Attenuation of Aβ-Induced Apoptosis of Plant Extract (Saengshik) Mediated by the Inhibition of Mitochondrial Dysfunction and Antioxidative Effect

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ABSTRACT: Recently, considerable attention has been focused on dietary manipulation of oxidative and/or nitrosative damage on neuronal cells. In this article, a neuroprotective effect of plant (Saengshik) extracts was investigated. Rat pheochromocytoma (PC12), cells treated with β-amyloid underwent apoptotic death as determined by positive *in situ* terminal end-labeling (TUNEL staining), decreased mitochondrial transmembrane potential, and elevated caspase-3 activity co-occurring with enhanced MDA accumulation and the reduction of GSH levels. Saengshik pretreatment attenuated β-amyloid-induced apoptosis in PC12 cells possibly by inhibiting mitochondrial dysfunction and exerting antioxidant properties. Saengshik pretreatment inhibited the loss of mitochondrial membrane potentials and reduced the activation of caspase-3. The *in vitro* antioxidant activities of Saengshik extracts were verified by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and superoxide dismutase (SOD) mimetic activity. In β-amyloid-challenged PC12 cells, Saengshik prevented the production of ROS, decreased the level of MDA, and elevated GSH. The potential of Saengshik as one of the neuroprotective regimens has been suggested through this article, and the combination with defined pharmaceuticals or other dietary antioxidants may provide a better therapeutic or preventive advantage for the management of Alzheimer's disease.

KEYWORDS: Saengshik; neuroprotective effects; antioxidant capacity; PC12 cells; Alzheimer's disease

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INTRODUCTION

Reactive oxygen or nitrogen species (ROS or RNS) are generated by the ordinary metabolic processes as well as by the external environmental stimuli, such as ultraviolet light, gamma radiation, and some kinds of xenobiotics in the living system.[1,2] A growing body of evidence suggests that oxidative stress may be implicated as a major cause of cellular injuries in a vast variety of clinical abnormalities including neurodegenerative disorders.[3,4] A decrease in mitochondrial energy charge and redox states, loss of transmembrane potential (depolarization), mitochondrial respiratory chain impairment, and release of substances, such as calcium and cytochrome *c*, all contribute to apoptosis.[5–8] Accumulation of amyloid beta (Aβ) in the form of senile plaques is also thought to play a central role in the pathogenesis of Alzheimer's disease (AD) mediated by oxidative stress.[9]

Many natural antioxidants, such as vitamin E, vitamin C, or many kinds of phytochemicals, are recognized as antioxidant agents that prevent oxidation *in vivo* and as free radical scavengers in lipophilic and water-soluble sites *in vitro*.[10–12] Recently, it has been reported that Aβ produces ROS, and antioxidants, such as vitamin E or melatonin, exert neuroprotective effects against Aβ-induced cytotoxicity.[13,14] Moreover, intracellular levels of antioxidative defense enzymes, such as superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase, are also changed during the progression of AD.[15,16] Several studies have addressed implications of apoptotic cell death in Aβ-mediated neurotoxicity.[17,18] Apoptosis is a tightly regulated process, which involves changes in the expression of distinct sets of enzymes and their substrate proteins.[19] AD is a neurodegenerative disorder characterized by progressive degeneration and loss of neurons in the brain. The appearance of neurofibrillary tangles and accumulation of senile plaques are two distinct neuropathological hallmarks of AD.[20,21] Several lines of evidence suggest that enhanced oxidative stress induced by ROS or RNS is associated with pathogenesis and progression of AD.[22,23]

The goal of this article was to examine the protective mechanisms of the standardized extract of Saengshik against Aβ-induced apoptosis in relation to neuroprotective effects as well as measuring *in vitro* antioxidant activities.

MATERIALS AND METHODS

Preparation of Saengshik Extracts

Dry samples (64 g) were extracted for 24 h with 7:3 EtOH and H_2O or 8:2 MeOH and H_2O (200 mL three times) at 80°C. For H_2O extraction, 10 g of sample was extracted for 24 h with 100 mL for three times at 80°C. The solvent

was evaporated in a rotary evaporator (Yamato Co., RE 47, Tokyo, Japan), and the remaining aqueous solution was freeze-dried, milled, and kept at −20°C

In vitro *Antioxidant Activity of Saengshik Extract*

The free radical scavenging activity was determined using the stable DPPH radical by a standardized method. The optical density was determined using a microplate reader, Spectra Max 340PC (Molecular Devices Co., Sunnyvale, CA) at 517 nm. SOD-like activity was determined using xanthine–xanthine oxidase and cytochrome *c*.

Cell Culture

Rat pheochromocytoma (PC12) cells were maintained routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37°C in a humidified atmosphere of 10% CO_2 / 90% air.

Measurement of Intracellular ROS Accumulation

To monitor net intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. Cells (1×10^6 cells/3 mL in 6-well plates) were rinsed with Kreb's ringer solution and 10 μM DCF-DA was loaded. After 15-min incubation at 37°C, cells were examined under a confocal microscope equipped with an argon laser (488 nm; 200 mW).

Assessment of Lipid Peroxidation

The extent of lipid peroxidation in PC12 cells treated with $A\beta_{25-35}$ was assessed using the commercially available colorimetric assay kit Bioxytech LPO-586 (OXIS Research International, Foster City, CA).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL)

The commercially available *in situ* death detection kit (product of Boehringer, Ingelheim, Germany) was used to detect DNA fragmentation.

Measurement of the Mitochondrial Membrane Potential

To measure the mitochondrial membrane potential ($\Delta\Psi m$), the lipophilic cationic probe TMRE was used. After treatment with $A\beta_{25-35}$ (25 μM) for 24 h

in the presence or absence of plant extract, cells (1×10^6 cells/3 mL in 6-well plates) were rinsed with PBS containing 1g/L glucose, and TMRE (150 nM) was loaded. After 30-min incubation at 37°C, cells were rinsed and examined under a confocal microscope (LEICA TCS SP; LEICA, Solms, Germany).

Assay for Caspase-3 Activity

The extent of caspase activation in PC12 cells treated with $A\beta_{25-35}$ was assessed using the commercially available colorimetric assay kit in accordance with the protocol supplied by the manufacturer (BioVision, Mountain View, CA).

RESULTS

Determination of Cell Viability of PC12 Cells in the Absence and Presence of Increasing Concentrations of Saengshik extracts

The possible cytotoxicity of Saengshik extracts in PC12 cells was evaluated based on their effects on cell growth (MTT assay) (FIG. 1 A, B, and C).

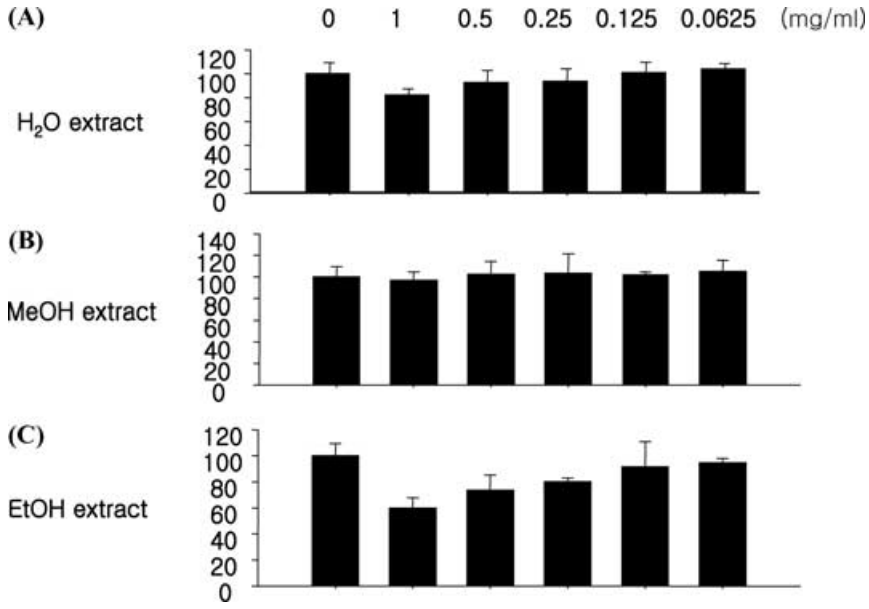

FIGURE 1. Cytotoxic effect of Saenkshik extract on cytotoxicity of PC12 cells. PC12 cells were treated with indicated concentrations of water (**A**), MeOH (**B**), and EtOH (**C**) extracts for 36 h at 37°C. Viable cells were determined using the MTT assay as described in the "Materials and Methods" section. The data are presented as mean ± SD ($n = 3$).

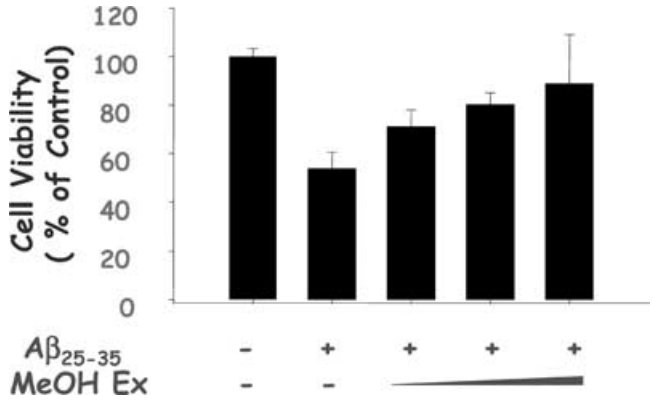

FIGURE 2. Protective effect of Saengshik extract on cytotoxicity of $A\beta_{25-35}$ in PC12 cells. PC12 cells were treated with indicated concentrations of $A\beta_{25-35}$ and MeOH extracts for 36 h at 37°C. Viable cells were determined using the MTT assay as described in the "Materials and Methods" section. The data are presented as mean ± SD ($n = 3$).

Results in FIGURE 1 A show that, at concentrations ranging from 0.0625 to 1 mg/mL, water extract of Saengshik slightly inhibited cell growth concentration dependently. MeOH extracts showed almost negligible cell cytotoxicity in all concentrations.

Saengshik Extract Prevents $A\beta_{25-35}$ -Induced Viability Loss of PC12 Cells

A toxicity was evaluated by the MTT assay upon incubation of PC12 cells for 36 h, and MeOH extract increased the cell survival rate in a concentration-dependent manner. Concentration of 0.05 mg/mL showed the highest protection rate among the three concentrations tested (0.0125, 0.025, and 0.05 mg/mL) (FIG. 2).

Saengshik Extract Inhibited the $A\beta_{25-35}$ -Induced Intracellular ROS Accumulation Accompanied with the Decrease in MDA and Increase in GSH Levels

PC12 cells treated with 25 μM $A\beta_{25-35}$ for 6 h displayed intense fluorescence after staining with DCF dye and intracellular ROS accumulation resulting from $A\beta_{25-35}$ treatment was significantly reduced when Saengshik extract was treated to the media (FIGS. 3 and 4). The GSH content in the control PC12 cells was significantly reduced by the treatment with $A\beta_{25-35}$, and this depletion of the GSH contents was recovered almost up to the level of the

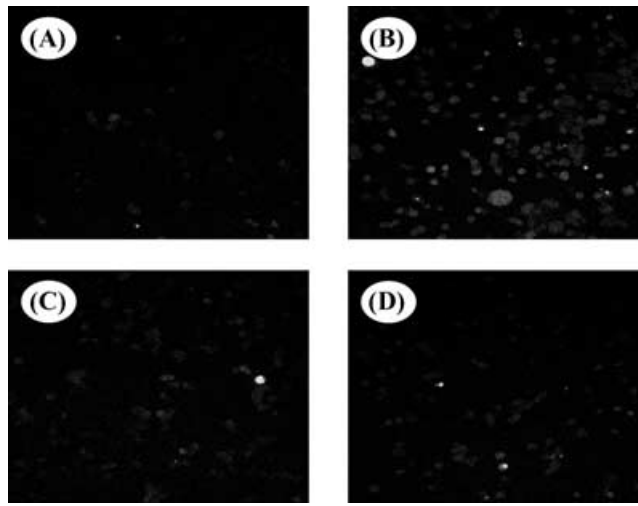

FIGURE 3. Microscopic analysis of $A\beta_{25-35}$-induced ROS and the protective effect of Saengshik on ROS accumulation. Intracellular ROS were determined based on the peroxide-sensitive DCF-fluorescence as described in the "Materials and Methods" section. (**A**) Control. (**B**) Treated with $A\beta_{25-35}$ (50 μM). (**C** and **D**) Treated with increasing concentration of Saengshik in the presence of $A\beta_{25-35}$ (50 μM).

control at the 0.05 mg/mL of MeOH extract (FIG. 5 A). FIGURE 5 B shows the effect of MeOH extract on the reduction of MDA. Saengshik MeOH extract was found to effectively scavenge free radicals generated by $A\beta_{25-35}$ treatment as measured by malondialdehyde (MDA) production. Oxidative damage has been involved in Aβ-induced cell death. PC 12 cells treated with $A\beta_{25-35}$ underwent peroxidation of their lipid bilayer, leading to increased levels of MDA derived from lipid peroxides.

Saengshik Extract Attenuates $A\beta_{25-35}$-Induced PC12 Apoptotic Cell Death

Apopototic cells were detected by TUNEL staining, which is a widely used immunostaining method in detecting DNA fragmentation *in situ*. In this histochemical technique, the appearance of intensely stained nuclei is indicative of terminal incorporation of labeled dUTP into fragmented DNA derived from apoptotic nucleus. Treatment with $A\beta_{25-35}$ significantly increased the proportion of TUNEL-positive cells and this was reduced by Saengshik extracts (FIG. 6 A, B).

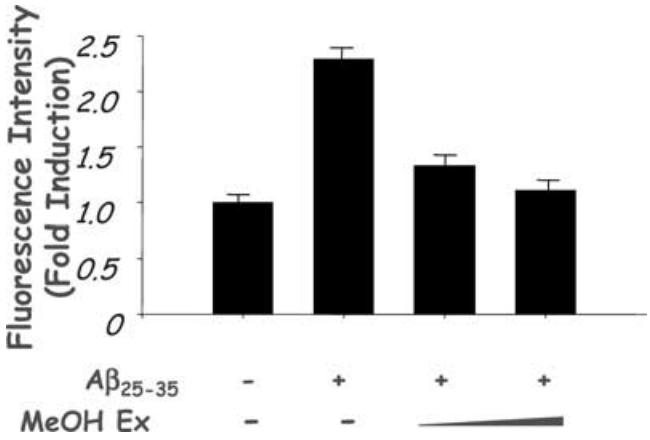

FIGURE 4. The graphical representation of the effect of Saengshik extract on the reduction of ROS accumulation in PC12 cells in the presence of $A\beta_{25-35}$ (50 μM).

Saengshik Extract Shows a Protective Effect on $A\beta_{25-35}$-Induced Dissipation of the Mitochondrial Membrane Potential

When PC12 cells were exposed to $A\beta_{25-35}$, the $\Delta\Psi m$ rapidly depolarized, as shown by the decrease in voltage-sensitive dye, TMRE compared to the control (FIG. 7 A upper panel) and pretreatment with Saengshik extract reduced the changes of $\Delta\Psi m$ as indicated by restoration of TMRE dye (FIG. 7 A lower panel). $A\beta_{25-35}$ -induced dissipation of the $\Delta\Psi m$ was blocked by the pretreatment with Saengshik extract in a concentration-dependent manner (FIG. 7 B).

Saengshik Decreases Caspase-3 Activity

There was approximately threefold induction of caspase-3 activity in PC12 cells treated with $A\beta_{25-35}$, which was suppressed by Saengshik extract. By treating 0.05 M MeOH extract, caspase-3 activity returned to the control level (FIG. 8).

Saengshik Has Antioxidant Activities as Assayed by DPPH and SOD Methods

The antioxidant activity of the Saengshik extract was assessed by means of two different *in vitro* tests. The Trolox equivalent antioxidant activities were determined using the stable DPPH*. The Trolox equivalent values of Saengshik extracts were evaluated and methanol extract of Saengshik showed highest

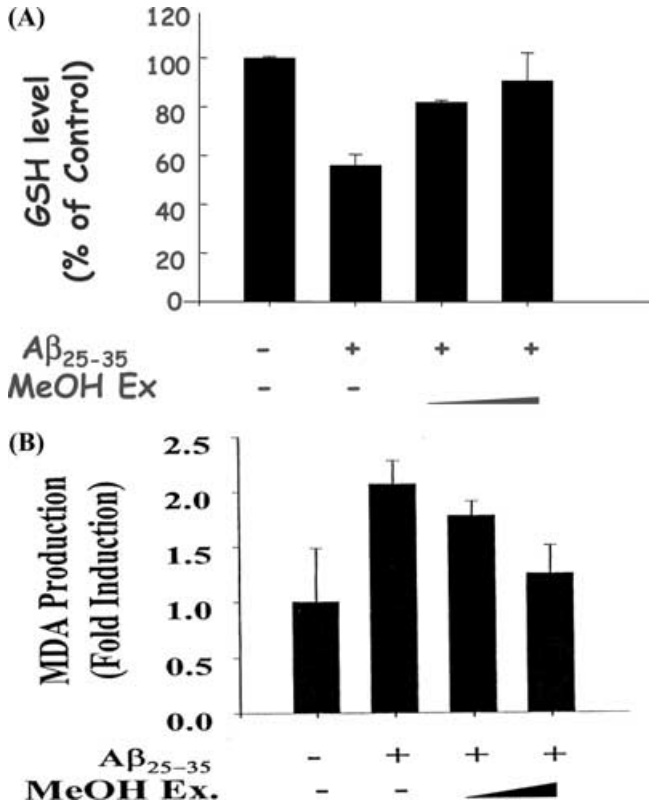

FIGURE 5. Saengshik MeOH extract increases GSH levels (**A**) and decreases MDA production (**B**) in $A\beta_{25-35}$ (50 μM)-treated PC12 cells.

value of 386.16 mg or 1.542 mM. The SOD-like activities of Saengshik EtOH and MeOH extract showed that 10.74% and 16.83% inhibition of oxidation process respectively, whereas water extract did not show any activity (FIG. 9).

DISCUSSION

The purpose of this study was to examine the neuroprotective effects and the antioxidant activity of Saengshik, which consisted whole grain, essential vegetables, seaweeds, mushrooms, and other minor ingredients. In this article Saengshik extract has been shown to protect cells from β-amyloid-induced toxicity, and it was also demonstrated that Aβ-induced apoptotic death via oxidative stress in PC12 cells was suppressed by the pretreatment with Saengshik extract. PC12 cells exposed to $A\beta_{25-35}$ showed characteristic morphological features of apoptosis, such as cell shrinkage and membrane blebbings

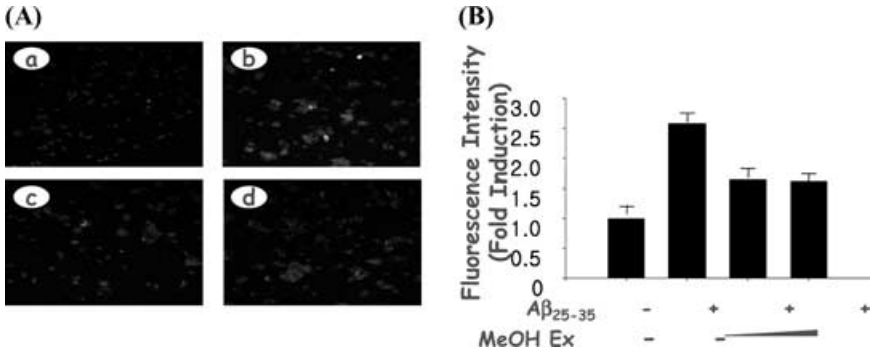

FIGURE 6. Microscopic analysis of $A\beta_{25-35}$ (50 μM)-induced cell death and the protective effect of Saengshik (**A**). DNA fragmentation was determined by TUNEL. (a) Control. (b) Treated with $A\beta_{25-35}$ for 4. (c and d) Treated with increasing concentration of Saengshik in the presence of $A\beta_{25-35}$ (50 μM). **B** is the graphical representation of fluorescence intensity of these treatments.

and positive TUNEL staining. $A\beta_{25-35}$ treatment also induced cleavage of PARP, which is the substrate of active caspase mediating apoptosis. Recently, considerable attention has been focused on dietary manipulation of oxidative and/or nitrosative damage. The various antioxidants are found to have the ability to scavenge free radicals. There has been a growing body of data implicating free radical toxicity, radical-induced mutations and oxidative enzyme impairment, mitochondrial dysfunctions, and excitotoxic mechanisms in the pathogenesis of neurodegeneration.[24,25] The brain may be particularly vulnerable to oxidative stress in that it consumes a large amount of the body's

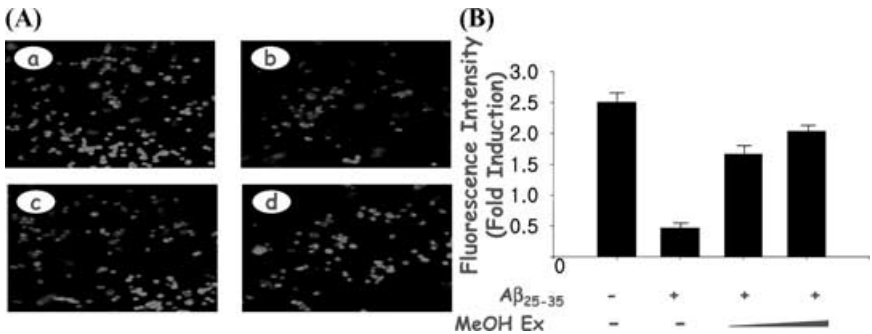

FIGURE 7. Saengshik MeOH extract increases mitochondrial membrane potential in $A\beta_{25-35}$ (50 μM)-treated PC12 cells (**A**). Mitochondrial membrane potential was determined by TMRE staining as described in the "Materials and Methods" section. (a) Control. (b) Treated with $A\beta_{25-35}$. (c and d) Treated with increasing concentration of Saengshik in the presence of $A\beta_{25-35}$ (50 μM). **B** is the graphical representation of fluorescence intensity of the treatments.

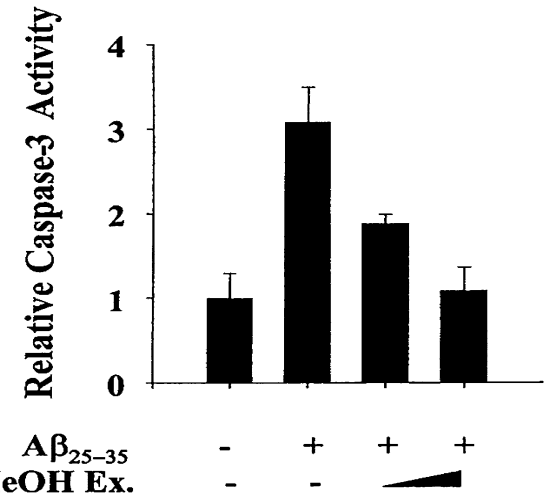

FIGURE 8. Saengshik extract decreases caspase-3 activity in $A\beta_{25-35}$-treated PC12 cells. Caspase-3 activity was determined as described in the "Materials and Method" section.

oxygen and has relatively poor antioxidant protection as shown by low levels of the antioxidant enzyme GSH peroxidase as well as antioxidants, such as GSH and vitamin E.[26] In this study, $A\beta_{25-35}$-induced intracellular accumulation of ROS was attenuated by Saengshik extract as revealed by reduced distribution of DCF-fluorescence in PC12 cells pretreated with Saengshik. The involvement of ROS in $A\beta_{25-35}$-induced cell death was further supported by increased accumulation of peroxides and the reduction of GSH in the treated cells. Saengshik extract not only suppressed $A\beta_{25-35}$-induced cytotoxicity, but also decreased ROS accumulation accompanied with the elevation of GSH and reduction of MDA in pretreated cells. It has been shown that reduction of GSH levels increases the sensitivity of neurons to the toxic effect of neurotoxins,[27] and is associated with mitochondrial dysfunction.[28] ROS contribute to cell death, in part, through modulation of various cellular signaling cascades.

The findings from these experiments demonstrate that the MeOH and EtOH fractions possess relatively strong antioxidant–free radical scavenging properties. It appeared that the increased apoptosis was protected by pretreatment with Saengshik. Further elucidation of intracellular signaling cascades involved in Aβ-induced cell death and their modulation may provide insights into the molecular basis for neuroprotective effects of naturally occurring phytochemical compounds found in Saengshik.

In summary, the cultures of PC12 pretreated with β-amyloid underwent apoptotic death as determined by positive *in situ* TUNEL staining, decreased mitochondrial transmembrane potential, elevated caspase-3 activity occurring

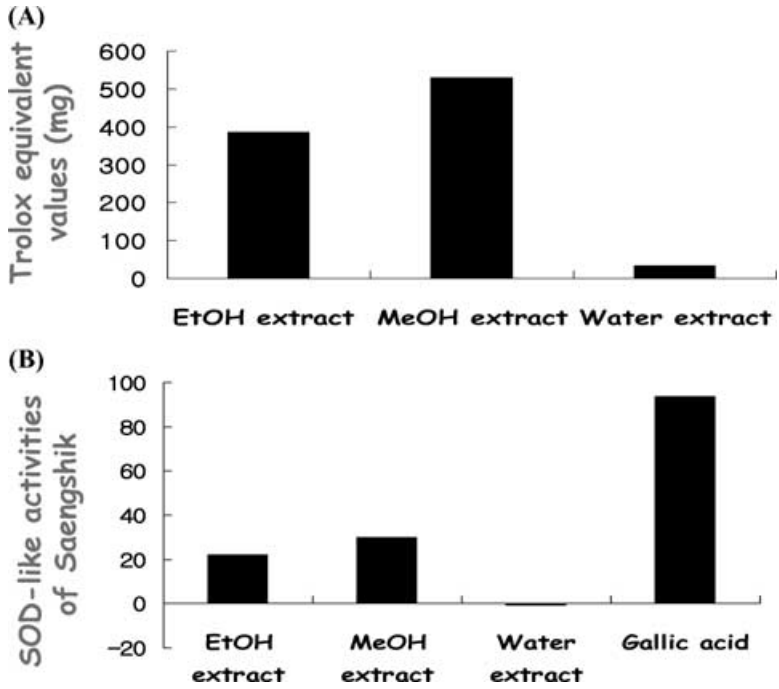

FIGURE 9. Trolox equivalent values (**A**) and SOD-like activities (**B**) of Saengshik EtOH, MeOH, and water extracts.

with enhanced MDA accumulation, and the reduction of GSH levels. Saengshik pretreatment attenuated Aβ-induced apoptosis in PC12 cells possibly by exerting antioxidant properties. The potential of Saengshik as one of the neuroprotective agents has been suggested through this study, and the combination with other dietary antioxidants possessing ROS or RNS scavenging activities could provide better therapeutic advantage for the management of AD.

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