

Neuroprotective and Neurotrophic Effects of Quinic Acids from *Aster scaber* in PC12 Cells

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Aster scaber T. (Asteraceae) has been used to treat bruises, snakebite, headache, and dizziness in traditional Chinese medicine. In the present study, the neuroprotective effect of four quinic acid derivatives from *A. scaber* on amyloid A β -induced PC12 cell toxicity was investigated. When cells were treated with quinic acid derivatives prior to A β , cell toxicity was significantly diminished. Among quinic acid derivatives, (–)4,5-dicaffeoyl quinic acid (1) gave the highest protection against A β -induced cell toxicity. In addition, the neurotrophic effects of compounds were evaluated by microscopically monitoring their potency to induce neurite outgrowth in PC12 cells. Four quinic acid derivatives from *A. scaber* promoted neurite outgrowth in PC12 cells. Interestingly, a novel quinic acid, (–)3,5-dicaffeoyl-*muco*-quinic acid (2) was more effective than the other compounds in promoting neurite outgrowth. Unlike nerve growth factor, the withdrawal of quinic acids did not result in any significant decrease in cell viability. The results suggest that quinic acid derivatives from *A. scaber* might potentially be used as a therapeutic agent in Alzheimer disease.

Key words quinic acids; neuroprotection; PC12 cells; Alzheimer disease

Dementia syndromes such as Alzheimer disease (AD) have their own pathologic characteristics including degeneration and loss of neurons in certain brain areas, such as the cholinergic neurons. To combat this disease, nerve growth factor (NGF) has held the promise of therapeutic efficacy, since NGF stimulates the outgrowth of neurites in neuronal cells and plays an important role in the survival and maintenance of neurons in central cholinergic neurons.^{2,3)} Considerable evidence from animal studies suggests that NGF may be useful in halting and slowing the progression of AD-related cholinergic basal forebrain atrophy.⁴⁾ Administration of NGF was reported to attenuate the degeneration of neurons and improve cognitive behavior in animals by stimulating central cholinergic neurons that are known to die during the development of AD.⁵⁾ A clinical trial using intracranial infusion of NGF was reported to improve the patients' verbal episodic memory.⁶⁾ Thus an attempt to counteract the degeneration of cholinergic neurons with NGF may be a reasonable approach to treat AD.

The amyloid β -protein (A β) is the principal component of the neuritic plaques characteristic of AD. The A β peptide is generated from a larger precursor, the amyloid precursor protein, by two sequential enzymatic activities called β -secretase and γ -secretase. This peptide has been shown to induce cytotoxic actions toward neuronal cells both *in vivo* and *in vitro*. A growing body of evidence suggests that the brains of AD patients are under increased oxidative stress.⁷⁾ This may play an important role in neuronal degeneration and death. Since free radicals in part mediate the neurotoxic effect of A β , the removal of free radicals or prevention of their formation may be beneficial in the treatment of AD.

Aster scaber (Compositae) is widespread in Korea. Aster species have been used for the treatment of bruises, headache, and dizziness. Some triterpene glycosides and volatile compounds were isolated from the *A. scaber*.^{8–10)} However, no precise correlation has been made between particular constituents of these herbs and observed pharmaco-

logical activity. Recently, four quinic acid derivatives were identified by Kwon *et al.*¹¹⁾

In order to provide a pharmacological basis for the neuroprotective actions of quinic acid derivatives isolated from *A. scaber*, we evaluated the effect of these quinic acid derivatives on A β -induced toxicity and their potentiating activity in the neurite outgrowth of PC 12 cells. We also tried to determine whether quinic acids are efficacious in the prevention and/or treatment of neurodegenerative diseases associated with A β toxicity and NGF deprivation.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin solution, and trypsin-EDTA solution were obtained from Gibco RBL (Grand Island, NY, U.S.A.). Fetal calf serum was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). Amyloid- β protein (A $\beta_{25–35}$) was from Bachem (Torrance, CA, U.S.A.). Poly-D-lysine, NGF, Hank's balance salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), and all the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Quinic acids were obtained from Professor Kang Ro Lee, SungKyunKwan University, Suwon, Korea.

Cell Culture PC12 cell lines were grown in high-glucose DMEM supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal calf serum, and 1% (v/v) penicillin/streptomycin at 37 °C in 5% CO₂ and 95% air in a humidified incubator. The cells were grown on culture dishes precoated with poly-D-lysine (50 μ g/ml in sterile water) overnight.

A β -Induced PC12 Cell Toxicity After cells were exposed to low-serum containing media (1% horse serum and 1% fetal bovine serum) for 16 h, 10 μ M of A $\beta_{25–35}$ (diluted in phosphate buffered saline [PBS], 0.05% as a final concentration) was added to the culture media and incubated for 48 h.

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Cells were treated with 5 μM (1–4) of quinic acid derivatives for 2 h before $A\beta_{25-35}$ treatment. To assess cell viability, $A\beta$ -containing media was aspirated the plates and each well was washed twice with PBS. The viability of the remaining PC12 cells was determined by the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Kwon *et al.*¹¹⁾ The net difference A570–A650 was used to express the viability of the cells. Lactate dehydrogenase (LDH) release assay was also carried out as described by Kim *et al.*¹²⁾

Neurite Measurement The neurotrophic effects of quinic acid derivatives (1–4) and NGF were evaluated by microscopically monitoring their potency in inducing neurite outgrowth of PC12 cells. Compounds (1 μM) were added to the media 24 h after PC12 cells (5×10^4 cells/well) were seeded on 6-well plates (Corning, New York, U.S.A.). The neurite outgrowth in PC12 cells was monitored under a microscope for the indicated times. The medium containing each compound was changed every 2 d. After 48 h, randomly selected fields were photographed using a camera attached to an Olympus optical inverted phase-contrast microscope (model CK-2; $\times 100$ magnification). Neurite extension was evaluated with lengths equivalent to one diameter of the cell body. More than 10 cells were examined for the measurement of neurite length at each data point.

Neuronal Cell Viability Following Withdrawal of NGF and Compounds PC12 cells were treated with compounds or NGF for 24 h after cells (1×10^6 cells/well) were seeded on 6-well plates. Cells were incubated for 48 h, washed with fresh medium to withdraw NGF and compounds, and subjected to an additional 12 h incubation. MTT and LDH release assays were carried out to determine the effects of withdrawal of NGF or compounds on neuronal cell viability.^{12,13)}

Statistical Analysis All data are expressed as the mean \pm S.D. The evaluation of statistical significance was determined by one-way ANOVA.

RESULTS

The protective effects of four quinic acid derivatives from *A. scaber* on $A\beta$ -induced PC12 cell toxicities were examined. A new quinic acid called (–)3,5-dicaffeoyl-mucoquinic acid (2) and three known compounds, 3,5-dicaffeoyl quinic acid (1), 4,5-dicaffeoyl quinic acid (3), and 5-caffeoyl quinic acid (chlorogenic acid, 4) were isolated and identified by Kwon *et al.* (Fig. 1).¹¹⁾

$A\beta$ -sensitive PC12 cells were used to screen the neuroprotective effects of quinic acids. As shown in Fig. 2, PC12 cells exhibited 51.8% viability when exposed to $A\beta_{25-35}$ 10 μM under our experimental conditions. However, pretreatment with quinic acid derivatives significantly diminished cell toxicity induced by $A\beta_{25-35}$. Among quinic acid derivatives, compound 1 showed the highest protective effect with 90.0% or 75% cell viability as measured by MTT (Fig. 2A) or LDH assay (Fig. 2B).

The neurite outgrowth activities of the four quinic acid derivatives from *A. scaber* were examined in PC12 cells by measuring the length of neurites (Fig. 3). All four compounds exhibited neurite outgrowth activity similar to or better than that of NGF. Compound 2 was the most potent in neurite outgrowth activity among them. NGF (50 ng/ml) or

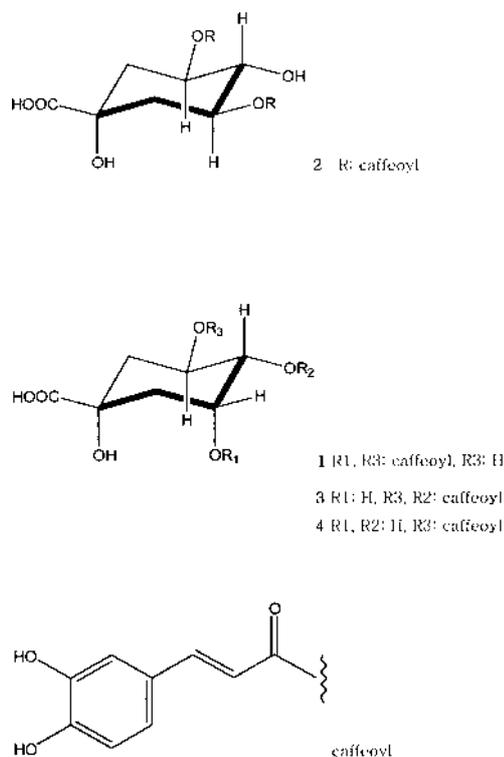


Fig. 1. Structures of Compounds 1–4

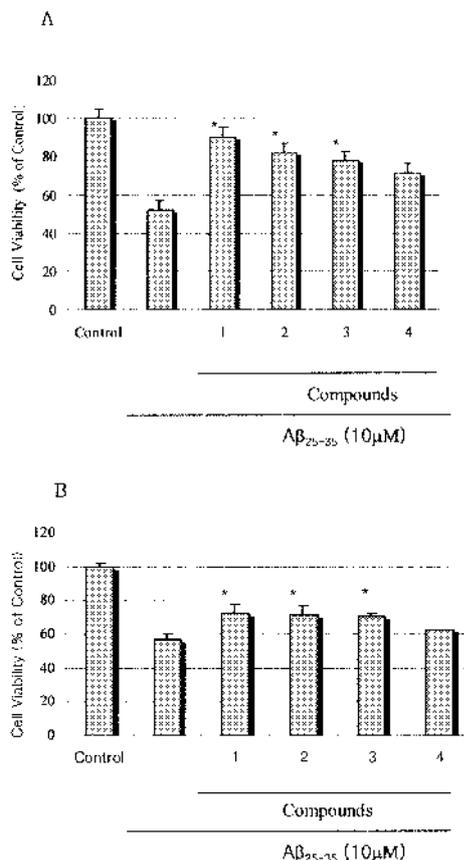


Fig. 2. The Effects of Compounds 1–4 on $A\beta$ -Induced PC12 Cell Damage

PC12 cells were incubated with $A\beta$ 10 μM for 48 h prior to pre-incubation of cells with quinic acid derivatives (1–4) 5 μM for 2 h. MTT (A) and LDH (B) assay were carried out to assess cell viability. These results are expressed as the mean \pm S.D. of an assay conducted three separate times with triplicate determinations. * $p < 0.01$ vs. $A\beta_{25-35}$ group.

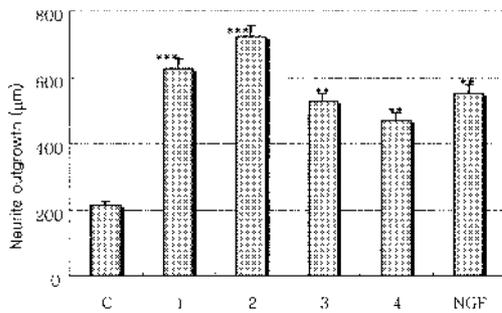


Fig. 3. The Effect of Compounds (1 µM each) and NGF (50 ng/ml) on Neurite Outgrowth

PC12 cells in 6-well plates were treated with compounds 1–4 and NGF, and neurite outgrowth was measured under a microscope at 6 d post-treatment. Fresh medium with compounds or NGF was changed every 2 d. Randomly selected fields were photographed with a camera attached to light microscope. Each value represents the mean ± S.D. (n=30). ** Significantly different from NGF value at the level of $p < 0.01$. *** Significantly different from NGF value at the level of $p < 0.001$.

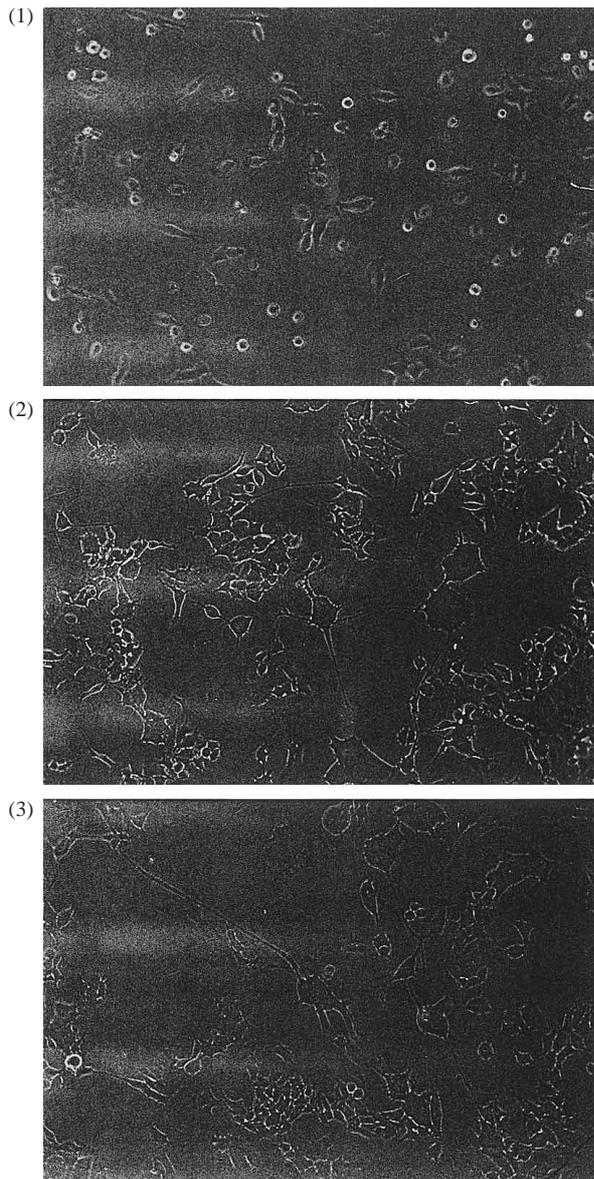


Fig. 4. Microscopic Observations of Neurite Outgrowth in PC12 Cells
PC12 cells were treated with compound 2 or NGF (100). (1) Control; (2) NGF 50 ng/ml; (3) 1 µM compound 2.

compound 2 (1 µM) was added to the culture medium of PC12 cells and neurite outgrowth was observed under the microscope after 6 d (Fig. 4). There was a significant increase in neurite outgrowth after the addition of compound 2. In addition, neurite outgrowth activity of compound 2 was investigated under different treatment conditions (Fig. 5). Compound 2 significantly increased the neurite extension of PC12 cells in a time-dependent manner. However, compound 2 did not show any significant dose-dependent increase in the ratio of cells to neurites. The neurite outgrowth activity of compound 2 at 1 to 10 µM was more potent than that of NGF at the concentration of 50 ng/ml.

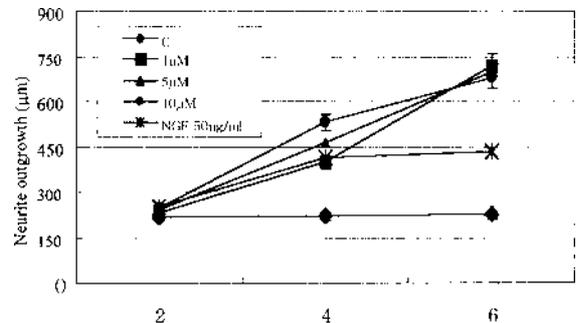


Fig. 5. Neurite Outgrowth of PC12 Cells at Various Concentrations of Compound 2

Compound 2 was added to PC12 cells in time- and concentration-dependent manners. The outgrowth of neurites from PC12 cells treated with compound 2 (1, 5, and 10 µM) or NGF (50 ng/ml) was measured under a microscope every 2 d after addition.

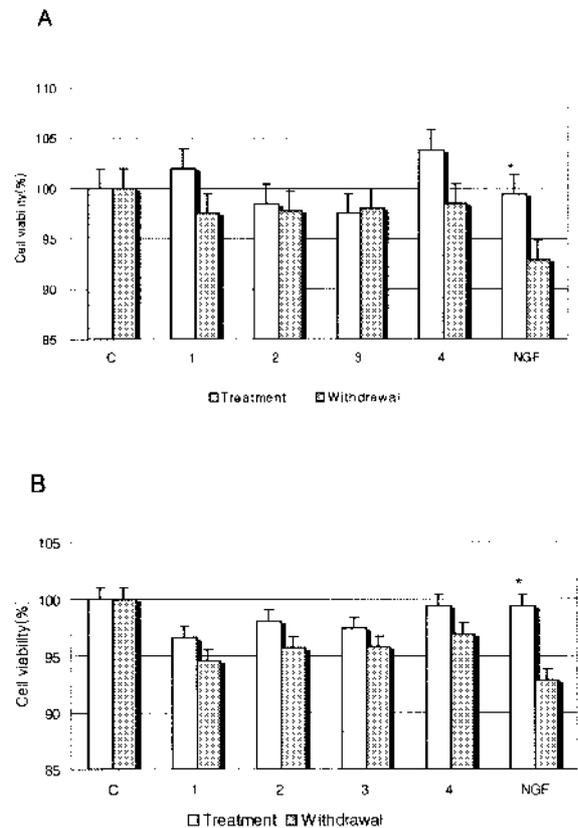


Fig. 6. Effect of Quinic Acids on Neuronal Cell Survival Following Withdrawal of Compounds and NGF

Cells were incubated with compounds and NGF for 2 d, washed twice with HBSS, and then further incubated for 12 h. Cell viability was determined by LDH release assay (A) and MTT assay (B).

The withdrawal of NGF from terminally differentiated PC12 cell culture has been shown to cause apoptotic death in terminally differentiated cells.¹⁴⁾ To determine whether quinic acids could be substituted for NGF, we further investigated neuronal cell viability after withdrawal of quinic acids (Fig. 6). Cells were treated with compounds or NGF for 2 d and washed with fresh medium. After incubation for 12 h, neuronal cell viability was assessed by MTT and LDH assays. Unlike NGF, withdrawal of quinic acids did not show any significant decrease in the cell viability.

DISCUSSION

We screened the neuroprotective activity of quinic acids isolated from *A. scaber*. Our present experiments using an *in vitro* model of cellular injury induced by amyloid A β demonstrated the neuroprotective effects of quinic acids. We also found that quinic acids are capable of promoting neurite outgrowth. Treatment of PC12 cells with quinic acids instead of NGF induced neuronal differentiation. These results suggest the possibility using quinic acid derivatives as NGF substitutes in the treatment of AD and other neurodegenerative diseases.

The degree of neuroprotection and neurite outgrowth activity differed among the compound tested. For example, compound **1** exhibited the highest neuroprotective effect against A β -induced toxicity, while compound **2** was the most potent promoter of neurite outgrowth activity. The results imply that the quinic acids described here exert different degrees of neuroprotective or neurotropic effect depending on their chemical structure. Since the cytotoxic action of A β on neurons results from free radical damage to susceptible cells¹⁵⁾ and neuroprotection of NGF against free radical insult has been well documented,¹⁶⁾ quinic acids might be involved in resistance against oxidative damage.

NGF can be used for treatment only when directly injected into the brain. Since it is a large molecular weight polypeptide, it does not cross the blood-brain barrier and is easily metabolized by peptidases when administered peripherally. Therefore extensive research is focusing on natural NGF substitutes for the development of new drug to treat illnesses such as dementia. Recently, a novel low molecular weight

neurotrophic factor has been isolated from human astrocytoma cells.¹⁷⁾ Our results suggest quinic acids be a good candidate for NGF substitutes.

In conclusion, the data presented here are the first to demonstrate the neuroprotective and neurotrophic effect of four quinic acids. These quinic acids may be available as a lead compound for anti-AD agents that can offer therapeutic protection as well as treatment of AD. However, further studies are required to determine the mechanism by which quinic acids inhibit A β toxicity and induce neurite extension in PC12 cells. The neuroprotective effects of quinic acids *in vivo* are currently under investigation.

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