Neuroprotective Effects of Korean Red Pine (*Pinus densiflora*) Bark Extract and Its Phenolics

Ji-Won Kim^{1,2}, Sungbin Im^{1,2}, Ha-Ram Jeong^{1,2}, Young Sung Jung^{1,2}, Inil Lee^{1,2}, Kwan Joong Kim^{1,2}, Seung Kook Park¹, and Dae-Ok Kim^{1,2*}

¹Department of Food Science and Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea

²Skin Biotechnology Center, Kyung Hee University, Suwon 16229, Republic of Korea

Received: January 29, 2018
Revised: February 12, 2018
Accepted: February 14, 2018

First published online
March 15, 2018

*Corresponding author
Phone: +82-31-201-3796;
Fax: +82-31-204-8116;
E-mail: DOKIM05@khu.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2018 by
The Korean Society for Microbiology
and Biotechnology

Korean red pine (*Pinus densiflora*) is one of the major *Pinus* species in Korea. Red pine bark is removed prior to the chipping process in the wood industry and discarded as waste. However, red pine bark contains a considerable amount of naturally occurring phenolics, including flavonoids, and therefore may have a variety of biological effects. In this study, we investigated if Korean red pine bark extract (KRPBE) could protect neuronal PC-12 cells from oxidative stress and inhibit cholinesterase activity. Analysis of reversed-phase high-performance liquid chromatography results revealed four phenolics in KRPBE: vanillin, protocatechuic acid, catechin, and taxifolin. The total phenolic and flavonoid contents of KRPBE were 397.9 mg gallic acid equivalents/g dry weight (DW) and 248.7 mg catechin equivalents/g DW, respectively. The antioxidant capacities of KRPBE measured using ABTS, DPPH, and ORAC assays were 697.3, 521.8, and 2,627.7 mg vitamin C equivalents/g DW, respectively. KRPBE and its identified phenolics protected against H₂O₂-induced oxidative cell death in a dose-dependent manner. Acetylcholinesterase and butyrylcholinesterase, which degrade the neurotransmitter acetylcholine to terminate neurotransmission in synaptic clefts, were inhibited by treatment with KRPBE and its identified phenolics. Taken together, these results suggest that KRPBE and its constituent antioxidative phenolics are potent neuroprotective agents that can maintain cell viability under oxidative stress and inhibit cholinesterase activity.

Keywords: Acetylcholinesterase, antioxidant capacity, butyrylcholinesterase, oxidative stress, PineXol®, taxifolin

Introduction

Alzheimer's disease (AD) is an age-dependent, chronic, neurodegenerative disorder characterized by symptoms of progressive decline in activities of daily living, cognitive impairment, and memory deficits [1]. Oxidative stress generated by reactive oxygen species (ROS), including hydrogen peroxide and superoxide, is associated with the apoptosis of neuronal cells in neurodegenerative disorders [2]. When not sufficiently scavenged, oxidative stress may cause lipid peroxidation of membranes and lead to excessive cell death, a characteristic of many neurological disorders. Antioxidants can reduce oxidative stress in the

body and may protect neuronal cells against oxidative stress. In addition, AD could potentially be treated by maintaining the level of acetylcholine, a neurotransmitter, by inhibiting the activity of cholinesterases, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), with reversible inhibitors. AChE exists mainly in neurons, whereas BChE is associated primarily with glial cells and white matter [3]. The principal role of AChE is termination of nerve transmission at cholinergic synapses by rapid hydrolysis of acetylcholine. AChE and BChE inhibitors improve endogenous levels of acetylcholine in the brain of AD patients, thereby prolonging cholinergic neurotransmission. However, synthetic drugs such as

donepezil, tacrine, and rivastigmine that are used to treat the cognitive functional disorders and memory loss associated with AD have side effects and can result in digestive disorders [4]. Hence, identifying antioxidants and cholinesterase inhibitors from natural resources may yield non-toxic treatments that can halt AD progression.

Pine bark is removed prior to the chipping process in the lumber industry [5]. Although pine bark complicates wood processing, it has high contents of phenolics and lignins [5, 6]. Lignins are cross-linked polymers with phenolic structures. Large amounts of pine bark are generated as waste, although pine bark is used as a boiler fuel. Pine bark has been reported to contain procyanidins, flavonoids such as taxifolin and catechin, and phenolic acids such as ferulic, caffeic, cinnamic, and *p*-hydroxybenzoic acids [6, 7]. Procyanidin, which is a condensed tannin, is a polymer made up of subunits of catechin and epicatechin with various chain lengths and is one of the major polymeric phenolics in pine bark [8]. Procyanidins, flavonoids, and phenolic acids have been reported to have potent antioxidant capacity, which is dependent on the number of hydroxyl groups in their structures [9, 10]. Catechins (flavan-3-ols) exert potent neuroprotective effects partly due to their iron-chelating and radical-scavenging properties [11]. Phenolics, including catechin and procyanidin B2, have been reported to have neuroprotective effects by inhibiting AChE and BChE [12, 13].

Natural phenolics show a wide variety of health-promoting effects, including antioxidative, anti-cholinesterase, anticancer, and anti-inflammatory activities [13]. Phenolics are used in the food industry in a variety of applications, including as food colorants and antioxidants [14]. Phenolics are also used in the fields of cosmetics and pharmaceuticals to protect against oxidative stress [15]. French maritime pine (*Pinus pinaster*) bark extract (Pycnogenol®) is a well-known commercial product utilized as a health-promoting supplement and as a phytochemical disease treatment worldwide [16]. Pycnogenol® has been reported to have photoprotective, antimicrobial, free radical scavenging, and neuroprotective properties against oxidative stress [15, 16]. Korean red pine (*Pinus densiflora* Sieb. et Zucc.) has been reported to have higher amounts of total phenolics and flavonoids as well as antioxidant capacity in bark than other pine species [5]. The total phenolic and flavonoid contents of Korean red pine bark extract (KRPBE; PineXol®) were reported previously [17]. PineXol® significantly reduced lipid accumulation in 3T3-L1 cells, suggesting that it has anti-adipogenic effects [17].

The biological activity of Pycnogenol®, including its

neuroprotective ability, has been studied extensively. However, little is known about the antioxidative and neuroprotective effects of KRPBE. In the present study, we evaluated the total phenolic content, total flavonoid content, antioxidant capacity, and cell protective effects of KRPBE on neuronal PC-12 cells exposed to oxidative stress. We also used a reversed-phase high-performance liquid chromatography (HPLC) system to determine the phenolic constituents of KRPBE, and its anti-cholinesterase activity was examined using AChE and BChE inhibition assays.

Materials and Methods

Samples and Reagents

KRPBE (PineXol®) was obtained from Nutrapharm Ltd. (Korea). Folin-Ciocalteu's phenol reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, catechin, vanillin, protocatechuic acid, taxifolin, ascorbic acid, hydrogen peroxide (H₂O₂), dimethyl sulfoxide (DMSO), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), AChE, BChE, acetylcholine iodide (ATCI), butyrylthiocholine chloride (BTCC), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (tacrine), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and phosphate-buffered saline (PBS) were purchased from Sigma Aldrich Co., LLC (USA). Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, Hank's balanced salt solution (HBSS), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Welgene Inc. (Korea). All other reagents used were of analytical or HPLC grade.

Determination of Total Phenolic Content

The total phenolic content of KRPBE was measured using a colorimetric method with Folin-Ciocalteu's phenol reagent [18, 19]. Two hundred microliters of appropriately diluted KRPBE or gallic acid standard was mixed with 2.6 ml of deionized water. At 0 min, 200 µl of Folin-Ciocalteu's phenol reagent was added to the mixture. At 6 min, 2 ml of 7% (w/v) Na₂CO₃ solution was added. At 90 min, the absorbance was measured at 750 nm using a spectrophotometer (Spectronic 200; Thermo Fisher Scientific Inc., USA). The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g dry weight (DW) of KRPBE.

Determination of Total Flavonoid Content

The total flavonoid content of KRPBE was measured using the method of Liu *et al.* [20]. Briefly, 500 µl of diluted KRPBE or catechin standard was mixed with 3.2 ml of deionized water, and then 150 µl of 5% (w/v) NaNO₂ was added. At 5 min, 150 µl of 10% (w/v) AlCl₃ was added. At 6 min, 1 ml of 1 M NaOH was added. Absorbance was measured immediately at 510 nm using a spectrophotometer (Spectronic 200). The total flavonoid content

was expressed as mg catechin equivalents (CE)/g DW of KRPBE.

Determination of the Antioxidant Capacity of KRPBE using ABTS and DPPH Radical Assays and the Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant capacity of KRPBE was also assessed using ABTS radicals [21] and expressed as mg vitamin C equivalents (VCE)/g DW of KRPBE. The ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm. The reaction between ABTS radicals and the appropriately diluted samples was allowed to proceed at 37°C for 10 min, and then the decrease in absorbance of the resulting solution was measured at 734 nm using a spectrophotometer (Spectronic 200).

The antioxidant capacity of KRPBE was also measured using DPPH radicals [21] and expressed as mg VCE/g DW of KRPBE. The absorbance of DPPH radicals in 80% (v/v) aqueous methanol was set to 0.650 ± 0.020 at 517 nm. DPPH radicals and appropriately diluted samples or vitamin C standard were reacted at 23°C for 30 min. The decrease in absorbance of the resulting solution was monitored at 517 nm using a spectrophotometer (Spectronic 200).

The ORAC assay was further performed to determine the antioxidant capacity of KRPBE [22]. Appropriately diluted KRPBE (25 µl) and 150 µl of 81.6 nM fluorescein solution were added to a 96-well plate and incubated at 37°C for 10 min with 3 min of shaking. We added 25 µl of 153 mM AAPH solution and then detected the fluorescence every minute for 90 min using a microplate reader (Infinite M200; Tecan Austria GmbH, Austria) with 485 nm excitation and 520 nm emission wavelengths. The antioxidant capacity measured by ORAC assay was expressed as mg VCE/g DW of KRPBE.

Quantification of Phenolics Using HPLC

KRPBE was quantitatively analyzed using a reversed-phase HPLC system (Agilent 1200; Agilent Technologies, USA) equipped with a diode-array detector, degasser, autosampler, and a reversed-phase column (Agilent Zorbax Eclipse XDB-C18, 250 × 4.6 mm, 5 µm). The injection volume was 20 µl, and the flow rate was 1.0 ml/min. Mobile phase A consisted of 0.1% (v/v) formic acid in deionized water, and mobile phase B was acetonitrile. The gradient elution profile was as follows: 95% A/5% B at 0 min, 5% A/95% B at 45 min, 95% A/5% B at 46 min, and 95% A/5% B at 50 min. The wavelengths for detection were set at 280 nm for protocatechuic acid and catechin and at 320 nm for vanillin and taxifolin. Phenolics were identified by comparison of UV-Vis spectra, retention times, and spiked inputs with commercial standards. Phenolics were quantified using calibration curves that relate different concentrations of authentic standards to the areas of their corresponding peaks.

Cell Culture

Neuronal PC-12 cells (ATCC, USA) were used to assess the effects of KRPBE on cell viability and intracellular oxidative stress. PC-12 cells were cultured in RPMI 1640 medium containing

10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml of streptomycin in a humidified incubator (BB 15; Thermo Fisher Scientific Inc., USA) with 5% CO₂ at 37°C.

Determination of Cell Viability

To determine the non-toxic maximal concentration of KRPBE, its cytotoxicity was assessed using an MTT reduction assay [23]. MTT is metabolized to insoluble purple formazan by mitochondrial dehydrogenases, which are active only in living cells. PC-12 cells were seeded at a density of 2×10^4 cells/well in a 96-well plate in RPMI 1640 medium containing FBS, for 12 h. After removing the medium, cells were treated with serum-free medium containing various concentrations of KRPBE at various non-toxic concentrations or a positive standard (200 µM of vitamin C). Following a 6-h incubation, the medium was removed from each well, and 200 µM of H₂O₂ was added to induce oxidative stress for 1 h. Then, MTT reagent was added, the plate was incubated for 3 h, and then 50 µl of DMSO was added to dissolve the purple formazan formed by reduction of MTT. The absorbance was measured using a microplate reader (Infinite M200) at 570 nm (test wavelength) and 630 nm (reference wavelength). Cell viability was expressed as the percentage (%) of viable cells relative to control cells cultured without test samples.

Measurement of Intracellular Oxidative Stress

Intracellular oxidative stress was determined by fluorescent assay using DCFH-DA. DCFH-DA is deacetylated in cells, and then 2',7'-dichlorofluorescein reacts with intracellular oxidative radicals and is converted into the fluorescent product 2',7'-dichlorofluorescein, which is retained within cells. PC-12 cells were seeded at a density of 2×10^4 cells/well in a 96-well plate in RPMI 1640 medium and incubated for 24 h. The cells were then treated with non-toxic concentrations (0.625–10 µg/ml) of KRPBE or positive standard (vitamin C) for 4 h. After removing the supernatant, 50 µM DCFH-DA in HBSS was added. At 30 min, oxidative stress was induced with 200 µM H₂O₂ for 1 h. Fluorescence was measured using a microplate reader (Infinite M200) with excitation at 485 nm and emission at 530 nm.

Determination of Cholinesterase Inhibition Activity

We determined anticholinesterase activity using AChE and BChE assays performed in a 96-well plate [24]. Substrates ATCI and BTCC were used for the AChE and BChE inhibitory assays, respectively. In both assays, DTNB was used as the color-developing reagent. For the AChE inhibition assay, 20 µl of KRPBE or phenolic compound was added to 150 µl of DPBS. Subsequently, 20 µl of ATCI substrate (15 mM) and 30 µl of DTNB (10 mM) were added to the mixture. After incubation for 10 min at 37°C, 20 µl of AChE (0.4 U/ml) was added. The BChE inhibition assay was performed using a similar protocol, except that instead of AChE and ATCI, we used 0.065 U/ml BChE and 10 mM BTCC. After 30 min at 37°C, we measured the absorbance at 415 nm using a microplate reader (Infinite M200). Tacrine was used as a positive

control. We made a tacrine standard curve relating various concentrations of tacrine to cholinesterase inhibition (%) for quantitative evaluation of the cholinesterase inhibitory activity in KRPBE. Inhibition of AChE and BChE in response to KRPBE was expressed as nM of tacrine equivalents (TE). Measurement of the AChE and BChE inhibitory activities of KRPBE was performed in triplicate.

Statistical Analysis

Data are expressed as the mean ± standard deviation of three replicate determinations. One-way analysis of variance was applied to determine the significance of differences among means. Statistical analyses were conducted using Duncan’s multiple range test using SAS software (version 9.3, SAS Institute Inc., USA), with significance set at $p < 0.05$.

Results and Discussion

Total Phenolic and Flavonoid Contents

The total phenolic content of KRPBE as a crude water extract powder was 397.9 mg GAE/g DW (Table 1). In a previous study, bark samples from five different maritime pine (*P. pinaster*) trees grown in France were reported to have a total phenolic content of 37.21–96.81 mg GAE/g dry bark, due to the differences in age of the trees [25]. *Pinus cembra* L. bark collected in Romania was reported to have a total phenolic content of 299.3 mg GAE/g freeze-dried extract [26]. *Pinus densiflora* extracted with hot water was reported to have a total phenolic content of 411 mg CE/g [5]. Inconsistent with our finding in this study, the total phenolic content of PineXol[®] was previously reported to be 717.4 mg GAE/g [17]; this discrepancy may be due to the manufacturing process, harvest season, growing location, and/or species. It was previously reported that *P. densiflora* bark extract had a higher total phenolic content than *P. thunbergii* and *P. pinaster* bark extracts [27].

The KRPBE used in this study had a total flavonoid content of 248.7 mg CE/g DW (Table 1). Consistent with this result, a *P. cembra* L. bark sample collected in Romania

was previously reported to have a total flavonoid content of 299.3 mg CE/g freeze-dried extract [26]. The total flavonoid content of PineXol[®] has been reported to be approximately 54.4 mg rutin equivalent/g [17].

Antioxidant Capacity

The antioxidant capacity of KRPBE was measured using ABTS, DPPH, and ORAC assays. Results are shown in Table 1. In the ABTS, DPPH, and ORAC assays, KRPBE had an antioxidant capacity of 697.3, 521.8, and 2,627.7 mg VCE/g DW, respectively. The bark extract of *P. morrisonicola* Hay. was reported to have the ability to scavenge superoxide radical anions and to inhibit lipid peroxidation, due in part to its antioxidant constituents [28]. It was previously reported that PineXol[®] had an antioxidant capacity of 693.97 μM Trolox equivalents/g in the ORAC assay [17]. In the DPPH assay, *P. densiflora* was reported to have the most potent antioxidant capacity among bark from 11 species of pine, and the main contributor to its antioxidant capacity was procyanidins [5]. KRPBE has been reported to have higher ABTS and DPPH radical scavenging activities than Pycnogenol[®] [29]. In contrast to our ABTS and DPPH assay results, PineXol[®] was reported to have higher antioxidant capacity than vitamin C [17].

Quantification of Phenolics in KRPBE Using a Reversed-Phase HPLC System

The concentrations of four phenolic compounds (catechin, protocatechuic acid, taxifolin, and vanillin) in KRPBE are presented in Table 2. The concentrations of the four phenolic compounds decreased as follows: catechin (24.5 mg/g) > taxifolin (7.7 mg/g) > protocatechuic acid (6.1 mg/g) > vanillin (2.3 mg/g). A previous study reported that KRPBE contained catechin, taxifolin, and oligomers of catechin and epicatechin [29]. Extract of the raw pine bark of *P. maritima* collected in France has been reported to contain a variety of phenolics, including catechin, protocatechuic acid, taxifolin, and vanillin [30]. Consistent with our findings in this

Table 1. Total phenolic and flavonoid contents and antioxidant capacity of Korean red pine bark extract (KRPBE).

	Total phenolic content (mg gallic acid equiv./g DW ^a)	Total flavonoid content (mg catechin equiv./g DW)	Antioxidant capacity (mg vitamin C equiv./g DW)		
			ABTS ^b	DPPH ^c	ORAC ^d
KRPBE	397.9 ± 5.9 ^c	248.7 ± 8.1	697.3 ± 22.5	521.8 ± 19.4	2,627.7 ± 108.8

^aDry weight

^b2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

^c2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

^dOxygen radical absorbance capacity assay

^eData are expressed as the mean ± standard deviation ($n = 3$).

Table 2. Concentrations of phenolics in Korean red pine bark extract.

Phenolics	Concentration (mg/g)
Catechin	24.5 ± 4.0
Protocatechuic acid	6.1 ± 0.1
Taxifolin	7.7 ± 0.5
Vanillin	2.3 ± 0.2

study, taxifolin, catechin, and protocatechuic acid were previously reported to be the major monomeric phenolics in *P. radiata* bark [6].

Protective Effects of KRPBE and Its Identified Phenolics on the Viability of PC-12 Cells

The cytotoxicity of KRPBE was examined to determine at what concentrations it was not toxic. Cell viability of 90% or above compared with the control (100%) was considered to be non-cytotoxic. KRPBE had no cytotoxicity against neuron-like PC-12 cells up to 10 µg/ml (Fig. 1A). PC-12 cells are derived from a pheochromocytoma of the rat adrenal medulla. Oxidative stress (200 µM of H₂O₂) decreased the viability of PC-12 cells up to approximately 60.5% (Fig. 1B). Pretreatment of PC-12 cells with KRPBE and vitamin C increased their viability in response to exposure to oxidative stress. Pretreatment with KRPBE at 10 µg/ml followed by oxidative stress treatment resulted in a cell viability of 77.7% compared with the control (100%), which was similar to the cell viability (78.1%) of the positive control, 200 µM vitamin C (Fig. 1B).

We measured the viability of neuronal PC-12 cells in the presence of vanillin, protocatechuic acid, catechin, and taxifolin (Fig. 2). These four phenolics had no cytotoxicity against PC-12 cells up to 100 µg/ml (data not shown). The viability of oxidatively stressed PC-12 cells was not significantly increased by pretreatment with vanillin at any concentration compared with the stress control (Fig. 2). PC-12 cells pretreated with vanillin, protocatechuic acid, catechin, and taxifolin at 100 µg/ml had viabilities of approximately 45%, 57%, 52%, and 87%, respectively (Fig. 2). Protocatechuic acid and taxifolin at a concentration of 100 µg/ml protected PC-12 cells significantly more against oxidative stress than did the stress control (40.3%).

Oxidative stress can damage cellular materials such as lipids, proteins, and DNA, leading to cellular malfunction and ultimately cell death. Antioxidants may protect cells, including neurons, against oxidative stress. Pine bark has been reported to have a neuroprotective effect, partly due

Fig. 1. (A) Cytotoxic effects of Korean red pine bark extract on neuronal PC-12 cells and (B) its protective effects against H₂O₂-induced oxidative stress as measured using the MTT assay.

Data are displayed as the mean ± standard deviation (bars) of three replicates. Different letters on the bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

to its antioxidant content [31]. Taxifolin has been reported to protect retinal pigment epithelial cells against H₂O₂-induced oxidative stress, partly due to the decrease of ROS production and inhibition of apoptosis [32]. *Pinus radiata* bark extract rich in procyanidins was demonstrated to significantly reduce protein oxidation in humans after 12 weeks of consumption [33]. Phenolics (catechin, taxifolin, vanillin, and protocatechuic acid) found in KRPBE have been reported to have antioxidant capacity [10, 34].

Fig. 2. Protective effects of vanillin (VAN), protocatechuic acid (PRO), catechin (CAT), and taxifolin (TAX), identified in Korean red pine bark extract, in neuronal PC-12 cells exposed to H₂O₂-induced oxidative stress, based on MTT assay results. Data are displayed as the mean $\pm$ standard deviation (bars) of three replicates. Different letters on the bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

Therefore, the increased viability of PC-12 cells pretreated with KRPBE in response to oxidative stress is likely due to the antioxidant activity of its phenolic constituents.

Fig. 3. Ability of Korean red pine bark extract to protect against intracellular oxidative stress in PC-12 cells exposed to H₂O₂-induced oxidative stress, as measured using the DCFH-DA assay.

Data are displayed as the mean $\pm$ standard deviation (bars) of three replicates. Different letters on the bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

Effects of KRPBE and Its Identified Phenolics on Intracellular Oxidative Stress in PC-12 Cells

Hydrogen peroxide can penetrate cell membranes via free diffusion through the lipid bilayer of membranes and function as intracellular ROS. The DCFH-DA assay was used to evaluate oxidative stress inside neuronal PC-12 cells. Hydrogen peroxide (200 μ M) increased oxidative stress inside PC-12 cells up to 304% of the control level (100%; Fig. 3). Vitamin C reduced oxidative stress to approximately 215% compared with that in stress control cells (Fig. 3). Pretreatment of PC-12 cells with KRPBE decreased oxidative stress in a dose-dependent manner compared with cells exposed to oxidative stress only (Fig. 3).

Pretreatment of PC-12 cells with the four identified phenolics at 100 μ g/ml decreased oxidative stress levels as follows: taxifolin (116%) > protocatechuic acid (212%) > catechin (234%) > vanillin (279%) (Fig. 4). Pretreatment with taxifolin at all concentrations decreased oxidative stress in PC-12 cells in a dose-dependent manner compared with the stress control (279%; Fig. 4). Protocatechuic acid and catechin at a concentration of 100 μ g/ml significantly reduced oxidative stress in PC-12 cells compared with the stress control.

These results suggest that phenolic antioxidants in KRPBE may decrease intracellular oxidative stress in neuronal PC-

Fig. 4. Ability of vanillin (VAN), protocatechuic acid (PRO), catechin (CAT), and taxifolin (TAX), identified in Korean red pine bark extract, to protect neuronal PC-12 cells against H₂O₂-induced oxidative stress, as measured using the DCFH-DA assay.

Data are displayed as the mean $\pm$ standard deviation (bars) of three replicates. Different letters on the bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

12 cells against ROS like H_2O_2 . It was previously reported that pine bark extract showed protective effects against oxidative stress in rats, partly due to increased antioxidant activity as well as inhibition of lipid peroxidation [35]. RBE-4 cells, which are rat cerebral capillary endothelial cells, were reported to be permeable to catechin in a time-dependent manner [36]. Catechins and procyanidins can reduce oxidative membrane damage by limiting access of oxidants to the hydrophobic region of the membrane bilayer [37]. Thus, compounds in the KRPBE may protect cell membranes from H_2O_2 penetration.

Effects of KRPBE and Its Identified Phenolics on AChE and BChE Activities

Inhibition of AChE and BChE by KRPBE is shown in Fig. 5. KRPBE at 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ had TE values of approximately 68.3 nM and 15.1 nM for inhibition of AChE and BChE, respectively. A higher TE means greater inhibition of enzyme activity; therefore, AChE was inhibited by KRPBE to a greater extent than BChE (Fig. 5). KRPBE inhibited the AChE and BChE activities in a dose-dependent manner. Tacrine, the positive control, had an IC_{50} for AChE and BChE of 1,530 nM and 181 nM, respectively (data not shown).

AChE and BChE are detected among neurofibrillary tangles and neuritic plaques in the brain, and the neuropathology of AD might result from a high ratio of BChE to AChE-positive glia [38, 39]. Cholinesterase inhibitors prolong neurotransmission in cholinergic synapses by increasing the brain acetylcholine level through relatively slower metabolism of released neurotransmitters such as acetylcholine, thereby enhancing neurotransmission at cholinergic synapses [40]. A BChE inhibitor has been reported to increase the level of acetylcholine and enhance cognitive function in rats [41]. Finding inhibitors for AChE and BChE to treat AD is therefore of great interest. Phenolics are known to inhibit AChE and BChE [19]. Therefore, we investigated if the phenolics found in KRPBE had AChE- and BChE-inhibitory effects.

We assessed the ability of vanillin, protocatechuic acid, catechin, and taxifolin to inhibit AChE and BChE. Protocatechuic acid did not inhibit either cholinesterase (data not shown). At 1,000 $\mu\text{g/ml}$, the AChE-inhibiting activities decreased as follows: taxifolin (103.6 μM TE) > catechin (86.4 μM TE) > vanillin (56.0 μM TE); whereas the BChE-inhibiting activities decreased as follows: taxifolin (16.4 μM TE) > vanillin (8.8 μM TE) > catechin (7.0 μM TE) (Fig. 6). Vanillin, catechin, and taxifolin inhibited AChE and BChE in a dose-dependent manner. Taxifolin inhibited both AChE and BChE to the greatest extent (Fig. 6).

Fig. 5. Inhibitory effects of Korean red pine bark extract on acetylcholinesterase (AChE; **A**) and butyrylcholinesterase (BChE; **B**) activities.

Data are displayed as the mean $\pm$ standard deviation (bars) of three replicates. Different letters on the bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

Catechin has been reported to bind easily to AChE and BChE and then inhibit their activity, resulting in prolonged neurotransmission [38]. Flavonoids, including catechin and quercetin, can traverse RBE-4 cells, suggesting that a wide range of flavonoids and their metabolites can be delivered to nerve cells across the blood-brain barrier to inhibit AChE and BChE [36]. KRPBE, which contains vanillin, catechin, and taxifolin, could therefore be a source of therapeutic agents for AD.

In conclusion, the KRPBE examined in this study had a high phenolic content and antioxidant capacity. Four phenolics (catechin, protocatechuic acid, taxifolin, and vanillin) were identified using a reversed-phase HPLC system. KRPBE and its identified phenolics protected neuronal PC-12 cells against oxidative stress and inhibited

Fig. 6. Inhibitory effects of vanillin (VAN), catechin (CAT) and taxifolin (TAX), identified in Korean red pine bark extract, on acetylcholinesterase (AChE; **A**) and butyrylcholinesterase (BChE; **B**) activities.

Data are displayed as the mean $\pm$ standard deviation (bars) of three replicates. Different letters on the bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

AChE and BChE activities, indicating that they can prolong termination of neurotransmission. KRPBE might therefore be a good natural source of antioxidative phenolics and can potentially be used as a functional material in food and pharmaceutical applications to reduce oxidative stress and inhibit the cholinesterases. Further studies of the neuroprotective effects and bioavailability of KRPBE and its individual phenolics in in-vivo models of cognitive impairment are warranted.

Acknowledgments

This study was financially supported by a grant (No. 20140322) from Kyung Hee University.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

- Jekel K, Damian M, Wattmo C, Hausner L, Bullock R, Connelly PJ, et al. 2015. Mild cognitive impairment and deficits in instrumental activities of daily living: a systematic review. *Alzheimers Res. Ther.* **7**: 17.
- Méndez-Armenta M, Nava-Ruiz C, Juárez-Rebollar D, Rodríguez-Martínez E, Gómez PY. 2014. Oxidative stress associated with neuronal apoptosis in experimental models of epilepsy. *Oxid. Med. Cell. Longev.* **2014**: 293689.
- Darvesh S. 2016. Butyrylcholinesterase as a diagnostic and therapeutic target for Alzheimer's disease. *Curr. Alzheimer Res.* **13**: 1173-1177.
- Mehta M, Adem A, Sabbagh M. 2012. New acetylcholinesterase inhibitors for Alzheimer's disease. *Int. J. Alzheimers Dis.* **2012**: 728983.
- Ku CS, Jang JP, Mun SP. 2007. Exploitation of polyphenol-rich pine barks for potent antioxidant activity. *J. Wood Sci.* **53**: 524-528.
- Ku CS, Mun SP. 2008. Antioxidant properties of monomeric, oligomeric, and polymeric fractions in hot water extract from *Pinus radiata* bark. *Wood Sci. Technol.* **42**: 47-60.
- D'Andrea G. 2010. Pycnogenol: a blend of procyanidins with multifaceted therapeutic applications? *Fitoterapia* **81**: 724-736.
- Jerez M, Pinelo M, Sineiro J, Núñez MJ. 2006. Influence of extraction conditions on phenolic yields from pine bark: assessment of procyanidins polymerization degree by thiolysis. *Food Chem.* **94**: 406-414.
- Floegel A, Kim D-O, Chung S-J, Song WO, Fernandez ML, Bruno RS, et al. 2010. Development and validation of an algorithm to establish a total antioxidant capacity database of the US diet. *Int. J. Food Sci. Nutr.* **61**: 600-623.
- Kim D-O, Lee CY. 2004. Comprehensive study of vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship. *Crit. Rev. Food Sci. Nutr.* **44**: 253-273.
- Mandel S, Youdim MBH. 2004. Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases. *Free Radic. Biol. Med.* **37**: 304-317.
- Szwajgier D. 2013. Anticholinesterase activities of selected polyphenols – a short report. *Pol. J. Food Nutr. Sci.* **64**: 59-64.
- Panche AN, Diwan AD, Chandra SR. 2016. Flavonoids: an overview. *J. Nutr. Sci.* **5**: e47.
- Giusti MM, Wrolstad RE. 2003. Acylated anthocyanins from edible sources and their applications in food systems. *Biochem. Eng. J.* **14**: 217-225.

15. Iravani S, Zolfaghari B. 2011. Pharmaceutical and nutraceutical effects of *Pinus pinaster* bark extract. *Res. Pharm. Sci.* **6**: 1-11.
16. Maimoona A, Naeem I, Saddiqe Z, Jameel K. 2011. A review on biological, nutraceutical and clinical aspects of French maritime pine bark extract. *J. Ethnopharmacol.* **133**: 261-277.
17. Lee YJ, Han OT, Choi H-S, Lee BY, Chung H-J, Lee O-H. 2013. Antioxidant and anti-adipogenic effects of PineXol®. *Korean J. Food Sci. Technol.* **45**: 97-103.
18. Singleton VL, Rossi JA Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **16**: 144-158.
19. 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 H₂O₂-induced oxidative stress in neuronal PC-12 cells. *Food Sci. Biotechnol.* **24**: 2219-2227.
20. Liu C-S, Nam T-G, Han M-W, Ahn S-M, Choi HS, Kim TY, et al. 2013. Protective effect of detoxified *Rhus verniciflua* Stokes on human keratinocytes and dermal fibroblasts against oxidative stress and identification of the bioactive phenolics. *Biosci. Biotechnol. Biochem.* **77**: 1682-1688.
21. 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.
22. Lim D, Kim W, Lee M-G, Heo HJ, Chun OK, Kim D-O. 2012. Evidence for protective effects of coffees on oxidative stress-induced apoptosis through antioxidant capacity of phenolics. *Food Sci. Biotechnol.* **21**: 1735-1744.
23. Heo H-J, Cho H-Y, Hong B, Kim H-K, Kim E-K, Kim B-G, et al. 2001. Protective effect of 4',5-dihydroxy-3',6,7-trimethoxyflavone from *Artemisia asiatica* against A β -induced oxidative stress in PC12 cells. *Amyloid* **8**: 194-201.
24. Hwang J-S, Cho CH, Baik M-Y, Park S-K, Heo HJ, Cho Y-S, et al. 2017. Effects of freeze-drying on antioxidant and anticholinesterase activities in various cultivars of kiwifruit (*Actinidia* spp.). *Food Sci. Biotechnol.* **26**: 221-228.
25. Chupin L, Motillon C, Bouhtoury FC-E, Pizzi A, Charriera B. 2013. Characterisation of maritime pine (*Pinus pinaster*) bark tannins extracted under different conditions by spectroscopic methods, FTIR and HPLC. *Ind. Crops Prod.* **49**: 897-903.
26. Apetrei CL, Tuchilus C, Aprotosoiaie AC, Oprea A, Malterud KE, Miron A. 2011. Chemical, antioxidant and antimicrobial investigations of *Pinus cembra* L. bark and needles. *Molecules* **16**: 7773-7788.
27. Kim SM, Kang S-W, Jeon J-S, Um B-H. 2012. A comparison of Pycnogenol® and bark extracts from *Pinus thunbergii* and *Pinus densiflora*: extractability, antioxidant activity and proanthocyanidin composition. *J. Med. Plant Res.* **6**: 2839-2849.
28. Hsu T-Y, Sheu S-C, Liaw E-T, Wang T-C, Lin C-C. 2005. Anti-oxidant activity and effect of *Pinus morrisonicola* Hay. on the survival of leukemia cell line U937. *Phytomedicine* **12**: 663-669.
29. Choi J-H, Choi M-K, Han O, Han S, Chung S-J, Shim C-K, et al. 2007. Evaluation of skin absorption of catechin from topical formulations containing Korean pine bark extract (PineXol®). *J. Korean Pharm. Sci.* **37**: 359-364.
30. Sarikaki V, Rallis M, Tanojo H, Panteri I, Dotsikas Y, Loukas YL, et al. 2004. In vitro percutaneous absorption of pine bark extract (Pycnogenol) in human skin. *J. Toxicol. Cutaneous Ocul. Toxicol.* **23**: 149-158.
31. Eftekhari S, Keshavarzi Z, Hadjzadeh MAR. 2016. Neuroprotective effects of pine bark and *Aloe vera* on the locomotor activity in focal cerebral ischemia: possible antioxidant mechanisms. *J. Biomed. Eng. Med. Devices* **1**: 121.
32. Xie X, Feng J, Kang Z, Zhang S, Zhang L, Zhang Y, et al. 2017. Taxifolin protects RPE cells against oxidative stress-induced apoptosis. *Mol. Vis.* **23**: 520-528.
33. Senthilmohan ST, Zhang J, Stanley RA. 2003. Effects of flavonoid extract Enzogenol® with vitamin C on protein oxidation and DNA damage in older human subjects. *Nutr. Res.* **23**: 1199-1210.
34. Tai A, Sawano T, Yazama F, Ito H. 2011. Evaluation of antioxidant activity of vanillin by using multiple antioxidant assays. *Biochim. Biophys. Acta* **1810**: 170-177.
35. Ko J-W, Lee I-C, Park S-H, Moon C, Kang S-S, Kim S-H, et al. 2014. Protective effects of pine bark extract against cisplatin-induced hepatotoxicity and oxidative stress in rats. *Lab. Anim. Res.* **30**: 174-180.
36. Faria A, Pestana D, Teixeira D, Azevedo J, de Freitas V, Mateus N, et al. 2010. Flavonoid transport across RBE4 cells: a blood-brain barrier model. *Cell. Mol. Biol. Lett.* **15**: 234-241.
37. Verstraeten SV, Keen CL, Schmitz HH, Fraga CG, Oteiza PI. 2003. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the bilayer structure. *Free Radic. Biol. Med.* **34**: 84-92.
38. Ali B, Jamal QMS, Shams S, Al-Wabel NA, Siddiqui MU, Alzohairy MA, et al. 2016. In silico analysis of green tea polyphenols as inhibitors of AChE and BChE enzymes in Alzheimer's disease treatment. *CNS Neurol. Disord. Drug Targets* **15**: 624-628.
39. Wright CI, Geula C, Mesulam M-M. 1993. Neuroglial cholinesterases in the normal brain and in Alzheimer's disease: relationship to plaques, tangles, and patterns of selective vulnerability. *Ann. Neurol.* **34**: 373-384.
40. Bartus RT, Dean RL, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217**: 408-414.
41. Greig NH, Utsuki T, Ingram DK, Wang Y, Pepeu G, Scali C, et al. 2005. Selective butyrylcholinesterase inhibition elevates brain acetylcholine, augments learning and lowers Alzheimer β -amyloid peptide in rodent. *Proc. Natl. Acad. Sci. USA* **102**: 17213-17218.