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The Neuroprotective and Neurotrophic Effects of Korean Gardenia (*Gardenia jasminoides* Ellis) in PC12h Cells

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Abstract We examined the neuroprotective and neurotrophic effects of genipin fractionated from gardenia (*Gardenia jasminoides* Ellis) originating from Korea. The neurotrophic effects of the genipin containing fraction was evaluated by microscopically monitoring its potency to induce neurite outgrowth in PC12h cells. The genipin containing fraction from Korean gardenia promoted neurite outgrowth in PC12h cells in this study, similar to previously reported effects by Wako Chemical, Japan. When cells were treated with the genipin containing fraction prior to β-amyloid peptide treatment (active domain of A peptide 25-35 treated), toxicity was significantly diminished ($p<0.01$). These results suggest that genipin prepared from Korean gardenia might potentially be used as a precautionary agent in neurodegenerative disease, such as Alzheimer's disease, etc.

Keywords: *Gardenia jasminoides* Ellis, genipin, PC12h cell, neuroprotective effect, neurotrophic effect

Introduction

With the increase in human life span and the decrease in cognitive functions of elderly individuals with Alzheimer's disease (AD) related dementia, AD has become a major health problem. There are currently more than 4 million AD patients in the USA, with AD having the 4th highest mortality rate. Patients with AD are expected to increase by 15 million in the next ten years. In Korea, the growing senior population now accounts for 7 percent of the population, with the prevalence of AD growing accordingly (1).

As the seriousness of AD grows, much global effort has been made to find compounds with preventative or therapeutic effects toward AD. Through these efforts, nerve growth factor (NGF), a product having a therapeutic effect on AD, was characterized.

NGF is an essential protein for supporting the growth and maintenance of peripheral sympathetic neurons as well as briefly facilitating the development of some sensory neurons during early development, and has also been reported as a potentially useful treatment for AD (2, 3). However, current clinical trials with NGF have encountered a number of problems, such as delivery, a short half-life, and poor penetration through the blood brain barrier. Therefore, new compounds with neurotrophic activity that have the potential to treat such diseases need to be developed (4-7).

β-Amyloid protein, a 40-42 amino acid peptide proteolytically derived from a larger β-amyloid precursor protein, accumulates as insoluble extracellular deposits in the senile plaques of AD patients. Several lines of evidence support a potentially important role for β-amyloid peptide in the pathogenesis of AD. The molecular mechanisms of β-amyloid peptide cytotoxicity are also closely involved in the generation of oxidative stress (8).

The fruit of gardenia (*Gardenia jasminoides* Ellis) is an oriental folk medicine included in traditional formulations. Its folkloric use has been for the treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders, and hypertension (9). Its pigments are also used as food colorants in oriental countries. The fruit of gardenia contains iridoid glycosides such as geniposide, geniposidic acid, genipin, and 10-acetylgeniposide as major compounds (10). Their pharmacological actions, such as protection from oxidative damage, cytotoxic effects, anti-inflammatory, and fibrolytic activity have already been elucidated (9-11). Genipin has been shown to have NGF-like activity (7) and to protect hippocampal neurons from β-amyloid protein toxicity (12).

Although many studies, such as those cited above, have been performed on the fruit of gardenia, there have been no studies examining the fruit of gardenia as a dietary supplement.

Therefore, in this study, the genipin containing fraction from Korean gardenia fruit was isolated to examine its potential as a functional food material. In order to separate the genipin containing fraction, a new method was set up using gel filtration chromatography. Previous methods for separating genipin have encountered problems in terms of food safety due to the use of organic solvent.

To confirm its usefulness as a precautionary agent in AD, the genipin containing fraction was tested for NGF-like activity and protection from neurotoxicity induced by β-amyloid protein in PC12h cells.

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Materials and Methods

Materials Genipin was purchased from Wako (Osaka, Japan) and dissolved in distilled water. NGF (7S, isolated from mouse submandibular gland) was obtained from Sigma (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. Dulbecco's modified minimum essential medium (DMEM) was purchased from Nissui (Tokyo, Japan), and horse serum, fetal calf serum, and kanamycin from Gibco BRL (Grand Island, NY, USA). Aβ25-35 was obtained from Sigma and β-glucosidase (Isorase) from the National Enzyme Company (Forsyth, MO, USA). All other chemicals were obtained from Sigma.

Extract of Korean gardenia fruit The fruit of gardenia was collected at Geoje-do, Gyeongnam, Korea, in November 2003. The dried fruit (100 g) was ground into powder and extracted with thirty volumes of distilled water at 100°C for 30 min. The liquid residue was filtered using Whatman paper No. 1 and the filtrate used to prepare the genipin containing fraction. After lyophilizing the filtrate, approximately 18.9 g of the fruit was obtained.

Preparation of the genipin containing fraction of gardenia fruit The genipin containing fraction was isolated from 100 mL of the gardenia fruit water extract (7 mg/mL) using enzymatic digestion. The gardenia fruit water extract was treated with 0.25% (v/v, mL-enzyme/mL-substrate) β-glucosidase/Isorase for 5 hr at 37°C to convert the geniposide contained in the extract to genipin. After digestion, the sample was immediately heated for 1 min in a boiling water bath, followed by centrifugation at 10,956×g for 10 min and filtration through a syringe filter (0.45 μm) to remove any denatured enzyme. The filtrate was lyophilized and subjected to gel filtration chromatography to separate the genipin containing fraction. The lyophilized filtrate (10 mg/mL in distilled water) was boiled for 1 min, applied to a Sephadex G-25 column (2× 110 cm) and eluted with distilled water at a flow rate of 1 mL/min, which produced three fractions as indicated by the gel chromatographic elution profile monitored at 238 nm absorbance.

Analysis of genipin The three fractions obtained from the gel filtration chromatography were analyzed using an HPLC system (Waters, Dublin, Ireland) to detect the genipin containing fraction. The detection of genipin was carried out using a Waters spherisorb ODS1 (4.6×25 mm, 5 μm, Ireland) with an 85%(v/v) acetonitrile mobile phase at a flow rate of 1 mL/min, and an oven temperature set at 40°C. Genipin was detected at a wavelength of 238 nm, and identified according to retention time and the UV spectra of a genipin standard.

Cell culture PC12h cells, a subclone of PC12 cells isolated by Dr. Hatanaka (13) and kindly donated by Dr. Chiba (Hokuriku University, Japan), were grown in DMEM medium supplemented with 5%(v/v) horse serum and 5%(v/v) fetal bovine serum in a 100 mm Petri dish under 10% CO_2 at 37°C.

PC12h cells also undergo certain NGF-responsive cellular events, including neurite outgrowth and the induction of tyrosine hydroxylase activity. PC12h cells are much more sensitive to NGF than PC12 cells (14).

Assay for neuritogenic activity in PC12h cells For morphological studies, cells were plated in 35 mm culture dishes coated with collagen (Type I; Sigma) at a density of 5×10^4 cells in 2 mL medium per dish. After 24 hr of culture, the medium was replaced with serum-free DMEM/Ham's F12 (1:1) medium supplemented with sodium selenate, transferrin, insulin, progesterone, and the vehicle or test compound. After 48 hr, the neuritogenic activity was evaluated by measuring the length of the longest neurite of individual cells using an image processor system (Leica Qwin, Germany) attached to a phase-contrast microscope. One hundred cells in at least 10 random fields in two culture dishes were measured, with the values averaged.

β-Amyloid protein induced toxicity in PC12h cells Cells were plated in 96 well plates coated with collagen (Type I; Sigma) at a density of 1×10^4 cells per 0.1 mL growth medium. After 24 hr of culture, the cells were exposed to low-serum containing media (0.5% horse serum and 0.5% fetal bovine serum) for 24 hr, either with or without the test compound (genipin containing fraction). The test compound was used at two concentrations; 1 and 2 μg/mL. Five μM of β-amyloid peptide 25-35 (diluted in phosphate buffered saline) was added to the culture media, which was then incubated for 60 hr. The viability of PC12h cells was determined using the 3-[4, 5-dimethyl-thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. To assess cell viability, β-amyloid peptide containing media was aspirated, and the PC12h cells were incubated with MTT (1 mg/mL) for 4 hr at 37°C. The MTT solution was then aspirated, and the formazan (MTT reduction product) was dissolved in 0.1 mL of dimethyl sulfoxide and quantified spectrophotometrically at 570 nm (reference 650 nm). The viability of the living cells, from three independent experiments, was expressed as a percentage of the control.

Statistical analysis The data were analyzed using analysis of variance (ANOVA) with the SAS statistical program and differences among the means were compared using Ducan's multiple range tests. All results were expressed as the mean±SE of triplicate determination, with each experiment repeated 3 times.

Results and Discussion

Preparation of genipin fraction To obtain the genipin containing fraction from the Isorase digested gardenia fruit water extract, the extract was applied to a Sephadex G-25 column for gel chromatography. The hydrolysate of the extract was separated into three fractions (Fig. 1). Each fraction was collected and analyzed for genipin content using HPLC. The third fraction (Fraction III) was observed to contain only genipin (Fig. 2). Conversely, the second fraction also contained some geniposide. Through this process, the total yield of the genipin containing fraction was determined to be about 2.52%. Only the genipin

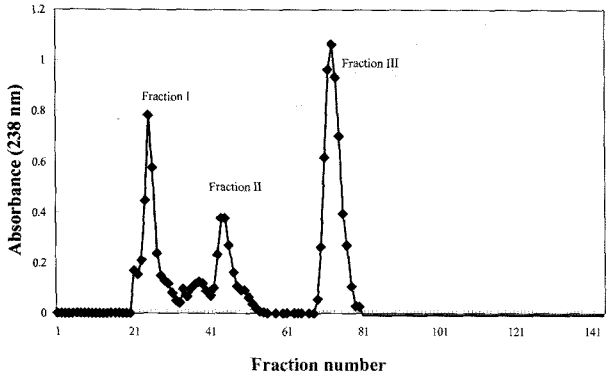

Fig. 1. Sephadex G-25 gel filtration chromatogram of the extract of Korean gardenia digested by Isolase.

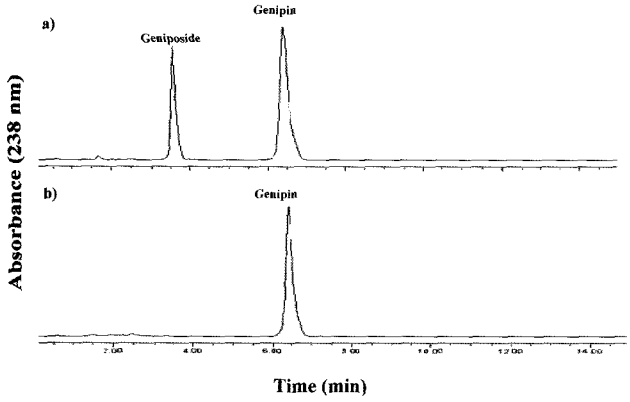

Fig. 2. HPLC chromatograms of standards (a) and Sephadex G-25 fraction III of the extract of Korean gardenia (b) digested by Isolase.

containing fraction (Fraction III) was used in subsequent experiments.

Neuritogenic activity of genipin fraction Nerve growth factor (NGF) has been reported to induce morphological changes in PC12h cells by improving neurite outgrowth (7). NGF was shown to improve the differentiation of PC12h cells in a dose-dependent manner, therefore longer neurite outgrowth was observed as the treatment dose increased.

After treatment of PC12h cells with the test compounds

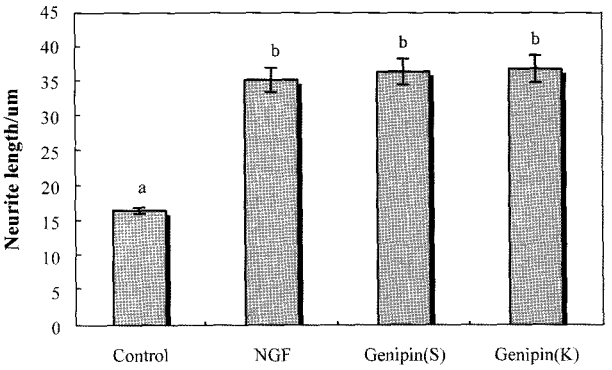

Fig. 4. Effects of the genipin containing fraction on PC12h neuritogenesis. n=100. Mean±SE. Means with different letters are significantly different ($p<0.05$). Control, negative vehicle; NGF, 0.1 μg/mL; Genipin (S), Genipin (Standard, Wako Chemical, Japan) 5 μg/mL; Genipin (K), (from Korean gardenia) 5 μg/mL.

at a concentration of 5 μg/mL for 48 hr, the longest neurite length of each cell was measured. The average neurite length of cells treated with the genipin containing fraction was 39.59±2.34 (μm), which was a significant increase in differentiation ($p<0.01$) compared to the neurite length of the control (Fig. 4). When PC12h cells were treated with the genipin containing fraction form Korean gardenia fruit, the observed neurite outgrowth was similar to those reported by Yamazaki *et al.* (7) for NGF and genipin. The elucidated mechanism of genipin-induced neuritogenesis in PC12h cells is through the NO/cGMP/PKG/ERK pathway (15, 16).

Prevention of β-amyloid peptide-induced neurotoxicity by the genipin fraction When PC12h cells were exposed to β-amyloid peptide 25-35 for 60 hr, the MTT reducing activity was significantly inhibited (Fig. 5). Pretreatment of cells with 1-2 μg/mL of the genipin fraction for 1 day significantly reduced the degree of β-amyloid peptide-induced inhibition of MTT reduction compared to the non treated control.

According to a previous report, genipin is capable of activating the mitogen-activated protein kinase cascade, which is an important signaling pathway implicated in the prevention of apoptosis (17). Yamamoto *et al.* (18) reported the suppression of Fas-induced apoptosis by genipin in cultured mouse liver cells via both the inhibition

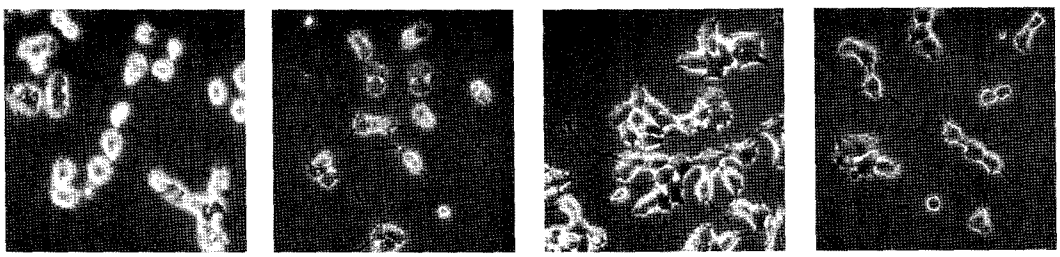

Fig. 3. Effects of the genipin containing fraction and/or NGF on the morphology of PC12h cells (×200). a) control (negative vehicle); b) NGF 0.1 μg/mL; c) Genipin (Standard, Wako Chemical, Japan) 5 μg/mL; d) Genipin containing fraction (from Korean gardenia) 5 μg/mL.

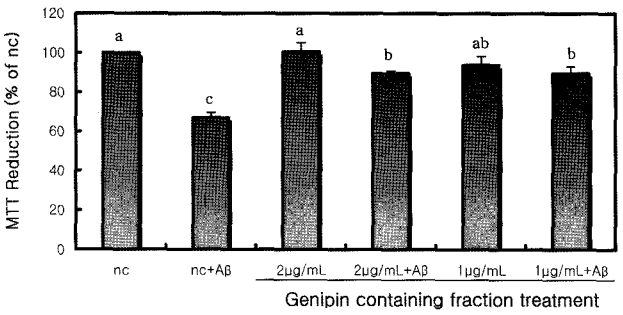

Fig. 5. Protective effects of the genipin containing fraction prepared from Korean gardenia on Aβ protein-induced cytotoxicity in PC12h cells. Data are the means±SE of three separate experiments performed in triplicate. Means with different letters are significantly different ($p<0.01$)

of caspase activation and the reduction of mitochondrial membrane potential.

These observations suggest a possible protective effect of genipin against β-amyloid peptide induced cytotoxicity, but further study is necessary to elucidate the precise mechanism.

In this study, the genipin containing fraction obtained from the Korean fruit of gardenia was shown to have a similar neurotrophic effects to that of commercially available genipin from Wako Chemicals, Japan. In addition, the genipin containing fraction had a satisfactory protective effect against neurotoxicity induced by β-amyloid peptide.

These results indicate that the Korean gardenia fruit might have potential as a dietary supplement for the prevention and treatment of Alzheimer's disease.

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