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Protective role of *Populus tomentiglandulosa* against hydrogen peroxide-induced oxidative stress in SH-SY5Y neuronal cells

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Abstract Oxidative stress caused by the overproduction of reactive oxygen species (ROS) is known as an etiology of neurodegenerative diseases. *Populus tomentiglandulosa* (PT), a member of the Salicaceae family, is widely grown in Korea and has been reported to exert protective effects on cerebral ischemia by attenuating of oxidative stress and neuronal damage. In the present study, we investigated the antioxidant activity and neuroprotective effects of an ethanol extract and four fractions [*n*-butanol, ethyl acetate (EtOAc), chloroform, and *n*-hexane] of PT under *in vitro* and cellular systems. The extract and four fractions of PT showed 1,1-diphenyl-2-picrylhydrazyl (DPPH), $^{\bullet}OH$, and O_2^- radical scavenging activities in a dose-dependent manner. In particular, the EtOAc fraction of PT had the strongest DPPH, $^{\bullet}OH$, and O_2^- radical scavenging activities among the extract and other fractions. Therefore, we further investigated the neuroprotective effect of the EtOAc fraction of PT against oxidative stress in H_2O_2-induced SH-SY5Y cells. Treatment with H_2O_2 significantly decreased cell viability and lactate dehydrogenase (LDH) release, and it also increased the ROS levels compared to the normal group. However, treatment with the EtOAc fraction of PT significantly increased cell viability. Moreover, the EtOAc fraction of PT-treated group significantly suppressed ROS production and LDH release compared to the H_2O_2-induced control group. In conclusion, our findings indicated that PT had *in vitro* antioxidant activity and neuroprotective effects against oxidative stress. Therefore, PT could be used as a natural agent for protection against oxidative stress.

Keywords Hydrogen peroxide · Neuronal damage · Oxidative stress · *Populus tomentiglandulosa* · Reactive oxygen species

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Introduction

Free radicals are typically unstable compounds containing a single unpaired electron, which is naturally generated in the body [1]. Free radicals have beneficial effects such as on the immune response, but the overproduction of free radicals can damage biological molecules such as lipids, proteins, and DNA in the body [2]. Reactive oxygen species (ROS) are representative free radicals and include hydroxyl radicals ($^{\bullet}OH$), superoxide ions (O_2^-), and hydrogen peroxide (H_2O_2) [3]. The overproduction of ROS can induce oxidative stress, an imbalance between the production of oxidants and their elimination, and oxidizes essential cell components such as cell membranes and cell organelles [4,5]. Therefore, oxidative stress is closely related to the pathogenesis of various diseases such as cancer, diabetes, cardiovascular disease, and neurodegenerative diseases [6,7].

The nervous system has relatively less well-developed protective mechanisms against oxidative stress compared to other tissues, thereby brain and neuronal cells are vulnerable to oxidative stress [8]. In the brain, several components such as excitatory amino acids and neurotransmitters induce oxidative stress by producing ROS. In addition, mitochondria in the brain use a large amount of oxygen in the process of generating energy, hence the brain

produces more free radicals than other tissues and is continually exposed to oxidative stress [9]. Oxidative stress in the brain is strongly associated with the progression of neuronal injury and death, leading to neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [7]. Many studies have investigated the development of antioxidants that can protect neuronal oxidative stress for preventing neurodegenerative diseases induced by oxidative stress.

Populus tomentiglandulosa (PT), a member of the Salicaceae family, is widely grown in Korea and is a hybrid between *P. alba* and *P. davidiana* [10]. *P. alba* and *P. davidiana* have been reported to have various biological activities such as antioxidant, anti-inflammatory, hepatoprotective, and vasorelaxant effects [11-13]. In addition, several studies have reported that the ethanol (EtOH) extract of PT had antioxidant activity demonstrated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities *in vitro*, and also elevated antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in rats [14,15]. The EtOH extract of PT contains several phenolic compounds such as catechin, caffeic acid, *p*-coumaric acid, chlorogenic acid, and gallic acid [14]. Furthermore, several neuroprotective effects of PT have been reported, which protected neurons from ischemic damage and cerebrovascular disorders [15,16]. However, the antioxidant activity and neuroprotective effects against oxidative stress of various extracts and fractions of PT have not been yet studied.

The present study investigated the *in vitro* antioxidant activity of an EtOH extract and four fractions such as *n*-butanol (BuOH), ethyl acetate (EtOAc), chloroform ($CHCl_3$), and *n*-hexane (hexane) of PT. In addition, we evaluated the neuroprotective effect of active fraction of PT against oxidative stress in H_2O_2-induced SH-SY5Y neuronal cells.

Materials and Methods

Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2-deoxyribose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and 2',7'-dichloro-fluorescein diacetate (DCF-DA) were purchased by Sigma Chemical Co. (St. Louis, MO, USA). EtOH was acquired from Duksan Co. Ltd. (Ansan, Korea). $FeSO_4 \cdot 7H_2O$ was obtained from Daejung Chemicals & Metals Co. Ltd. (Gyeonggi, Korea). Phosphoric acid was obtained from Samchun Pure Chemical Co. Ltd. (Gyeonggi, Korea). Hydrochloric acid and H_2O_2 were purchased from Junsei Chemical Co. (Tokyo, Japan). Trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were supplied by Biosesang Inc. (Seongnam, Korea) and Acros Organics, Inc. (Morris Plains, NJ, USA). Phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), and nicotinamide adenine dinucleotide (NADH) were obtained from Bio Basic, Inc. (Amherst, NY, USA). Tris was purchased from the LPS Solution Co. (Daejeon, Korea). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), and trypsin-EDTA solution were purchased from Welgene, Inc. (Daegu, Korea).

Preparation of the samples

The dried leaves of PT (1442.6 g) were extracted with EtOH under reflux three times (86 °C 3 h), and this extract was combined and evaporated to afford a brown residue (278.3 g). Then, the residue was suspended in distilled water and partitioned sequentially with BuOH (59.9 g), EtOAc (36.0 g), $CHCl_3$ (35.5 g), and hexane (45.7 g). Subsequently, the extract and four fractions were dissolved in DMSO and used for the experiments.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

We investigated the DPPH radical scavenging activity of PT following the method of Hatano et al. [17]. The extract and four fractions of PT were diluted in EtOH, and the diluted solutions were mixed with 60 μM DPPH in a 1:1 ratio in a 96-well plate. The plate was incubated in the dark at room temperature for 30 min, the absorbance was read at 540 nm using a microplate reader (Thermo Fisher Scientific, Vantaa, Finland). Using the absorbance value, the DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{control} - A_{PT})/A_{control}] \times 100$$

(A_{PT} means the absorbance of PT and $A_{control}$ means the absorbance of the control)

Hydroxyl radical (˙OH) scavenging assay

The ˙OH radical scavenging activity of PT was evaluated using the method described by Chung et al. [18]. In the dark, 1400 μL of extract and four fractions from PT were mixed with 200 μL of 10 mM $FeSO_4 \cdot 7H_2O$-EDTA, 200 μL of 10 mM 2-deoxyribose solution, and 200 μL of 10 mM H_2O_2. The mixed solutions were incubated at 37 °C for 4 h, and then 1 mL of 1% TBA and 1 mL of 2.8% TCA were added. The mixtures were boiled for 20 min and cooled on ice. The absorbance of the mixture in a 96-well plate was read at an absorbance of 490 nm using a microplate reader. The ˙OH radical scavenging activity was calculated as follows:

$$\text{˙OH radical scavenging activity (\%)} \ [(A_{control} - A_{PT})/A_{control}] \times 100$$

(A_{PT} means the absorbance of PT and $A_{control}$ means the absorbance of the control)

Superoxide radical (O_2^-) scavenging assay

The O_2^- radical scavenging activity of PT was determined according to the method of Ewing and Janero [19]. Briefly, 500 μL of the extract and four fractions of PT were added to 100 μL of 0.1 M Tris-HCl (pH 7.4), 200 μL of 0.1 mM PMS, 200 μL of 0.5 mM

NBT, and 400 μL of 0.5 mM NADH, and then reacted at room temperature for 10 min. The mixed solutions were measured at an absorbance of 560 nm using a microplate reader. The O_2^- radical scavenging activity was calculated as follows:

$$O_2^-\text{ radical scavenging activity (\%)} [(A_{control}-A_{PT})/A_{control}]\times 100$$

(A_{PT} means the absorbance of PT and $A_{control}$ means the absorbance of the control)

Cell culture

SH-SY5Y neuronal cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). The SH-SY5Y cells were cultured in T-75 flasks containing DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The incubator used for cell culture maintained an atmosphere of 5% CO_2 at 37 °C. The cells were fed once every two days with fresh medium. The cells were sub-cultured at 80% confluency using 0.05% trypsin-EDTA.

Measurement of cell viability

Cell viability was measured using the MTT assay [20]. The SH-SY5Y cells were plated at a density of 2.5×10^5 cells/mL onto 96-well plates, and incubated for 24 h. The cells were treated with the EtOAc fraction of PT at various concentrations (1, 5, and 10 μg/mL), and incubated for 2 h. Afterward, 500 μM H_2O_2 was added. After 24 h of incubation, 200 μL of MTT solution (5 mg/mL) was added to the cells to form crystals. After 4 h, 200 μL of DMSO was added to the cells to dissolve the incorporated formazan crystals. The absorbance of each well was measured at 540 nm using a microplate reader.

Measurement of reactive oxygen species (ROS) production

ROS production was measured using the DCFH-DA assay [21]. SH-SY5Y cells were seeded at 2.5×10^5 cells/mL in a 96-well black plate and incubated for 24 h. Subsequently, the cells were pretreated with the EtOAc fraction of PT at different concentrations (1, 5, and 10 μg/mL) for 2 h. After, the cells were treated with 500 μM H_2O_2 for 24 h. Then, the cells were treated with 80 μM of DCF-DA and incubated for 30 min and the absorbance of each well was read at at excitation and emission wavelengths of 480 nm and 535 nm, respectively, for 60 min using a fluorescence spectrophotometer (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany).

Measurement of lactate dehydrogenase (LDH) release activity

The LDH release assay was conducted using the LDH Cytotoxicity Detection Kit (Takara Bio, Shiga, Japan). The SH-SY5Y cells were seeded at a density of 2.5×10^5 cells/mL in a 96-well plate and incubated for 24 h. Then, the cells were pretreated with the EtOAc fraction of PT at various concentrations (1, 5, and 10 μg/mL) and treated with 500 μM H_2O_2. After 24 h of incubation, the supernatant of mixture was reacted with reaction mixture of kit in a ratio of 1:1 at room temperature for 30 min. The absorbance of each well was measured at 490 nm using a microplate reader.

Statistical analysis

The results are presented as the mean ± standard deviation (SD). The statistical significance was analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test using the IBM SPSS program (IBM Corporation, Armonk, NY, USA). Statistical significance was set at *P*-values of less than 0.05.

Results and Discussion

DPPH radical scavenging activity of the EtOH extract and four fractions of PT

The DPPH radical scavenging assay is a widely used method for evaluating *in vitro* antioxidant activity [22]. DPPH is a stable free radical with a violet color that is absorbed by EtOH solution. When DPPH solution is mixed with a substance that can donate a hydrogen atom such as an antioxidant, it is converted to the reduced form with a loss of color [23]. As shown in Table 1, we confirmed the DPPH radical scavenging activity of the EtOH extract and four fractions of PT. The EtOH extract and four fractions of PT showed dose-dependent elevations in DPPH radical scavenging activity. In particular, the EtOAc fraction of PT showed DPPH radical scavenging activities of 83.43 and 87.12% at concentrations of 50 and 100 μg/mL, respectively. Meanwhile, ascorbic acid is widely used as a positive control for evaluation of antioxidant activity [24]. Previous study reported that ascorbic

Table 1 DPPH radical scavenging activity of *Populus tomentiglandulosa*

Treatment (μg/mL)	DPPH radical scavenging activity (%)				
	EtOH ext.	BuOH fr.	EtOAc fr.	$CHCl_3$ fr.	Hexane fr.
1	1.28±1.73[d]	12.66±2.10[d]	19.65±3.52[d]	9.41±2.96[c]	-
10	30.01±1.73[c]	27.33±3.57[c]	56.90±2.82[c]	10.43±2.75[c]	11.48±1.96[c]
50	56.99±2.86[b]	72.83±1.94[b]	83.43±2.68[b]	32.82±1.23[b]	33.66±0.71[b]
100	76.84±1.11[a]	81.61±1.72[a]	87.12±1.37[a]	41.54±1.33[a]	51.35±2.11[a]
IC_{50} (μg/mL)	25.57±0.60	15.75±0.70	6.98±0.44	300.97±10.03	132.63±6.37

Values are means ± SD. The different letters (a-d) are significantly different (p <0.05) by Duncan's multiple range test. IC_{50} means the concentration (μg/mL) required to scavenge DPPH radical formation by 50%. EtOH ext.: ethanol extract, BuOH fr.: *n*-butanol fraction, EtOAc fr.: ethyl acetate fraction, $CHCl_3$ fr.: chloroform fraction, Hexane fr.: *n*-hexane fraction

Table 2 ˙OH radical scavenging activity of *Populus tomentiglandulosa*

Treatment (μg/mL)	˙OH radical scavenging activity (%)				
	EtOH ext.	BuOH fr.	EtOAc fr.	$CHCl_3$ fr.	Hexane fr.
1	14.48±1.30[d]	13.18±0.54[c]	33.53±0.30[c]	33.78±1.59[d]	23.24±0.59[d]
10	72.93±1.47[c]	73.88±1.43[b]	74.46±0.47[b]	69.85±1.28[c]	70.93±0.40[c]
50	86.69±0.44[b]	89.58±1.57[a]	85.38±0.88[a]	86.00±0.20[b]	89.44±0.81[b]
100	89.02±1.43[a]	88.41±1.40[a]	86.02±0.49[a]	89.81±1.08[a]	97.89±0.74[a]
IC_{50} (μg/mL)	6.18±0.15	6.23±0.09	3.11±0.02	3.12±0.08	4.35±0.04

Values are means ± SD. The different letters (a-d) are significantly different (p <0.05) by Duncan's multiple range test. IC_{50} means the concentration (μg/mL) required to scavenge ˙OH radical formation by 50%. EtOH ext.: ethanol extract, BuOH fr.: *n*-butanol fraction, EtOAc fr.: ethyl acetate fraction, $CHCl_3$ fr.: chloroform fraction, Hexane fr.: *n*-hexane fraction

Table 3 O_2^- radical scavenging activity of *Populus tomentiglandulosa*

Treatment (μg/mL)	O_2^- radical scavenging activity (%)				
	EtOH ext.	BuOH fr.	EtOAc fr.	$CHCl_3$ fr.	Hexane fr.
1	-	-	11.10±1.67[c]	-	-
10	15.25±1.60[c]	4.36±1.99[c]	12.83±3.58[c]	2.32±3.05[c]	10.56±1.83[b]
50	41.48±2.55[b]	42.00±1.83[b]	50.72±4.74[b]	13.08±4.62[b]	38.85±1.43[a]
100	44.07±1.21[a]	64.46±1.65[a]	66.07±1.64[a]	25.20±1.48[a]	-
IC_{50} (μg/mL)	120.53±4.47	57.65±2.37	39.99±2.14	4817.04±507.77	-

Values are means ± SD. The different letters (a-d) are significantly different (p <0.05) by Duncan's multiple range test. IC_{50} means the concentration (μg/mL) required to scavenge O_2^- radical formation by 50%. EtOH ext.: ethanol extract, BuOH fr.: *n*-butanol fraction, EtOAc fr.: ethyl acetate fraction, $CHCl_3$ fr.: chloroform fraction, Hexane fr.: *n*-hexane fraction

acid showed IC_{50} value of 8.21 μg/mL in DPPH radical scavenging activity [24]. The EtOAc fraction of PT had the lowest IC_{50} value (6.98 μg/mL) for DPPH radical scavenging activity among the extract and other fractions. These results indicated that the EtOAc fraction of PT had higher DPPH radical scavenging activity than the extract and other fractions.

˙OH radical scavenging activity of the EtOH extract and four fractions of PT

The photo-Fenton reaction is a reaction that photoproduced Fe (II) reacts with H_2O_2 to reduce to Fe (III) and generate ˙OH radicals [25,26]. ˙OH radicals have a highly reactive property and can cause damage to essential cell components such as mitochondria, DNA, and cell membranes [27]. Therefore, ˙OH radicals have been known to play a role in many diseases such as aging, rheumatoid arthritis, and neurodegenerative diseases [28]. The ˙OH radical scavenging activity of the EtOH extract and four fractions of PT is shown in Table 2. The EtOH extract and four fractions of PT dose-dependently increased the ˙OH radical scavenging activity at concentrations of 1, 10, 50, and 100 μg/mL. In particular, the IC_{50} value of the EtOAc and $CHCl_3$ fractions of PT were 3.11 and 3.12 μg/mL, respectively, showing the strongest ˙OH radical scavenging activity compared to the extract and other fractions. Moreover, these values are comparable to ascorbic acid, which showed the IC_{50} value of 1.06 μg/mL for ˙OH radical scavenging activity in previous study [29]. These findings suggested that the EtOAc and $CHCl_3$ fractions of PT showed higher ˙OH radical scavenging activity than the extract and other fractions.

O_2^- radical scavenging activity of the EtOH extract and four fractions of PT

O_2^- radicals are formed by NADH phosphate oxidase, which is a membrane-bound enzyme that reduces the one-electron of free molecular oxygen [30]. Nishikimi et al [31] reported that NADH and PMS formed O_2^- radicals *in vitro* and then reacted with NBT to form a blue color. O_2^- radicals are involved in the production of other ROS such as ˙OH radicals and H_2O_2, leading to oxidative damage in the body [32,33]. The O_2^- radical scavenging activity of the EtOH extract and four fractions of PT is shown in Table 3. The BuOH and EtOAc fractions of PT showed more than 60% O_2^- radical scavenging activity at a concentration of 100 μg/mL. Especially, the EtOAc fractions of PT showed the lowest IC_{50} value of 39.99 μg/mL, which suggests that it had the highest O_2^- radical scavenging activity among the extract and other fractions. In previous study, ascorbic acid exhibited an IC_{50} value of 20.72 μg/mL in O_2^- radical scavenging activity [24]. Therefore, our findings indicated that EtOH extract and four fractions of PT has antioxidant activity by indicating the DPPH, ˙OH, and O_2^- radical scavenging activities under *in vitro*. Particularly, the EtOAc fraction of PT showed the strongest *in vitro* antioxidant activity among the extract and other fractions, suggesting that it was an active fraction of PT.

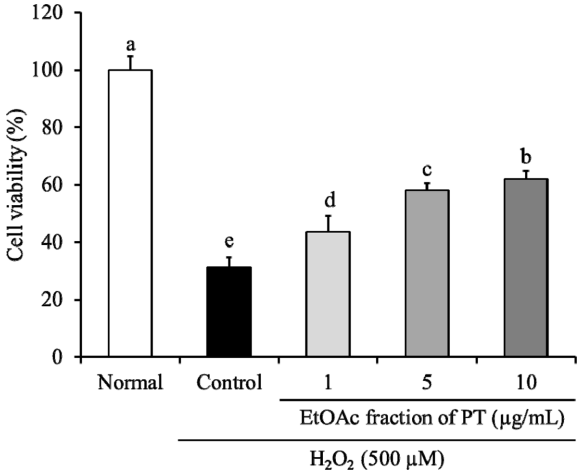

Fig. 1 Effect of the EtOAc fraction of *Populus tomentiglandulosa* on cell viability in H_2O_2-treated SH-SY5Y cells. The results are expressed as the mean ± SD. The different letters (a-e) among the groups indicate significant differences (p <0.05) by Duncan's multiple range test

Effect of the EtOAc fraction of PT on cell viability in H_2O_2-treated SH-SY5Y cells

SH-SY5Y cells are widely used for studying neuronal damage induced by oxidative stress under cellular system [34]. H_2O_2, a ROS, is broadly used as an inducer of oxidative stress [35]. Kang et al. [36] reported that treatment with H_2O_2 significantly reduced cell viability in SH-SY5Y neuronal cells, showing that oxidative stress induced by H_2O_2 caused neuronal cell death. To evaluate the neuroprotective effect of the EtOAc fraction of PT on neuronal cell death caused by oxidative stress, we measured the cell viability in H_2O_2-treated neuronal SH-SY5Y cells. As shown in Fig. 1, cell viability in the H_2O_2-treated control group significantly declined to 31.31% compared to the non-treated normal group (100%). This result showed that treatment with H_2O_2 induced neuronal oxidative stress in SH-SY5Y cells. However, treatment with the EtOAc fraction of PT increased the cell viability in a dose-dependent manner compared to the H_2O_2-treated control group. Especially, the cell viability increased to 57.99 and 61.89% at concentrations of 5 and 10 μg/mL, respectively. Our results showed that the EtOAc fraction of PT had a neuroprotective effect on neuronal cell death by increasing cell viability in oxidative stress-induced SH-SY5Y cells.

Effect of the EtOAc fraction of PT on ROS production in H_2O_2-treated SH-SY5Y cells

DCFH-DA is a non-fluorescent molecule, but when it reacts with ROS, it is oxidized and converted to dichlorofluorescein (DCF) [37]. DCF is highly fluorescent and can be measured by fluorescence analysis. The DCFH-DA assay estimates the level of intracellular ROS using this principle [37]. Overproduction of ROS induces oxidative stress in neuronal cells, resulting in neuronal apoptosis, which directly contributes to neurodegenerative diseases [38]. In addition, increases in ROS levels were observed in the brain of patients with neurodegenerative diseases such as Alzheimer's disease [39]. According to Law et al. [35], H_2O_2-treated SH-SY5Y cells had significantly elevated ROS levels compared to non-treated cells. To confirm the inhibition of the production of ROS by the EtOAc fraction of PT, we measured the production of ROS in H_2O_2-treated neuronal SH-SY5Y cells. As shown in Fig. 2A, treatment with 500 μM H_2O_2 increased ROS production for 60 min compared to the non-treated normal group. Moreover, the production of ROS at 60 min was significantly increased in the H_2O_2-induced control group compared to the normal group, suggesting that H_2O_2 induced oxidative stress by increasing ROS

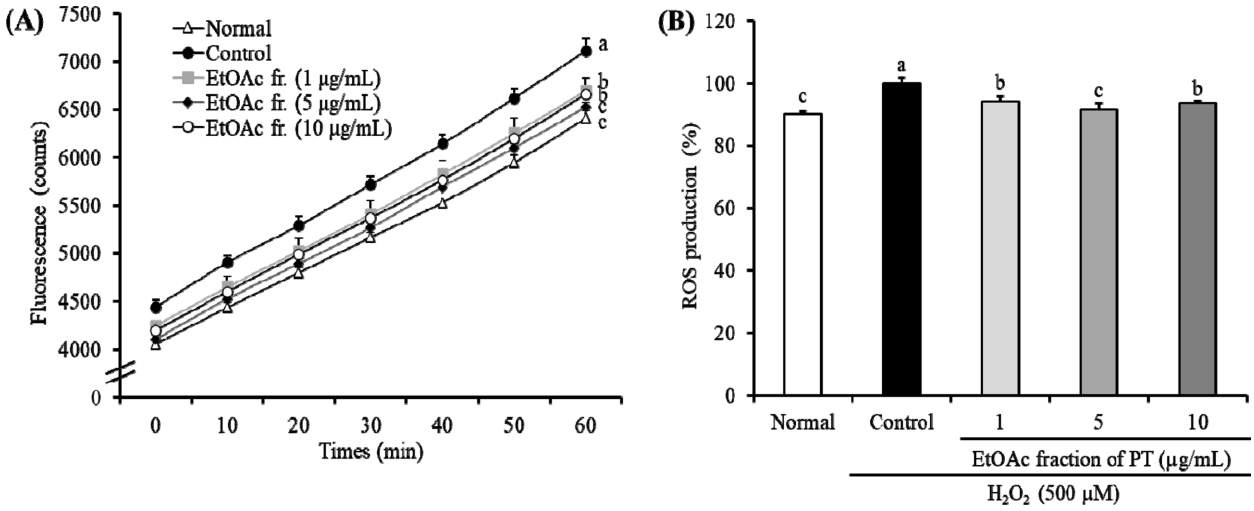

Fig. 2 Effect of the EtOAc fraction of *Populus tomentiglandulosa* on ROS production in H_2O_2-treated SH-SY5Y cells. (A) Time-course of changes in DCFH fluorescence intensity over 60 min. (B) Percentage of ROS production at 60 min. The results are expressed as the mean ± SD. The different letters (a-c) among groups indicate significant differences (p <0.05) by Duncan's multiple range test

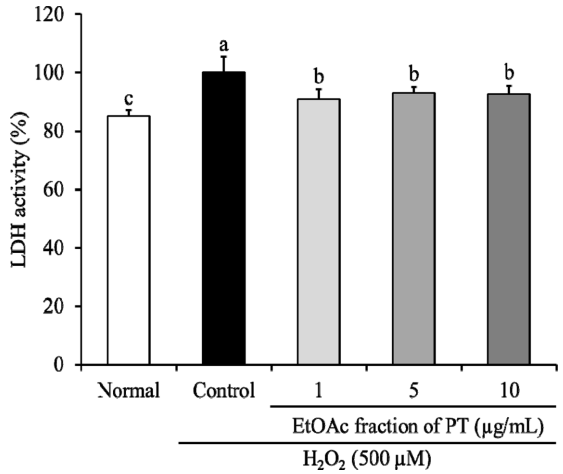

Fig. 3 Effect of the EtOAc fraction of *Populus tomentiglandulosa* on LDH release in H_2O_2-treated SH-SY5Y cells. The results are expressed as the mean ± SD. The different letters (a-c) among groups indicate significant differences (p <0.05) by Duncan's multiple range test

levels in the SH-SY5Y cells (Fig. 2B). In contrast, treatment with the EtOAc fraction of PT at concentrations of 1, 5, and 10 μg/mL significantly attenuated the production of ROS compared to the H_2O_2-treated control group. In particular, treatment with the EtOAc fraction of PT at 5 μg/mL decreased ROS production to 91.66%, similar to that of the normal group. These findings suggested that the EtOAc fraction of PT suppressed the oxidative stress induced by H_2O_2 by reducing the ROS levels in SH-SY5Y cells.

Effect of the EtOAc fraction of PT on LDH release in H_2O_2-treated SH-SY5Y cells

LDH is an enzyme present in the cytoplasm of most tissues including the brain. The organ and extracellular levels of LDH are used as an indicator of cell damage [40]. A previous study demonstrated that H_2O_2 exposure increased the LDH release induced by oxidative damage in SH-SY5Y cells [41]. To confirm the neuroprotective effect on oxidative stress, we investigated the effect of the EtOAc fraction of PT on LDH release in H_2O_2-treated SH-SY5Y cells (Fig. 3). The H_2O_2-treated control group had significantly increased LDH release (100%) compared to the non-treated normal group of 85.13%. Treatment with the EtOAc fraction of PT at doses of 1, 5, and 10 μg/mL significantly decreased the LDH release compared to the H_2O_2-treated control group. Therefore, we suggested that PT prevented the neuronal damage caused by oxidative stress by decreasing LDH release.

Previous studies have reported that EtOH extract of PT contains several active compounds including catechin, caffeic acid *p*-coumaric acid, chlorogenic acid, and gallic acid, and these compounds are widely known to have antioxidant and neuroprotective activities [42-45]. According to Cho et al. [42], catechin and gallic acid have been shown to exhibit DPPH radical scavenging activity (IC_{50} values of 0.85 and 0.28 μg/mL, respectively). In addition, *p*-coumaric acid has *in vitro* antioxidant activity by DPPH and ABTS radical scavenging activities [43]. Moreover, catechin protected neuronal oxidative stress by decreasing ROS production and had radical scavenging activity in H_2O_2-induced neuronal cells [44]. Chlorogenic acid ameliorated oxidative stress-induced neuronal toxicity by inhibition of ROS and up-regulation of antioxidant enzymes in neuronal cells [45]. Therefore, PT could show *in vitro* antioxidant and neuroprotective effect against oxidative stress by their active compounds.

In summary, the EtOH extract and four fractions of PT showed *in vitro* antioxidant activity in the DPPH, $^{\bullet}OH$, and O_2^{-} radical scavenging assays. In particular, the EtOAc fraction of PT had the strongest *in vitro* antioxidant activity among the extract and other fractions of PT. Furthermore, the EtOAc fraction of PT had a neuroprotective effect against oxidative stress by regulating ROS production and LDH release in H_2O_2-induced SH-SY5Y cells. Therefore, this study supported that PT can be used as a natural agent for protection against oxidative stress in the brain.

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