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Isolation and Identification of an Autophagy-inducing Compound from Raphani Semen

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Abstract – The autophagy-lysosomal pathway is an important protein degradation system, and its dysfunction has been implicated in a number of neurodegenerative diseases, including Parkinson's disease. Raphani Semen, one of the herbs of Yeoldahanso-tang (YH), has neuroprotective effects via the autophagy pathway. The activity-guided method was used to isolate and identify the components of Raphani Semen. In this experiment, the total extract of Raphani Semen was partitioned to n-butanol, methylene chloride, and water fractions. Flow cytometry data showed that only the water fraction showed autophagy-inducing activity *in vitro*. Compounds **1** and **2** were isolated from this water fraction by preparative HPLC separation. The structures of compounds **1** and **2** were identified as stachyose and raffinose, respectively, by the analysis of various spectral data (^{1}H NMR, ^{13}C NMR, and MS) and comparisons with standard stachyose and raffinose. Of these two compounds, raffinose showed autophagy-inducing activity in PC12 cells through the mTOR pathway.
Keywords – Autophagy, Raffinose, Raphani semen, *Raphanus sativa* L., Cruciferae

Introduction

Parkinson's disease (PD) is a degenerative disorder of the central nervous system. While the initial cause is undetermined, PD results from the death of the dopaminergic neurons of the substantia nigra, which is a region of the midbrain. Insufficient protein degradation, caused by various factors, may lead to the dopaminergic neuronal cell death that is related to PD and other neurodegenerative diseases (Cho, 2012).

The autophagy-lysosome pathway (ALP) is a mechanism that cleans the misfolded proteins and organelles in the cell (Ciechanover, 2005; Rubinsztein, 2006; Kim *et al*., 2012). "Autophagy", literally meaning "self-eating", describes a catabolic process in which cell constituents such as organelles and proteins are delivered to the lysosomal compartment for degradation (Nedlsky *et al*., 2008). Autophagy plays an important role in Parkinson's disease. Microtubule-associated protein light chain 3 (LC3), a mammalian homologue of autophagy-related gene 8 (Atg8) in yeast, is recruited to the autophagosome membrane during autophagy and is considered a specific marker of autophagy (Kabeya *et al*., 2000).

Rapamycin, which is a lipophilic and macrolide antibiotic, induces autophagy by inactivating the protein mammalian target of rapamycin (mTOR), and as such, it is an autophagy enhancer (Berger *et al*., 2006). Several studies have shown that rapamycin, acting through the mTOR pathway, is neuroprotective in various neurological diseases (Erlich *et al*., 2007; Parker *et al*., 2000; Wu *et al*., 2008; Zemke *et al*., 2007). In the present study, we used rapamycin (200 nM) as a positive control in experiments to examine autophagy induction (Pan *et al*., 2008).

A previous study showed that Yeoldahanso-tang (YH), a Chinese herbal medicine, had neuroprotective effects via autophagy enhancement in Parkinsonian model systems, both *in vivo* and *in vitro* (Bae *et al*., 2011). Raphani Semen is one of the herbs of YH, and it was chosen for further study because its extract showed autophagy enhancement in PC12 cells.

Raphani Semen belongs to the Cruciferae, meaning "cross-bearing," family of flowering plants (Angiosperms), which contains over 330 genera and approximately 3,700 species. The largest genera are *Draba* (365 species), *Cardamine* (200 species, although the definition of this genus is controversial), *Erysimum* (225 species), *Lepidium*

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(230 species) and *Alyssum* (195 species). The family contains well-known species such as *Brassica oleracea* (broccoli, cabbage, cauliflower, etc.), *Brassica rapa* (turnip, Chinese cabbage, etc.), *Brassica napus* (rapeseed, etc.), *Raphanus sativus* (common radish), *Armoracia rusticana* (horseradish), *Matthiola* (stock), *Arabidopsis thaliana* (model organism) and many others. The family is cosmopolitan but is concentrated in the northern temperate regions and reaches its maximum diversity around the Mediterranean area.

Experimental

Plant material – In this study, Raphani Semen (RS) was provided by the Botany and Drug Department of the Oriental Hospital of Daejeon University (Daejeon, Korea), according to the Korean herbal pharmacopoeia (The Korea Food and Drug Administration, 2002).

Chemicals and instruments – Monodansylcadaverine (MDC), Raffinose, stachyose and rapamycin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). NMR experiments were performed on a Varian NMR System (Unity Plus 500 MHz). Low resolution ESI-MS (electrospray ionization mass spectrometry) was measured on an Agilent Technologies 1200 series HPLC system using a 6120 Quadrupole. An YL9100 HPLC (YL instrument Co., Ltd, Anyang, South Korea) system and YMC-ODS-AQ column (150 × 1.6 mL, 5 μm YMC, Tokyo, Japan) were used for analysis. Semi-preparative HPLC 321 Pumps (Gilson Inc, Middleton, WI, USA) and Delta-Pak C18 column (300 × 30.00 mm, 15 microns, Waters, Tokyo, Japan) were used to isolate compounds. In bioassay experiments, microplate reader (BIO-TEKR, Dower Wave XS, Winooski, VT, USA) was used to measure cell viability. Western immunoblotting assay used LAS-4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan). Flow cytometry (BD FACSCalibur, San Jose, CA, USA) was used to analyze MDC staining cells.

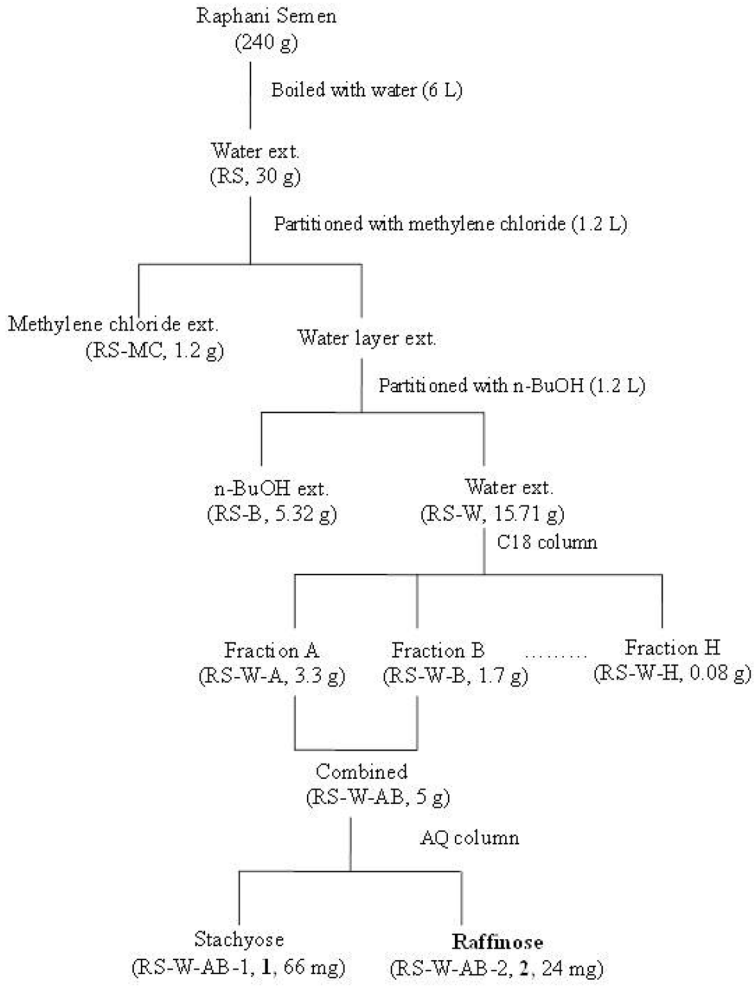

Fig. 1. Extraction scheme for the isolation of active compound from Raphani Semen.

Extraction and isolation – Dried Raphani Semen, 240 g, was immersed in a total volume of distilled water (6 L) and boiled for 1 h. The water extract was vacuum-concentrated at 40 ºC to obtain the total water extract (30 g, RS). The concentrated total extract was partitioned with methylene chloride (1.2 L) to yield a methylene chloride-soluble residue (1.2 g, RS-MC). Then, the water layer was partitioned again with n-butanol (1.2 L) to yield a n-butanol-soluble residue (5.3 g, RS-B) and a water-soluble extract (15.7 g, RS-W). A summary of the present partition scheme is shown in (Fig. 1). The water extract (RS-W) was then fractionated by preparative HPLC using a Delta-Pak C18 and a CH_3CN-H_2O (0.05% TFA, 10% to 100%, 12 mL/min) solvent system to yield fractions A through H. Bioassay results demonstrated that fractions A (RS-W-A) and B (RS-W-B) could induce autophagy in PC12 cells. Fractions A and B were combined (RS-W-AB) and further purified by repeated HPLC using a YMC-ODS-AQ column (250 × 10.00 mm, 10 μm, YMC, Tokyo, Japan) with a water solvent system yielding compound **1** (RS-W-AB-1) and compound **2** (RS-W-AB-2).

Compound 1 – Compound **1** exhibited the following features: ^{1}H NMR (D_2O, 500 MHz) δ 4.97 (2H, dd, J = 3.5, 2.1 Hz), 3.79 (1H, m), 3.82 (1H, m), 3.96 (1H, m), 3.98 (1H, m), 3.72 (2H, m), 3.81 (1H, m), 3.89 (1H, m), 4.02 (1H, m), 4.12 (1H, dd, J = 7.5, 4.8 Hz), 3.70 (1H, m), 3.85 (1H, m), 5.40 (1H, d, J = 3.9 Hz), 3.55 (1H, m), 3.73 (1H, m), 3.51 (1H, m), 4.04 (1H, m), 3.65 (1H, m), 4.03 (1H, m), 3.65 (2H, m), 4.20 (1H, d, J = 8.8 Hz), 4.04 (1H, m), 3.87 (1H, m), 3.75 (1H, m), 3.81 (1H, m); ^{13}C NMR (D_2O, 500 MHz) δ 98.00, 68.11, 69.31, 69.04, 70.73, 60.94, 98.00, 68.01, 69.11, 69.11, 68.58, 66.15, 91.93, 70.72, 72.45, 69.20, 71.01, 65.56, 61.19, 103.67, 76.09, 73.77, 81.16, 62.10.

Compound 2 – Compound **2** exhibited the following features: ^{1}H NMR (D_2O, 500 MHz) δ 4.97 (1H, d, J = 3.8 Hz), 3.80 (1H, m), 3.87 (1H, m), 3.98 (1H, d, J = 3.2 Hz), 3.93 (1H, dd, J = 12.1, 5.8 Hz), 3.72 (2H, m), 5.40 (1H, d, J = 3.8 Hz), 3.55 (1H, m), 3.74 (1H, m), 3.52 (1H, m), 4.03 (1H, m), 4.02 (1H, m), 3.67 (1H, m), 3.65 (2H, m), 4.20 (1H, dd, J = 8.8, 0.4 Hz), 4.04 (1H, m), 3.87 (1H, m), 3.82 (1H, ddd, J = 10.2, 6.7, 3.7 Hz), 3.76 (1H, m); ^{13}C NMR (D_2O, 500 MHz) δ 98.31, 68.33, 69.27, 68.90, 70.88, 60.90, 91.88, 70.73, 72.35, 69.06, 71.09, 65.61, 61.22, 103.58, 76, 73.81, 81.12, 62.32.

Cell culture – PC12 cells (pheochromocytoma cells, which can be differentiated by nerve growth factor stimulation) were obtained from the RIKEN BRC Cell Bank (catalog number RCB0009) (Tsukuba, Ibaraki, Japan). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Hyclone) and penicillin (100 U/mL), and kept at 37 ºC in a humidified incubator containing 95% air and 5% CO_2. The PC12 cells were seeded at densities appropriate to each experimental scale.

Sample treatment – RS fractions were dissolved in DMSO at a concentration of 100 mg/mL, and compounds isolated from RS were dissolved in DMSO at a concentration of 30 mM as stock solutions. The stock solutions were diluted in treatment medium at the desired final concentrations prior to use.

Cell viability – Cell viability was measured using the Cell Counting Kit-8 (CCK-8, WST-8, Kamimashikigun, Kumamoto, Japan). Briefly, the cells (1.5×10^4 per well) were seeded into 96-well plates and incubated for 24 h. After treatment with the different drugs for the indicated time periods, the medium (90 μL) was incubated with 10 μL of WST-8 solution for 1 h at 37 ºC to form water dissoluble formazan. Absorbance was read at 450 nm on a microplate reader, and cell viability was expressed as the percentage of viable cells relative to untreated control cells.

Western immunoblotting assay – Cells were exposed to different fractions or compounds for 24 h, harvested by centrifugation at 2500 rpm for 5 min, and washed in PBS (PH 7.2). The pellets were incubated in RIPA buffer (CST, Boston, MA, USA) and supplemented with protease inhibitor cocktail (Roche, Penzberg, Germany) according to the manufacturer's instructions. Protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Then, total cell lysates (18 μg of protein) were separated by SDS-PAGE (15%) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk; primary antibodies against LC3B-I and LC3B-II or mTOR (1 : 1000 dilution; Cell Signaling) were then added and incubated for 2 h at room temperature. This was followed by incubation with the corresponding anti-rabbit IgG, HRP-linked secondary antibodies (Cell Signaling) at room temperature for 1 h. Extensive washes were preformed between each step. The bound antibodies were visualized using ECL advance western blotting detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Imaging and densitometry analyses were performed using LAS-4000 Luminescent Image. GAPDH was also immunoblotted to demonstrate equal protein loading.

Flow cytometry – Autophagy induction was detected with an Annexin-V-FLUOS Staining Kit (Roche, Penzberg,

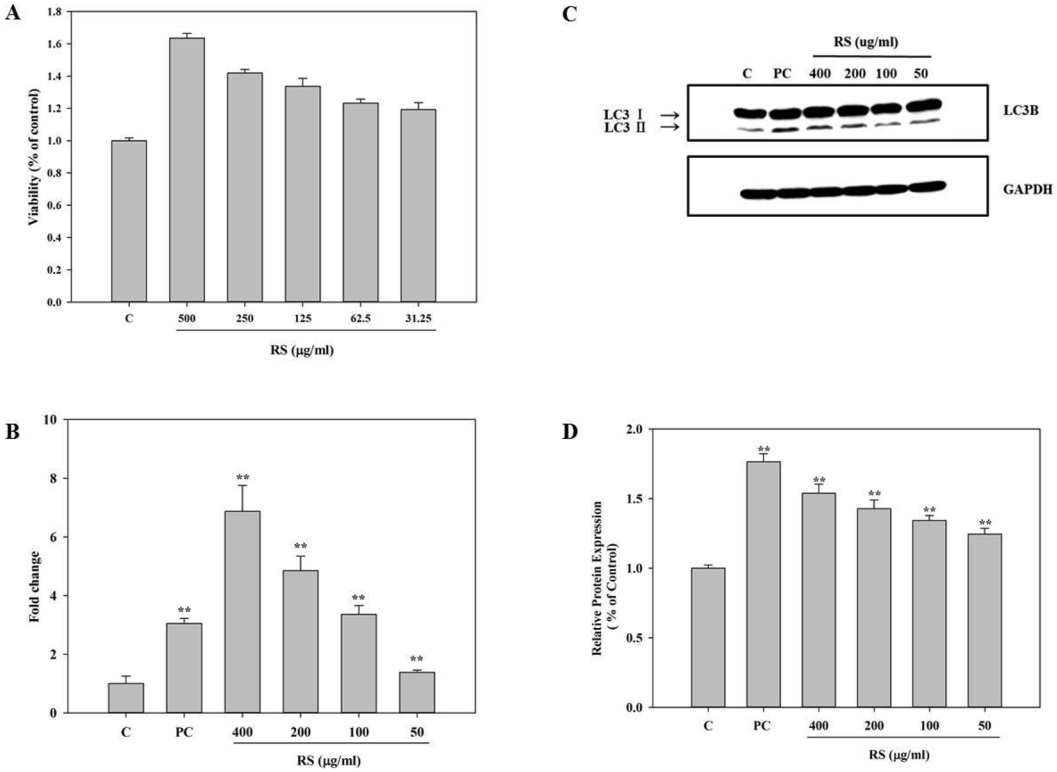

Fig. 2. The autophagy-inducing effect of total water extract of RS in NGF-differentiated PC12 cells. In MTT assay NGF-differentiated PC12 cells were treated with RS at various concentrations (31.25, 62.5, 125, 250 and 500 μg/ml) for 24 h. For autophagy induction, NGF-differentiated PC12 cells were treated with RS at various concentrations (50, 100, 200 and 400 μg/ml) for 24 h. A: Cell viability was measured by MTT assays, and the results were expressed as the percentile of absorbance of treated samples compared to that of the vehicle control sample. B: Flow cytometric analysis of autophagy activity in control and RS-treated cells was performed after MDC staining. C: A representative result of Western blot analysis for LC3B-I and LC3B-II expression; induction of autophagy by the various concentrations of RS in differentiated PC12 cells was determined by measuring the LC3B protein expression levels by immunoblotting with an antibody against LC3B. D: The ratio of LC3B-II/LC3B-I protein levels in RS-treated cells compared with that of the control; $*p < 0.05$, $**p < 0.01$ relative to the control group (C: control, untreated cells; PC: positive control, cells treated with 200 nM of rapamycin).

Germany). Briefly, cells (5×10^5) were collected, re-suspended in PBS, and then stained with MDC labeling solution for 60 min at room temperature in the dark. Then, the cells were quantitatively analyzed by flow cytometry. The minimum number of events counted per sample was 10,000.

Statistical analysis – Statistical data were expressed as mean ± S.D. Student's t-test and analysis of variance (ANOVA) were used to determine statistical significance.

Results

Effect of RS on cell viability – To examine the effects of RS on PC12 cell viability, a MTT assay was performed after 24 h of exposure to RS at the various concentrations of 31.25, 62.5, 125, 250 and 500 μg/mL. Viability was expressed as the fold change in absorbance of the treated sample over that of the vehicle control sample. RS had a positive effect on the survival of PC12 cells (Fig. 2A).

Induction of the autophagy-lysosomal pathway by RS – Flow cytometry with MDC dye was employed to assess the extent of mature autophagic vesicle formation in PC12 cells (Munafo and Colombo, 2001; Mizushima, 2004; Biederbick *et al*., 1995). Our data showed that, after treatment with 50, 100, 200 and 400 μg/mL of RS for 24 h, the incidence of autophagy increased, respectively, to 1.39, 3.36, 4.85, and 6.87-fold over that of control. These results showed that RS was able to induce autophagy in PC12 cells (Fig. 2B).

To determine the effect of RS on the induction of LC3-II protein in differentiated PC12 cells, these cells were treated with 50, 100, 200 and 400 μg/mL of RS for 24 h. Western blot analyses revealed that treatment with 50 μg/mL of RS increased the ratio of LC3B-II/LC3B-I in these cells, and the ratio increased with RS treatment in a dose-dependent manner (Fig. 2C, 2D).

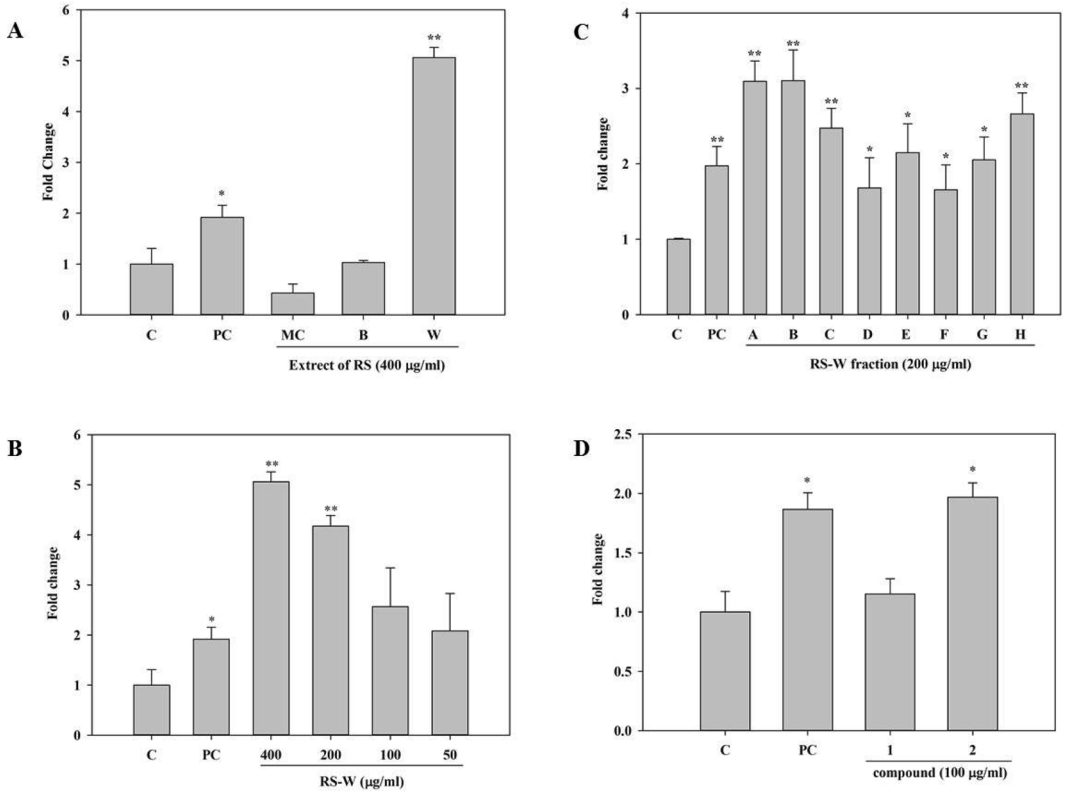

Fig. 3. The activity guided purification of RS on autophagy-inducing effect in NGF-differentiated PC12 cells. NGF-differentiated PC12 cells were treated with RS samples for 24 h. Flow cytometric analyses of autophagy activity in control and RS-treated cells were performed after MDC staining. A: The water fraction of the RS extract (RS-W) showed autophagy induction in the PC12 cells, but the two other fractions (RS-MC and RS-B) did not. B: The RS-W fractions at various concentrations (50, 100, 200 and 400 μg/ml) induced autophagy in PC12 cells in a dose-dependent manner. C: The RS-W-A and RS-W-B fractions induced more autophagy in PC12 cells at 200 μg/ml concentration. D: The RS-W-AB-2 compound induced autophagy in PC12 cells at 100 μg/ml concentration, but the RS-W-AB-1 compound did not. *$p < 0.05$, **$p < 0.01$ relative to the control group (C: control, untreated cells; PC: positive control, cells treated with 200 nM of rapamycin).

Determination of the autophagy-inducing effect of extracts and fractions through flow cytometry – For further study, RS was partitioned first with methylene chloride and then with n-butanol to obtain the RS-MC, RS-B, and RS-W extracts. PC12 cells were treated with 400 μg/mL of each extract for 24 h. While neither RS-MC nor RS-B induced autophagy in PC12 cells, the RS-W extract induced autophagy at a rate that was 5.06-fold higher than in the control samples (Fig. 3A). Moreover, the RS-W extract induced autophagy in PC12 cells in a dose-dependent manner (Fig. 3B), so this extract was then fractionated to yield fractions A through H. Treating PC12 cells with 200 μg/mL of water-soluble fractions A, B, C, D, E, F, G, or H for 24 h, increased autophagy rates, respectively, to 3.09, 3.10, 2.47, 1.68, 2.15, 1.65, 2.05, and 2.66 times higher than the control rates. RS-W fractions A and B induced more autophagy in PC12 cells (Fig. 3C).

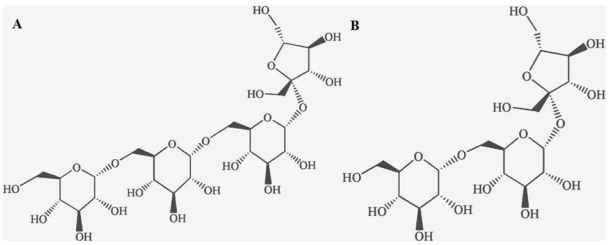

Fig. 4. The structures of stachyose (compound 1) and raffinose (compound 2). A: Stachyose, B: Raffinose.

Isolation and structure elucidation of active compounds – Fractions A and B were combined (RS-W-AB) and further purified to yield compounds **1** (RS-W-AB-1) and **2** (RS-W-AB-2). PC12 cells were treated with RS-W-AB-**1** and **2** for 24 h, and the autophagy levels increased to 1.15 and 1.96-fold higher, respectively, than that of the control samples (Fig. 3D).

Compound **1** was isolated with HPLC using a YMC-

ODS-AQ column. Its molecular weight was 666.578, as determined by Low resolution ESIMS. A comprehensive analysis of 1D and 2D NMR data from ^{1}H, gCOSY, gHSQC, gHMBC and ^{13}C NMR experiments allowed us to assemble the planar structure of compound **1**. The ^{1}H data for compound **1** was the same as the data for the compound stachyose, which we purchased from Sigma, and it was consistent with a previous report on stachyose (Laidlaw and Wylam, 1953) (Fig. 4A).

Compound **2** was isolated with HPLC using a YMC-ODS-AQ column. Its molecular weight was 504.51, as determined by Low resolution ESIMS. A comprehensive analysis of 1D and 2D NMR data from ^{1}H, gCOSY, gHSQC, gHMBC and ^{13}C NMR experiments allowed us to assemble the planar structure of compound **2**. The ^{1}H data of compound **2** was the same as the data for the compound raffinose, which we purchased from Sigma, and it was consistent with a previous report on raffinose (Neubauer *et al*., 2001) (Fig. 4B).

Effect of raffinose on cell viability – Raffinose did not induce cytotoxicity, even at the high 400 μM concentration, and cell viability was expressed as the fold change of absorbance of the treated sample over that of the vehicle control sample (Fig. 5A).

Induction of the autophagy-lysosomal pathway by raffinose – NGF-differentiated PC12 cells were treated with increasing concentrations of raffinose (50, 100, 200 and 400 μM) for 24 h. A Western blot assay revealed that the ratio of LC3B-II/LC3B-I was higher in cells treated with 50 μM of raffinose than in the control cells, and the

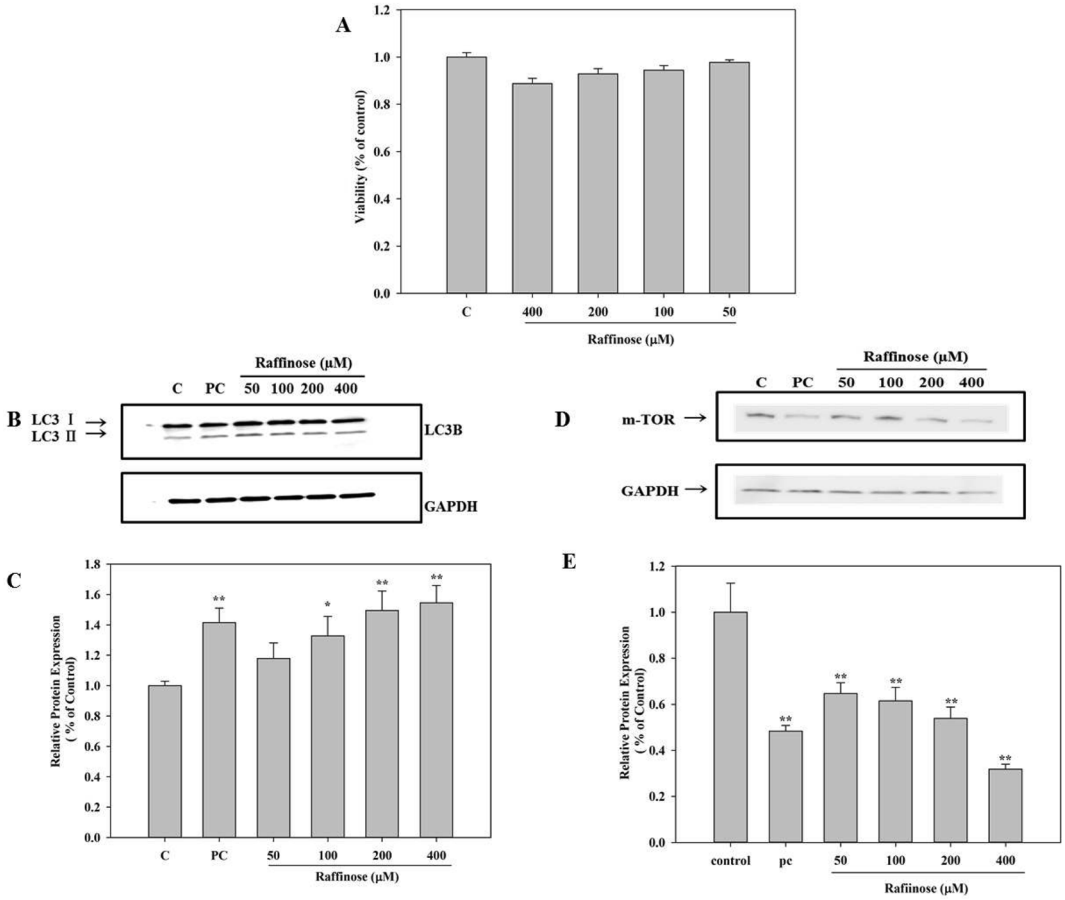

Fig. 5. The autophagy-inducing effect of Raffinose in NGF-differentiated PC12 cells. NGF-differentiated PC12 cells were treated with raffinose (Sigma) at various concentrations (50, 100, 200 and 400 μM) for 24 h. The changes in autophagy induction were determined by measuring the LC3B protein expression levels via Western blot analysis with an antibody against LC3B. And the changes in mTOR inhibition were determined by measuring the mTOR protein expression levels via Western blot analysis with an antibody against mTOR. A: The cell viability was measured by MTT assays, and the results were expressed as the percentile of absorbance of treated samples compared to that of the vehicle control sample. B: A representative result of a Western blot analysis for LC3C-I and LC3B-II expression. C: The ratio of LC3B-II/LC3B-I protein levels in RS treated cells compared to that of the control; $*p < 0.05$, $**p < 0.01$ relative to the control group. D: A representative result of a Western blot analysis for mTOR expression. E: Result of densitometric analysis of the representative Western blot. Each value was normalized against that of the control; $*p < 0.05$, $**p < 0.01$ relative to the control group (C: control, untreated cells; PC: positive control, cells treated with 200 nM of rapamycin).

ratio increased with raffinose treatment in a dose-dependent manner (Fig. 5B, 5C).

Inhibition of mTOR by raffinose – mTOR, the upstream protein in the autophagy pathway, can inhibit autophagy, and rapamycin is the inhibitor of mTOR. In this study, we used rapamycin (200 nM) as the positive control, and NGF-differentiated PC12 cells were treated with raffinose at various concentrations (50, 100, 200 and 400 μM). Raffinose was able to inhibit mTOR significantly, even at the 50 μM concentration, and its effect increased in a dose-dependent manner (Fig. 5D, 5E).

Discussion

Protein turnover is essential for both removing defective proteins and contributing to the pool of amino acids required for continued protein synthesis, particularly in times of limited nutrient availability. Therefore, dysregulated or impaired protein degradation can lead to a wide variety of disease states. The role of protein catabolism in protecting cells from defective, misfolded proteins has been the subject of increased attention as its relevance to human disease has become apparent (Nedelsky *et al*., 2008).

Many neurodegenerative diseases are characterized by accumulation of misfolded protein deposits in affected brain regions, suggesting a failure of the cell's degradative capacity. Neurons are particularly vulnerable to the toxic effects of mutant or misfolded proteins. Increased understanding of the effect of toxic protein accumulation on neuronal survival may allow the development of rational and effective treatments for these disorders (Taylor *et al*., 2002).

The pathological hallmark of Parkinson's disease is the deposition of Lewy bodies within dopaminergic neurons. Lewy bodies are cytoplasmic inclusions composed largely of α-synuclein, a protein that can be degraded by the UPS (ubiquitin-proteasome system), macroautophagy and chaperon-mediated autophagy (Webb *et al*., 2003). Many reports have addressed a potential neuroprotective role of autophagy (Pan *et al*., 2008). Augmentation of autophagy is neuroprotective by (1) maintaining the overall rate of catabolism, (2) eliminating specific protein substrates that would otherwise accumulate, aggregate and acquire toxic properties, or (3) a combination of these (Nedelsky *et al*., 2008).

In this study, we demonstrated that RS is an effective herb in the modulation of autophagic activity in PC12 cells. The increased expression of lipidated LC3-B, in LC3-II form, is a hallmark of autophagy, and it was found in the RS-treated cells. The present immunoblot assay showed that the ratio of LC3B-II/LC3B-I in RS treated cells was approximately 1.41, 1.32, 1.28, and 1.21-fold higher than that of control at 400, 200, 100 and 50 μg/mL, respectively (Fig. 2C, 2D). It has been reported that MDC is a specific marker for autolysosomes which form post-autophagy. We performed flow cytometry after MDC staining of RS-treated PC12 cells. Data showed that the autophagy rates increased in a dose-dependent manner when treated with 50, 100, 200 and 400 μg/mL of RS for 24 h (Fig. 2B). Therefore, these findings further suggested that RS might have a neuroprotective effect in PC12 cells by activating the autophagic pathway and indicated that MDC can detect autophagy *in vitro*.

One of the aims of this study was to elucidate the compounds in RS that exhibited autophagy-inducing activity. During this procedure, two compounds, Compound **1** and **2,** were isolated from the water-soluble RS extract (RS-W), and their chemical structures were characterized as stachyose and raffinose, respectively, by comparing their physical and spectroscopic data with previous reports. Stachyose is naturally found in numerous vegetables (e.g., green beans, soybeans and other beans) and plants. Raffinose can be found in beans, cabbage, sprouts, broccoli, asparagus, and other vegetables and whole grains, and it is the first member of a family of α-galacto-oligosaccharides (α-GOS). In the present study, we reported the isolation of raffinose and stachyose from Raphani Semen for the first time.

Nondigestible oligosaccharides, e.g., α-GOS, present important physiological functions and have been used extensively as both food ingredients and pharmacological supplements to aid weight control, regulate glucose, thrombi, reduce serum lipid levels, and control some acute and chronic diseases (Matsukawa *et al*., 2009; Ariefdjohan *et al*., 2008; Lim *et al*., 2011). It is an excellent dietary fiber and prebiotic as well as one of the best known and most commonly applied oligosaccharides. Therefore, the application of α-GOS as a food ingredient has increased rapidly.

Pharmacologically, considerable evidence has accumulated, showing that agaro-oligosaccharides have the antioxidant activity and hepatoprotective potential *in vitro* and *in vivo* (Chen *et al*., 2006). Another oligosaccharide, chitosan, can attenuate the inflammation and oxidative stress (Qiao *et al*., 2011). There are some oligosaccharides which have neuroprotective activity, such as hyaluronan oligosaccharides (Wakao *et al*., 2011) and chitosan (Pangestuti and Kim, 2010). However, to date, there had been no studies on raffinose, which can cross the BBB

(blood brain barrier), as a therapeutic target of neurodegenerative diseases (Lucchesi and Gosselin, 1990).

In the Western blot assays, raffinose showed autophagy-inducing activity; the ratio of LC3B-II/LC3B-I was higher after 50 μM raffinose treatment, and it increased in a dose-dependent manner (Fig. 5B, 5C). This result was consistent with RS. Our present study demonstrated that RS and raffinose can be promising therapeutics for the treatment of neurodegenerative diseases, including Parkinson's disease.

In the present work, RS enhanced the pharmacological induction of the autophagy-lysosomal pathway (ALP) in NGF-differentiated PC12 cells. Activity-guided isolation of RS resulted in the isolation of two compounds, which were identified as raffinose and stachyose. Of these two compounds, raffinose showed potent autophagy-inducing activity in PC12 cells through the mTOR pathway. Further studies will be conducted in the near future to investigate the underlying mechanism of autophagy enhancement by raffinose.

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