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Protective Effect of *Acanthopanax senticosus* on Oxidative Stress Induced PC12 Cell Death

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Abstract Epidemiologic studies have shown important relationships between oxidative stress and Alzheimer's disease (AD) brain. In this study, free radical scavenging activity and neuronal cell protection effect of aqueous methanol extracts of *Acanthopanax senticosus* (*A. senticosus*) were examined. H_2O_2-induced oxidative stress was measured using 2',7'-dichlorofluorescein diacetate (DCF-DA) assay. Pretreatment with the phenolics of *A. senticosus* prevented oxidative injury against H_2O_2 toxicity. Since oxidative stress is known to increase neuronal cell membrane breakdown, leading to cell death, lactic dehydrogenase release, and trypan blue exclusion assays were utilized. We found that phenolics of *A. senticosus* have neuronal cell protection effects. It suggests that the phenolics of *A. senticosus* inhibited H_2O_2-induced oxidative stress and *A. senticosus* may be beneficial against the oxidative stress-induced risk in AD.

Keywords: *Acanthopanax senticosus*, Alzheimer's disease (AD), oxidative stress

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder associated with aging, characterized by selective neuronal loss in discrete regions of the central nerve system. The two neuropathological hallmarks of AD are neurofibrillary tangles (NFT) and senile plaques (SP) (1). Amyloid β protein (Aβ) is derived from the amyloidal transmembrane precursor protein (APP) through cleavage by β- and γ-secretases. The Aβ is the major component of NFT and considered to have a crucial role in the development and progress of AD (2). A large number of evidences from both *in vivo* and *in vitro* experiments indicated that different molecular forms of Aβ affect a broad influence of neuronal and glial functions, thereby leading to neuronal cell death (3, 4). Several lines of evidence suggest the involvement of oxidative stress in the pathogenesis of AD with abnormalities observed in neurons (5). In particular, it has been reported that Aβ produces hydrogen peroxide (H_2O_2) through metal ion reduction, with concomitant release of thiobarbituric acid-reactive substances (TBARS), a process probably mediated by formation of hydroxyl radicals (6). The increase of free radicals results in lipid peroxidation producing alteration of cellular homeostasis and cell functions (7). Antioxidants such as vitamin E and vitamin C have demonstrated neuroprotective effects on Aβ-induced neurotoxicity (8).

Phytochemicals, including phenolic compounds in natural plant sources, have been known due to many biological properties such as antiproliferative and antioxidative activities (9, 10). *Acanthopanax senticosus*, a member of the Araliaceae family, is an herbal medicine and has long been used as ingredients for the treatment of human diseases such as ischemic injury, hypertension, tumor, and rheumatism (11, 12). The extract of *A. senticosus* has been reported to possess a biological activity including antioxidant effect (12). In the present study, we have examined the possible role of *A. senticosus* against oxidative cell death induced by H_2O_2 in cultured PC12 cells

Materials and Methods

Materials RPMI 1640 medium, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). All other chemicals were the products of Sigma (St. Louis, MO, USA).

Extraction of *Acanthopanax senticosus* phenolics *A. senticosus* was purchased in a local market for Oriental medicines in Seoul, Korea in 2003 and was authenticated by Institute of Biotechnology, Korea University where voucher specimens are maintained. The phenolics in *A. senticosus* were extracted from 10 g of dried sample using 80% aqueous methanol by the ultrasound-assisted method

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(13). The mixture was sonicated for 20 min with a continual stream of nitrogen gas purging to prevent possible oxidative degradation of phenolics. The mixture was filtered through Whatman no. 2 filter paper (Whatman International Limited, Kent, UK) using a chilled Buchner funnel and rinsing with 50 mL of absolute methanol. Extraction of the residue was repeated using the same conditions. The 2 filtrates were combined and transferred into a 1 L evaporating flask with an additional 50 mL of 80% aqueous methanol. The solvent was removed using a rotary evaporator at 40°C. The remaining phenolic concentrate was first dissolved in 50 mL of absolute methanol and diluted to a final volume of 100 mL using deionized distilled water (ddH_2O). The mixture was centrifuged at refrigerated temperatures at 12,000×g for 20 min and stored at -20°C until analysis.

Determination of total phenolics Total phenolics were determined by the spectrophotometric analysis (14). In brief, a 1 mL portion of appropriately diluted extracts was added to a 25 mL volumetric flask containing 9 mL of ddH_2O. A reagent blank using ddH_2O was prepared. One mL of Folin-Ciocalteu's phenol reagent was added to the mixture and then shaken. After 5 min, 10 mL of a 7% Na_2CO_3 solution was added with mixing. The mixed solution was then immediately diluted to volume (25 mL) with ddH_2O and mixed thoroughly. After 90 min at 23°C, the absorbance was read at 750 nm. The standard curve for total phenolics was made using gallic acid standard solution (0-100 mg/L) under the same procedure as above. Total phenolics in *A. senticosus* were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dried sample.

Cell culture PC12 cells were propagated in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, and 50 units/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 5% CO_2 (15, 16). The PC12 cell line was derived from a transplantable rat pheochromocytoma. The cells respond reversibly to nerve growth factor (NGF) by induction of the neuronal phenotype.

Measurement of intracellular oxidative stress Levels of intracellular reactive oxygen species (ROS) were determined by 2',7'-dichlorofluorescein diacetate (DCF-DA; Fluorescent probe) assay (17). In brief, cells (10^4 cells/well on 96-well) were treated for 10 min with the indicated concentrations of the *A. senticosus* or vitamin C. The cells were then treated with or without 400 μM H_2O_2 for 2 hr. At the end of the treatment, cells were incubated in the presence of 50 μM DCF-DA in phosphate buffered saline (PBS). Fluorescence was then quantified using TECAN SER-NR 94572 fluorometer (San Jose, CA, USA) using 485 nm excitation and 530 nm emission filters.

MTT reduction PC12 cells were plated at a density of 10^4 cells/well on 96-well plates in 100 μL RPMI and the cell viability was determined by the conventional [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT)] reduction assay (17, 18). The cells were incubated with 0.25 mg MTT /mL (final concentration) for 2 hr at 37 °C, and the reaction was stopped by adding solution containing 50% dimethylformide and 20% sodium dodecyl sulfate (pH 4.8). The amount of MTT formazan product was determined by measuring absorbance using a microplate reader (Bio-Rad, Hercules, CA, USA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Lactate dehydrogenase (LDH) release PC12 cells were precipitated by centrifugation at 2,000×g for 2 min at room temperature, 50 μL of the supernatants was transferred into new wells, and LDH was determined using Sigma *in vitro* toxicology assay kit. Total cellular LDH activity was determined by solubilizing the cell with 0.2% Triton X-100 (17). Damage of the plasma membrane was evaluated by measuring the amount of the intra-cellular enzyme LDH released into the medium.

Trypan blue exclusion assay The trypan blue exclusion assay was based on the capability of viable cells to exclude the dye. Since viable PC12 cells maintained membrane integrity, the cells did not allow trypan blue dye to pass through the cell membrane. Cells with damaged membrane appeared blue due to their accumulation of dye, and were counted as dead. The dye of 0.4% trypan blue was added to PC12 cells, and after 10 min cells were loaded into a hematocytometer and counted for the dye uptake. At least 600 cells were counted in 5 different fields, and the number of viable cells was calculated as percent of the total cell population. PC12 cells with ≥98.0% viability were employed in all treatments (17).

Statistical analysis All data were expressed as mean±SD. Statistical analysis was performed by Student's *t* test. $p<0.05$ was considered significant.

Results and Discussion

Total phenolics in *A. senticosus* and their antioxidant activity Phenolic compounds have long been studied to possess many properties including antioxidative activity and suggested for reducing the oxidative stress associated with AD (19). Total phenolics in *A. senticosus* were 366.4 mg GAE/100 g dry weight. To examine intracellular accumulation of ROS, the DCF-DA probe, which is freely permeable to the cell membrane, was used. In PC12 cells, the compound is hydrolyzed by the cellular esterase to 2', 7'-dichlorofluorescein (DCF), which interacts with peroxides forming fluorescent DCF. Exposure of PC12 cells to H_2O_2 for 2 hr resulted in a 407±8.5% increase of ROS levels compared to control (Fig. 1). Intracellular ROS accumulation resulting from H_2O_2 treatment was significantly reduced when the phenolic extracts of *A. senticosus* were present in the media compared to PC12 cells treated with H_2O_2 only. Vitamin C is one of naturally occurring major nutrients having antioxidant activity (20). At the level of 60 μM vitamin C, as a positive control, PC12 cells had significantly lower oxidative stress than PC12 cells with treatments of H_2O_2 only (Fig. 1). Oxidative stress in AD may result from aging, energy deficiency, inflammation or excessive production of Aβ. Aβ protein can induce cell

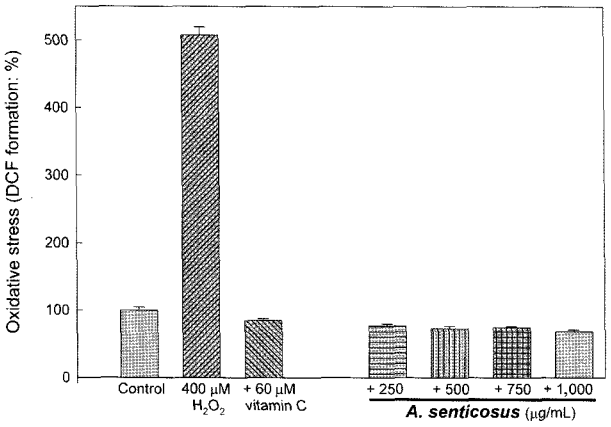

Fig. 1. Effect of the phenolics of *Acanthopanax senticosus* on free radical production determined in the presence and absence of H_2O_2 in PC12 cell. DCF value was not changed by vitamin C or the phenolics of *A. senticosus* (data not shown). Results shown are means±SD (n=3). Statistical analysis indicated that the influence of the compounds used had significant effect on the H_2O_2-induced oxidative stress ($p<0.05$ vs. vitamin C).

death through a mechanism involving H_2O_2 (21). In this respect, this result suggests that *A. senticosus* phenolics with antioxidant activity may play an important role to reduce the oxidative stress-induced risk of AD.

Neuronal cell protective effect of phenolics in *A. senticosus* The oxidative stress-induced neurotoxicity was examined by determining the percentage of MTT reduction after incubation of PC12 cells for 2 hr with H_2O_2. Hydrogen peroxide caused a decrease in cell viability (31±2.4%), but pretreatment of PC12 cell with increasing concentrations of the phenolics of *A. senticosus* dose-dependently inhibited oxidative stress-induced cytoxicity (Fig. 2). Cell protection effect of vitamin C on oxidative injury was less than that of 1,000 μg/mL

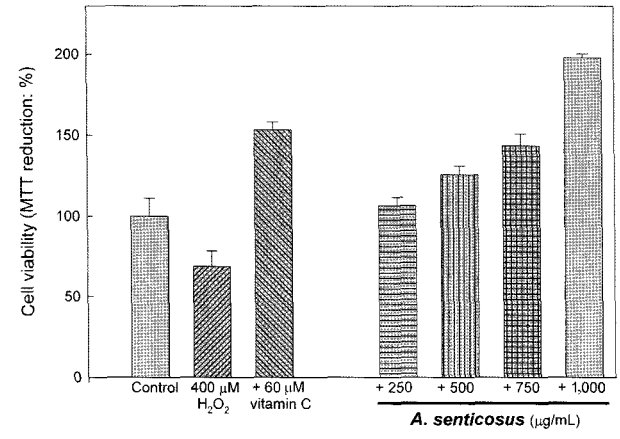

Fig. 2. Protective effect of the phenolics of *Acanthopanax senticosus* on H_2O_2-induced cytotoxicity in PC12 cell. Cell viability was not changed by vitamin C or the phenolics (data not shown). Results shown are means±SD (n=3). Significant difference ($p<0.05$ vs. vitamin C) was observed on the H_2O_2-induced cell death.

phenolics. This study demonstrated that PC12 cell apoptosis through oxidative stress was suppressed by pretreatment with the phenolics of *A. senticosus*. MTT dye reduction assay is based on the catalytic activity of some metabolic enzymes in intact mitochondria (22). Hence, these result show that PC12 cell protection by the phenolics of *A. senticosus* is partially due to the mechanisms with mitochondrial protection.

Several markers of excess oxidative stress, which is resulting in accumulation of oxidized products such as protein carbonyls from protein oxidation and aldehydes from lipid peroxidation, serve to establish the direct role of Aβ in the oxidative damage associated with cell membrane (23, 24). The resulting protein damage by oxidative stress may appear in the form of physical, chemical, or functional changes. Free radical-induced oxidation of proteins may damage enzymes critical to neuronal function. Consistent with above notions, membrane bilayer resident phospholipid unsaturated fatty acids are especially defective to free radical stress. Hydrogen atom abstraction from unsaturated fatty acid and the subsequent immediate reaction of the C-centered radicals with molecular oxygen result in the formation of lipid peroxyl radicals or hydroperoxides (25). To examine the probability of membrane attack, we have assessed the protective effect of the phenolics of *A. senticosus* on H_2O_2-induced cytotoxicity using the LDH assay, measuring the activity of this stable enzyme released into the medium from dead cells. A quantitative analysis of LDH activity can determine what percentage of cells is dead. Figure 3A shows that treatment with H_2O_2 caused an increase in LDH release into the medium and a decrease in the number of viable cells (63±1.6%). Pretreatment with the phenolics presented more efficient inhibition activity of LDH release in PC12 cells, while vitamin C was less effective on LDH release. To confirm if the phenolics of *A. senticosus* block the H_2O_2-induced membrane damage, the trypan blue exclusion assay, which directly measures the viable cells maintaining the capability of excluding the dye and may reflect more precisely the integrity of viable cell membrane, was also used. H_2O_2-induced oxidative stress increased plasma membrane damage and the phenolics protected the PC12 cells from neurotoxicity (Fig. 3B). In this study, the protective effect of the phenolics of *A. senticosus* seemed to be a greater efficiency than it did at the same concentrations in the MTT assay. These results suggested that PC12 cell protective effect of *A. senticosus* phenolics might be largely due to the inhibition of oxidative stress-induced membrane damage compare to mitochondrial protection. Therefore, it is not strange that the neuronal cell protective activity of the phenolics is not strong in MTT assay, while the phenolics in the same concentration range was more effective in terms of inhibiting morphological or biochemical features of apoptosis.

Among present results (Fig. 1-3), *A. senticosus* phenolics showed obvious dose-dependent pattern in only MTT assay (Fig. 2). As above mentioned, used assays in our experiments have various and individual principles for detection of H_2O_2-induced stress. *A. senticosus* phenolics may have many different phenolic compounds, too. Therefore, we just guess that is the interfering effect of

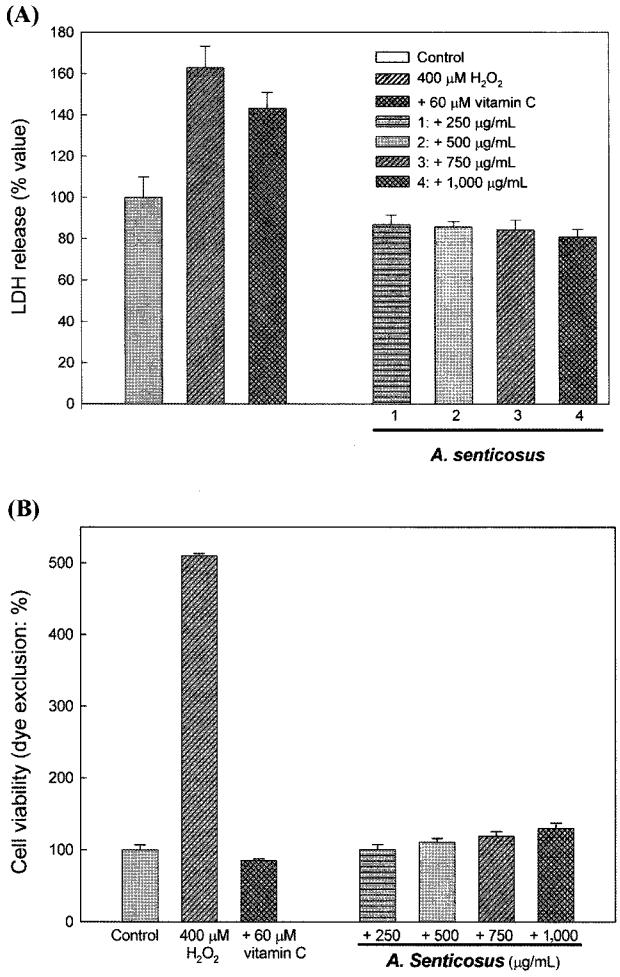

Fig. 3. Cell membrane protective effect of the phenolics of *Acanthopanax senticosus* on H_2O_2-induced membrane damage in PC12 cells. (A) LDH activity in culture supernatants was measured with a colorimetric LDH assay kit. Basal and total LDH activities were determined in intact cells and cell solubilized with 0.2% Triton X-100, respectively, and LDH release was calculated as [(sample LDH - basal LDH) / (total LDH - basal LDH)] × 100 (%). (B) Inhibition of H_2O_2-induced cytoxicity in PC12 cells after pretreatment with the phenolics as assessed by trypan blue exclusion staining followed by cell counting. Data are presented as mean±SD for one representative triplicate determination and are expressed as the percent survival compared to the corresponding controls. Statistical analysis indicated that the influence of the compounds used had significant effect on H_2O_2-induced membrane toxicity (LDH release) ($p<0.05$).

phenolic compounds of *A. senticosus*. For finding exact reasons, more studies will be needed.

The amount of ROS production is about 2% of the total oxygen consumed during respiration, but it may vary depending on several parameters (26). The human brain operates the cognitive and behavioral functions through an intricate network of about 100 billion neurons and supporting cell (glial cell: astrocytes etc.). Despite only 2% of the adult body weight, it requires and utilizes a plentiful amount of energy. This high demand of energy may be required to maintain efficient functioning of its complex and various chemical processes. Since brain consumes an excessive fraction (20%) of the total oxygen consumption, is not abundant in antioxidant defenses, and is enriched in the more easily peroxidizable fatty acids (arachidonic acid and docosahexaenoic acid; DHA) in the neuronal cell membrane, the membrane of brain is regarded abnormally sensitive to oxidative damage (26). Membrane lipid peroxidation in the brain of AD patients is considered to be increased and especially arachidonic acid and DHA are more defective to attack by ROS (27). Consequently, loss of membrane integrity leads to cellular dysfunction, such as inhibition of ion-motive ATPases, loss of Ca^{2+} homeostasis, inhibition of glial cell Na^{+}-dependent glutamate uptake system with consequences on neuronal excitatory NMDA receptors, loss of protein transporter function, disruption of signaling pathways, and activation of nuclear transcription factors and apoptotic pathways (28, 29). Our results suggest that the phenolics of *A. senticosus* might be inhibiting the brain neuronal apoptosis, which is the ultimate consequence of these cellular dysfunctions.

Previous *in vitro* studies in neuronal and non-neuronal cell systems indicate that caspase (aspartate-specific cysteine protease) is effectors of apoptosis (30). Especially, in neurons, several evidences show that caspase-3 (a 32-kDa cytosolic protein) plays a pivotal role in the practical phase of apoptosis (31). Furthermore, neuronal cell death in experimental models of several acute and chronic neurodegenerative disorders has been associated with activation of caspase-3 (32-34).

The two well-known pathways of caspase activation include the surface death receptor pathway and the mitochondrion-initiated pathway (35). In the second pathway, varied pro-apoptotic signals collect at the mitochondrial level. As a result of various apoptotic inducers, cytochrome c becomes translocated from the mitochondrial inter-membrane into the cytosol (36, 37). Released cytochrome c binds to Apaf-1 and triggers activation of caspase-9 (38), which in turn cleaves and activates caspase-3 and -7 (39). Moreover, other studies have shown that H_2O_2 stimulates mitochondrial cytochrome c release and then activates caspase-3 (40). Gruss-Fischer and Fabian (41) reported that treating of human leukemia cells with ascorbic acid decreases denauration of cytochrome c and the activation of the H_2O_2-induced apoptotic cascade. Consequently, our results suggested that neuronal cell protective effect of *A. senticosus* phenolics may participate in apoptotic caspase signaling system.

The inflammatory reaction hypothesis on AD has been interesting when it was demonstrated that the products of inflammatory reaction, such as cytokines (42) and free radicals (43), were neurotoxic in experimental neuron models. These products of inflammatory reactions may represent extracellular signals, which initiate and promote neuronal degeneration in AD. A mediates the actions of these extracellular signals (44). This hypothesis was further supported by clinical studies in which administration of non-steroidal anti-inflammatory drugs (NSAIDs) reduced the rate of retrogression of cognitive function in reasonable AD patients (45). These drugs ameliorate the levels of neurotoxins by inflammatory reactions and hence protect neuronal cells. In AD patients, senile plaques, which are made from A and one of the neuropathological

hallmarks of AD, provide as a stable source of inflammatory reactions. Therefore, anti-inflammatory agents may play an important role in degeneration of neurons in AD brain. It has been reported that a number of phenolics including syringin, syringaresinol were isolated from *A. senticosus* (46) and oral administration of the phenolics of *A. senticosus* showed an anti-inflammatory effect (47). Especially, the phenolics inhibited the production of inflammatory reaction-induced nitric oxide (NO: inflammatory mediator) through the blocking of the expression level of nitricoxide synthase (iNOS), COX-2 gene in a concentration-dependent manner. Therefore, these results suggest that the phenolics of *A. senticosus* with anti-inflammatory activity may be used to reduce the risk of oxidative stress-induced neuronal cell death in AD. In conclusion, the phenolics of *A. senticosus* with anti-inflammatory effect protected neuronal cell membrane by free radical scavenging activity, which provided higher cell viability to PC12 cells.

Acknowledgments

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