

Quantitative analyses of the flavonoids detected in the EtOAc fraction were performed using reversed-phase HPLC. We measured 13.8 mg fustin, 10.7 mg sulfuretin, and 8.3 mg butein in the RVS EtOAc fraction (g solid fraction).

Intracellular Oxidative Stress

To determine whether H_2O_2-induced cell death is linked to upregulation of intracellular ROS, we measured ROS production using DCFH-DA fluorescence and quantified the intracellular oxidative stress in the presence of the EtOAc fraction, fustin, sulfuretin, and butein (Fig. 3). A decrease in cell viability by more than 10% compared with control cells was considered to be cytotoxic. The EtOAc fraction, fustin, sulfuretin, and butein showed no cytotoxicity in PC-12 cells at the concentrations applied (data not shown). ROS formation in cells pretreated with the EtOAc fraction (10 μg/ml) was approximately 54% that of cells treated with H_2O_2 only. ROS levels were significantly attenuated in cells pretreated with 25 μM fustin, 25 μM sulfuretin, and 10 μM butein to approximately 90%, 80%, and 83%, respectively, compared with those of cells treated with H_2O_2 only. Butein at the 10 μM concentration resulted in significantly ($p < 0.05$) higher intracellular oxidative stress than sulfuretin or fustin (Fig. 3).

Table 2. Concentrations (mg/g solid fraction) of individual flavonoids present in the ethyl acetate fraction of *Rhus verniciflua* Stokes.

Flavonoids	λ_{max} (nm)	$[M–H]^-$ (*m/z*)[a]	Concentrations
Fustin	232, 278	287.0570	13.8 ± 1.4[b]
Sulfuretin	257, 394	269.0449	10.7 ± 1.1
Butein	260, 378	271.0614	8.3 ± 1.0

[a]Negative-ion mode of Q-TOF MS of flavonoids in the ethyl acetate fraction of *Rhus verniciflua* Stokes.

[b]Each value is presented as the mean ± standard deviation ($n = 3$).

Determination of Intracellular Antioxidant Enzymes

SOD enzyme activity was determined in cells treated with the EtOAc fraction and individual flavonoids. SOD activity was significantly higher in PC-12 cells treated with the EtOAc fraction, fustin, sulfuretin, and butein at the highest concentration tested than that of control cells (Fig. 4A).

To determine whether the enhanced SOD activity provided protective effects in H_2O_2-induced PC-12 cell injury, an MTT assay was performed (Fig. 4B). Cells were co-treated with DETC (as a SOD inhibitor), the EtOAc fraction, and individual flavonoids for 24 h. DETC (1.0 mmol/l) was not cytotoxic in PC-12 cells (data not shown). When cells were co-treated with DETC and test samples, the protective effects were significantly less than those observed in cells without DETC co-treatment. These results indicate that the EtOAc fraction and its flavonoids protect PC-12 cells

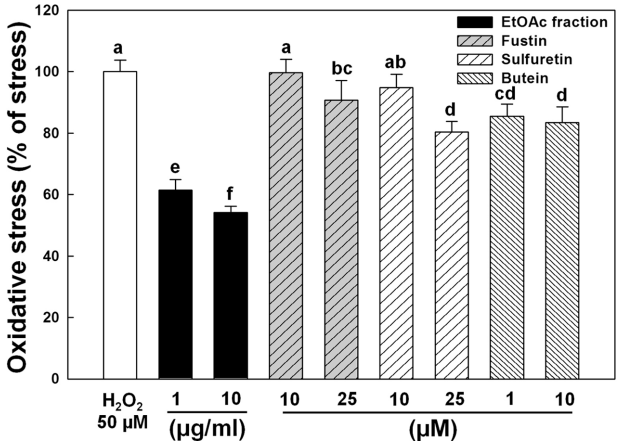

Fig. 3. Effect of the ethyl acetate fraction from *Rhus verniciflua* Stokes and its flavonoids (fustin, sulfuretin, and butein) on intracellular oxidative stress determined by DCFH-DA assay. Different letters on the bars indicate significant difference by Duncan's multiple range test ($p < 0.05$).

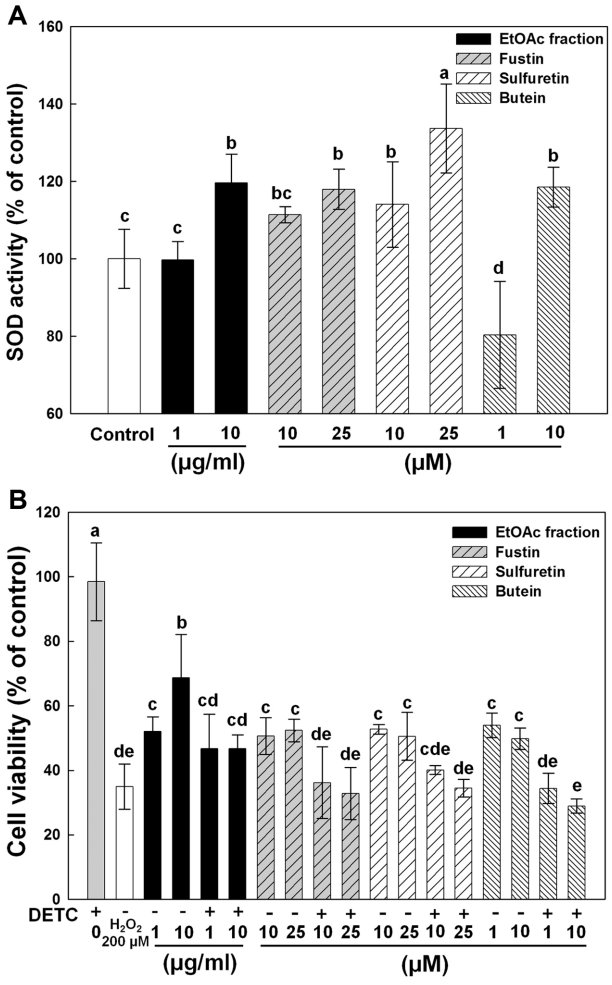

Fig. 4. Effects of the ethyl acetate fraction from *Rhus verniciflua* Stokes and its flavonoids (fustin, sulfuretin, and butein) on superoxide dismutase (SOD) activity (**A**) and PC-12 cell viability (**B**) in the absence and presence of diethyldithiocarbamic acid (DETC) determined using the MTT assay.

Different letters on the bars indicate significant difference by Duncan's multiple range test at $p < 0.05$.

against H_2O_2-induced oxidative damage by upregulating SOD activity.

Discussion

Overproduction of ROS and impaired antioxidative defenses usually induce neuronal cell death. It is well known that brain neuronal cells are vulnerable to ROS, which leads to the development of neurodegenerative diseases [19]; therefore, regulating ROS may be a critical intervention in maintaining cellular functions. In this study, we demonstrate that the RVS EtOAc fraction and its flavonoids effectively inhibit H_2O_2-induced neuronal cell death. The protective effects of the EtOAc fraction and its flavonoids were attributed to their antioxidant capacity, as evidenced by intracellular ROS scavenging and upregulation of SOD activity (Figs. 3 and 4).

Apoptosis is generally characterized by morphological changes such as cell shrinkage, nuclear fragmentation, and formation of membrane pores. Apoptosis is critically mediated by activation of a group of cysteine proteases, including caspase-3, an apoptosis executer enzyme [20]. Caspase-3 is a predominant enzyme involved in H_2O_2-induced apoptosis in the PC-12 cell model [21]. ROS generation resulting in apoptosis can be blocked by antioxidants. Cordycepin has been reported to effectively reduce 6-OHDA-induced expression of caspase-3 through its antioxidant properties [22]. Additionally, the protective antioxidant effects of 7,8-dihydroxyflavone on 6-OHDA-induced apoptosis in PC-12 cells have been investigated [11]. Our results indicate that H_2O_2 significantly increases caspase-3 activation in PC-12 cells. However, caspase-3 activity was attenuated by pretreatment with the RVS EtOAc fraction. Therefore, suppression of caspase-3 activity by the EtOAc fraction may be partially attributable to its antioxidant capacity.

Several reports have indicated that RVS extracts and their flavonoids act as potent antioxidants [23, 24]. The antioxidant capacities of sulfuretin, fustin, and butein are directly associated with adjacent hydroxyl groups as electron donors, resulting in direct radical scavenging properties. Accumulation of ROS can induce molecular damage in neuronal cells connected to the stress response and apoptosis. Therefore, intracellular ROS scavenging activity plays an important role in preventing oxidative injury. Antioxidants such as flavonoids attenuate intracellular ROS levels in H_2O_2-induced oxidative stress [25]. To determine the oxidative damage induced by H_2O_2, intracellular ROS production was measured using DCFH-DA fluorescence. In this study, we found that H_2O_2 markedly increases intracellular ROS levels, whereas ROS production was attenuated by pretreatment with the RVS EtOAc fraction and its flavonoid constituents, indicating their direct antioxidant activities (Fig. 3).

Endogenous antioxidant enzymes can prevent premature aging as well as neuronal cell death [26]. SOD catalyzes dismutation of the superoxide anion, a primary antioxidant defense mechanism. Oxidases present in human vascular endothelial cells primarily produce superoxide anions. The presence of extracellular superoxide anions induces apoptosis in cultured cells [27]. It has been found that

treatment with RVS extracts increases intracellular SOD activity in hepatic cells [28]. Butein and sulfuretin provide enhanced neuroprotective effects in HT22 cells treated with glutamate by maintaining antioxidant defense mechanisms such as SOD and glutathione reductase activities [29]. In this study, we demonstrated that pretreatment with the RVS EtOAc fraction and its flavonoid constituents markedly enhanced SOD activity in PC-12 cells (Fig. 4). Moreover, the enhanced SOD activity provides further protective effects in H_2O_2-induced PC-12 cell injury.

In conclusion, the RVS EtOAc fraction had the highest total phenolic content and antioxidant capacity among all solvent fractions tested. Furthermore, the RVS EtOAc fraction and its flavonoids effectively protected PC-12 cells against H_2O_2-induced cytotoxicity by decreasing caspase-3 activity and reducing H_2O_2-induced oxidative damage, as well as directly scavenging intracellular radicals and enhancing SOD activity. Thus, our results suggest that the RVS EtOAc fraction and its flavonoids are powerful antioxidants that represent an important source for preventing ROS-related neurological disorders.

Acknowledgments

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References

1. Reth M. 2002. Hydrogen peroxide as second messenger in lymphocyte activation. *Nat. Immunol.* **3:** 1129-1134.
2. Ray PD, Huang B-W, Tsuji Y. 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **24:** 981-990.
3. Alzheimer's Association. 2010. 2010 Alzheimer's disease facts and figures. *Alzheimers Dement.* **6:** 158-194.
4. Magliaro BC, Saldanha CJ. 2009. Clozapine protects PC-12 cells from death due to oxidative stress induced by hydrogen peroxide via a cell-type specific mechanism involving inhibition of extracellular signal-regulated kinase phosphorylation. *Brain Res.* **1283:** 14-24.
5. Maher P, Salgado KF, Zivin JA, Lapchak PA. 2007. A novel approach to screening for new neuroprotective compounds for the treatment of stroke. *Brain Res.* **1173:** 117-125.
6. Nichols M, Zhang J, Polster B, Elustondo P, Thirumaran A, Pavlov E, Robertson G. 2015. Synergistic neuroprotection by epicatechin and quercetin: activation of convergent mitochondrial signaling pathways. *Neuroscience* **308:** 75-94.
7. Wolfe KL, Liu RH. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.* **55:** 8896-8907.
8. Martín MÁ, Serrano ABG, Ramos S, Pulido MI, Bravo L, Goya L. 2010. Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *J. Nutr. Biochem.* **21:** 196-205.
9. Park S, Kim JA, Choi S, Suh SH. 2010. Superoxide is a potential culprit of caspase-3 dependent endothelial cell death induced by lysophosphatidylcholine. *J. Physiol. Pharmacol.* **61:** 375-381.
10. Sarker KP, Obara S, Nakata M, Kitajima I, Maruyama I. 2000. Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. *FEBS Lett.* **472:** 39-44.
11. Han X, Zhu S, Wang B, Chen L, Li R, Yao W, *et al*. 2014. Antioxidant action of 7,8-dihydroxyflavone protects PC12 cells against 6-hydroxydopamine-induced cytotoxicity. *Neurochem. Int.* **64:** 18-23.
12. Jung CH, Jun C-Y, Lee S, Park C-H, Cho K, Ko S-G. 2006. *Rhus verniciflua* Stokes extract: radical scavenging activities and protective effects on H_2O_2-induced cytotoxicity in macrophage RAW 264.7 cell lines. *Biol. Pharm. Bull.* **29:** 1603-1607.
13. Jeong EJ, Sung SH, Kim JW, Kim SH, Kim YC. 2008. *Rhus verniciflua* Stokes attenuates glutamate-induced neurotoxicity in primary cultures of rat cortical cells. *Nat. Prod. Sci.* **14:** 156-160.
14. Sapkota K, Kim S, Park S-E, Kim S-J. 2011. Detoxified extract of *Rhus verniciflua* Stokes inhibits rotenone-induced apoptosis in human dopaminergic cells, SH-SY5Y. *Cell. Mol. Neurobiol.* **31:** 213-223.
15. Kwon S-H, Ma S-X, Hwang J-Y, Lee S-Y, Jang C-G. 2015. Involvement of the Nrf2/HO-1 signaling pathway in sulfuretin-induced protection against amyloid beta 25–35 neurotoxicity. *Neuroscience* **304:** 14-28.
16. Park BC, Lee YS, Park H-J, Kwak M-K, Yoo BK, Kim JY, *et al*. 2007. Protective effects of fustin, a flavonoid from *Rhus verniciflua* Stokes, on 6-hydroxydopamine-induced neuronal cell death. *Exp. Mol. Med.* **39:** 316-326.
17. Singleton VL, Rossi JA Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **16:** 144-158.
18. Kim D-O, Lee KW, Lee HJ, Lee CY. 2002. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J. Agric. Food Chem.* **50:** 3713-3717.
19. Singh RP, Sharad S, Kapur S. 2004. Free radicals and oxidative stress in neurodegenerative diseases: relevance of dietary antioxidants. *J. Indian Acad. Clin. Med.* **5:** 218-225.
20. Elmore S. 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35:** 495-516.
21. Jiang B, Liu JH, Bao YM, An LJ. 2003. Hydrogen peroxide-induced apoptosis in pc12 cells and the protective effect of

puerarin. *Cell Biol. Int.* **27:** 1025-1031.
22. Olatunji OJ, Feng Y, Olatunji OO, Tang J, Ouyang Z, Su Z. 2016. Cordycepin protects PC12 cells against 6-hydroxydopamine induced neurotoxicity via its antioxidant properties. *Biomed. Pharmacother.* **81:** 7-14.
23. Lee J-C, Lim K-T, Jang Y-S. 2002. Identification of *Rhus verniciflua* Stokes compounds that exhibit free radical scavenging and anti-apoptotic properties. *Biochim. Biophys. Acta* **1570:** 181-191.
24. Lim K-T, Hu C, Kitts DD. 2001. Antioxidant activity of a *Rhus verniciflua* Stokes ethanol extract. *Food Chem. Toxicol.* **39:** 229-237.
25. Choi J, An X, Lee BH, Lee JS, Heo HJ, Kim T, *et al.* 2015. Protective effects of bioactive phenolics from jujube (*Ziziphus jujuba*) seeds against H_2O_2-induced oxidative stress in neuronal PC-12 cells. *Food Sci. Biotechnol.* **24:** 2219-2227.
26. Greenlund LJ, Deckwerth TL, Johnson EM. 1995. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron* **14:** 303-315.
27. Shen H-M, Yang C-F, Ding W-X, Liu J, Ong C-N. 2001. Superoxide radical-initiated apoptotic signalling pathway in selenite-treated HepG2 cells: mitochondria serve as the main target. *Free Radic. Biol. Med.* **30:** 9-21.
28. Son Y-O, Lee K-Y, Lee J-C, Jang H-S, Kim J-G, Jeon Y-M, *et al*. 2005. Selective antiproliferative and apoptotic effects of flavonoids purified from *Rhus verniciflua* Stokes on normal versus transformed hepatic cell lines. *Toxicol. Lett.* **155:** 115-125.
29. Cho N, Choi JH, Yang H, Jeong EJ, Lee KY, Kim YC, *et al*. 2012. Neuroprotective and anti-inflammatory effects of flavonoids isolated from *Rhus verniciflua* in neuronal HT22 and microglial BV2 cell lines. *Food Chem. Toxicol.* **50:** 1940-1945.