Original paper

Evaluation of Phenolic Compounds and Neurotrophic/neuroprotective Activity of Cultivar Extracts Derived from *Chrysanthemum morifolium* Flowers

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Petal extracts of the edible flowers of two typical cultivars, Aboukyu and Enmeiraku, of Japanese *Chrysanthemum* spp. (*Chrysanthemum morifolium* Ramat. forma *esculentum* Makino) were analyzed for their bioactive characteristics, including neuroprotective and neurotrophic-like activity in relation to the chemical profile of phenolic components. Phenolic components of petals differed significantly between the Aboukyu and Enmeiraku cultivars. The level of luteolin, an antioxidant polyphenol that possesses neurotrophic and neuroprotective activities, was higher in Enmeiraku than in Aboukyu. Extracts of Enmeiraku protected neuroblastoma SH-SY5Y cell viability against oxidative stress-induced injury in a concentration-dependent manner (12.5 – 100 µg/mL). By contrast, the neuroprotective effect of Aboukyu extracts was biphasic and was weakened at concentrations > 25 µg/mL. Individually, the Aboukyu and Enmeiraku extracts only slightly induced neurite outgrowth; however, in combination with nerve growth factor, the extracts acted synergistically to induce full neurite outgrowth in PC12 cells. Our findings suggest that differences in the neuroprotective effect of Aboukyu and Enmeiraku extracts are attributable to differences in the composition of the constituent phenolic compounds.

Keywords: edible chrysanthemum flower, flavonoids, neuroprotective activity, neurotrophic-like activity

Introduction

Aboukyu and Enmeiraku, two cultivars of edible *Chrysanthemum* spp. (*Chrysanthemum morifolium* Ramat. forma *esculentum* Makino), are cultivated in the northeastern region of Honshyu Island, Japan. Enmeiraku is also known by the flower names of Mottenohoka, Omoinohoka or Kakinomoto depending on the region of production. These cultivars are abundantly produced and constitute the major cultivars of edible *Chrysanthemum* in Japan. In traditional medicine, *Chrysanthemum* flowers have been used for the treatment of cold symptoms, fever alleviation and detoxification, and brightening of the eyes. Many components of *Chrysanthemum* flowers have been studied, which have been

reported to exhibit various significant pharmacological activities (Ranxin *et al.*, 2004), including anti-inflammatory, antipyretic, and anti-allergic effects (Xie *et al.*, 2012), protective effects against brain ischemia, anti-tumorigenic effects, and many other activities, including anti-toxic effects in cancer cells. It has traditionally been held that flavonoid compounds (including luteolin and apigenin) and their glycosides (Chen *et al.*, 1983; Li and Jiang, 2006), caffeoylquinic acids (Kim and Lee, 2005), and terpenes are the major active components in *Chrysanthemum* flowers. Specifically, chlorogenic acid and luteolin are considered to be the most characteristic components of *Chrysanthemum* flowers (Ranxin *et al.*, 2004; Xie *et al.*, 2012).

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There are over 50 edible *Chrysanthemum* cultivars in Japan, and numerous studies comparing the various components of *Chrysanthemum* cultivars have been conducted (Sugawara and Igarashi, 2009; Lin and Harnly, 2010; Xie *et al.*, 2012). However, to date, comprehensive chemical profiles that characterize cultivar bioactivity have been obscure. In the present study, neuroprotective and neurotrophic effects of the flowers of two typical cultivars of *Chrysanthemum*, which quantitatively differed in phenolic composition, were compared to examine the correlation between chemical profile and bioactivity.

Living organisms are constantly exposed to oxygen free radicals. Thus, antioxidant defense systems play a role in preventing oxidative damage induced by oxygen free radicals. However, breakdown of the antioxidant defense system or overproduction of free radicals can result in oxidative damage to biomolecules, leading to various degenerative diseases such as atherosclerosis, cardiovascular disease, and cancer (Uttara *et al.*, 2009). It has been well established that nerve cells are extremely sensitive to oxidative stress. Toxicity of free radicals contributes to glial cell and neuronal injury, leading to disorders such as Alzheimer's disease, Parkinson's disease, and many other neural disorders. Nutritional studies have shown that the regular consumption of polyphenolic phytochemicals has positive effects on the treatment and prevention of a wide range of oxidative stress injuries and associated pathologies, including cancer, coronary heart disease, and neurodegenerative disease (Bonfili *et al.*, 2008). Some of these properties are related to the antioxidative or free radical-scavenging effects of flavonoids. Recently, luteolin, which is a widely distributed flavone in many fruits, vegetables, and *Chrysanthemum* flowers, has been shown to exhibit protective effects against oxidative stress in neuroblastoma cells and to promote the induction of neurite outgrowth (Lin *et al.*, 2011; Lin *et al.*, 2012). In light of these findings, we sought to compare the bioactive characteristics of two cultivars of edible *Chrysanthemum* by analyzing the composition of phenolic components and assessing the neuroprotective and neurotrophic effects of these compounds.

Materials and Methods

Reagents Apigenin, luteolin, tertiary-butyl hydroperoxide (t-BOOH), and MEK inhibitor (U0126) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acacetin was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), luteolin-7-*O*-glucoside was purchased from Extrasynthese SA (Lyon, France), SOD Assay Kit-WST and Cell Counting Kit-8 (CCK-8) were purchased from Dojindo Molecular Technologies Inc. (Kumamoto, Japan). BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL, USA). p38MAPK inhibitor (SB203580) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Sample preparation Aboukyu and Enmeiraku flowers were

cultivated at the same farm in Aomori Prefecture (northeastern region of Japan) and were harvested from mid-October to the beginning of November. The raw *Chrysanthemum* petals were vacuum dried, powdered, and defatted with hexane at 40°C. The residue was extracted with 80% methanol at 70°C for 2 h. The filtrate of the extract solvent was evaporated to an adequate concentration under reduced pressure.

Measurement of flavonoid and total phenolic compounds contents The total phenolic content of the extracts was determined by the Folin-Denis method. Briefly, 0.1 mL of the diluted sample extract was transferred to tubes containing 0.2 mL of Folin-Denis reagent and 3.3 mL of water. After a 10 min rest, 0.4 mL of a saturated sodium carbonate solution was added to the mixture. The tubes were then allowed to stand at room temperature for 30 min before measuring absorbance at 700 nm. The total phenolic content of the extracts was calculated as gallic acid equivalents from the standard graph, and the results were expressed as mg per petal gram (dry weight).

HPLC-DAD analysis High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-10 HPLC coupled to a diode array detector (DAD; Shimadzu Corp., Kyoto, Japan). The data were acquired and processed using CLASS-LC10 software (Shimadzu Corp.). The extract was analyzed using a reverse-phase HPLC column: Mightysil RP-18 (150 mm × 4.6 mm, 3 μm) column (Kanto Corp., Tokyo, Japan). The oven temperature was 40°C and the flow rate was 0.7 mL/min. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The column was developed with 20% B (v/v) for 20 min, then developed by a linear gradient from 20% to 65% B in 20 min, and held at 65% B to 55 min. Spectral data was recorded at 230, 250, 270, 290, 310, 330, 350 and 370 nm during the entire run.

Superoxide radical scavenging activity Superoxide radical scavenging activity was determined using a commercial SOD Assay Kit-WST according to the manufacturer's instructions. This method uses the xanthine/xanthine oxidase system to generate superoxide radicals, which react with an electron acceptor 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST) to generate formazan. Absorbance of formazan was detected and SOD activity was measured by the degree of inhibition of this reaction. Briefly, 20 μL of sample solution diluted at various concentrations was placed in a 96-well microplate. A 200 μL aliquot of the WST working solution containing WST in 50 mmol/L carbonate buffer (pH 10.2) and 20 μL of enzyme working solution containing xanthine oxidase in the same buffer were added. Then, the samples were incubated at 37°C for 20 minutes, and absorbance at 450 nm was measured. The SOD activity (inhibition percentage) was calculated as: Inhibition (%) = $(1 - A_{\text{sample}} / A_{\text{blank}}) \times 100$, where A_{blank} is the absorbance of the blank (dilution buffer instead of sample solution).

PC12 cell culture PC12 cells were obtained from the

American Type Culture Collection ATCC (Cat. No. 88022401, Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco Cat. No. 11885) supplemented with 10% horse serum (HS; Gibco, Cat. No. 26050) and 5% fetal bovine serum (FBS; Corning Inc., Corning, NY, USA, Cat. No. 35-010-CV) and maintained at 37°C in a 5% CO₂/air atmosphere. PC12 cells were seeded in collagen-coated 6-well plates with normal serum medium (10% HS and 5% FBS) at a density of $2 - 5 \times 10^5$ cells/mL, and cultured for 24 h. The cells were serum-starved by changing the medium to low serum medium (1% HS and 0.5% FBS); 16 h later, the medium was changed to low serum (1% HS and 0.5% FBS) medium and cells were treated with the sample. After 24 – 72 h incubation, the cells were photographed under phase-contrast microscopy at 200× magnification using an inverted microscope (Olympus IX70, Tokyo, Japan). The quantification of neurite outgrowth in PC12 cells was performed according to the procedures described by Lin *et al.* with some modifications (Lin *et al.*, 2012). The percentage of neurite-induced cells was scored. Neurite positive cells, identified as having one or more neurites, were counted in three randomly selected microscopic fields with an average of 100 cells per field. Data are expressed as a percentage of the total cells in the field.

SH-SY5Y cell culture and cell viability assay SH-SY5Y cells were obtained from ATCC (Cat. No. 94030304). The cells were grown in DMEM/F12 (Gibco, Cat. No. 11330) supplemented with 10% FBS, and maintained at 37°C in a 5% CO₂/air atmosphere. To assess the protective effect of the *Chrysanthemum* petal extracts or flavonoid samples, SH-SY5Y cells were plated at a density of 5×10^4 cells per well in 96-well plates with DMEM/F12 medium supplemented with 10% FBS. One day after plating, the cells were treated with various concentrations of the samples in DMEM/F12 medium supplemented with 1% FBS, or vehicle as the control. t-BOOH was added to the medium at 0.13 – 0.15 mM after 2 h of pre-treatment with test samples, and cultured for 16 h (Maher and Hanneken, 2005). Cell viability was evaluated using CCK-8 according to the manufacturer's instructions. The number of viable cells was determined by colorimetric assay using water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfohenyl)-2H-tetrazolium, monosodium salt] (Ishiyama *et al.*, 1997). WST-8 is reduced by dehydrogenases in cells to give an orange formazan dye, and absorbance of the dye is directly proportional to the number of live cells. After washing, the cells were incubated with 10 (v/v)% of CCK-8 solution in DMEM/F12 medium and incubated for an additional 3 h. The absorbance was determined at 450 nm. Results are expressed as a percentage of the control.

Western blotting analysis of ERK, phosphorylated ERK, p38 and phosphorylated p38 To assess the activation of extracellular signal-regulated kinase (ERK) and p38 in mitogen activated protein kinase (MAPK) signaling pathway by *Chrysanthemum* petal extracts, PC12 cells were seeded in collagen-coated 6-well plates

at a density of 5×10^5 cells/mL in DMEM supplemented with 10% HS and 5% FBS, and cultured for 24 h. Cells were serum-starved by changing the medium to low serum medium (1% HS and 0.5% FBS), and the cells were incubated for 16 h before treatment with the test samples. The cells treated under different experimental conditions were washed twice with ice-cold phosphate-buffered saline and lysed in RIPA buffer (Santa Cruz Biotechnology Inc., TX, USA). Cell lysates were collected using a cell scraper and centrifuged at 15000×g for 30 min at 4°C. The obtained supernatant was subjected to determination of protein concentration using the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), with BSA as the standard. Samples with equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 12% SDS-PAGE, and proteins in the gel were then transferred to polyvinylidene difluoride membranes (ATTO, Tokyo, Japan). Immuno-blotting analysis was performed by using monoclonal antibodies according to the manufacturer's instructions, against p44/42 ERK, p-p44/42 ERK, p38MAPK, p-p38MAPK, (Cell Signaling Technology, Lake Placid, NY, USA) as the primary antibodies, followed by reaction with alkaline phosphatase-linked anti-rabbit IgG antibodies (Cell Signaling Technology) as the secondary antibody. The blots were washed three times with TBST (10 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and the secondary antibody was stained using 1-StepTM NBT/BCIP (Thermo Scientific). Images were obtained using the Electrophoresis Documentation and Analysis System (Kodak EDAS 290, Kodak 1D Image Analysis Software, Eastman-Kodak Co., Rochester, NY, USA).

Statistical analysis Quantitative data were presented as the mean ± SD. Between group comparisons were analyzed using a Student's t-test.

Results and Discussion

Flavonoid and total phenolic compounds contents of *Chrysanthemum* flowers Several studies have characterized the flavonoid components of the flowers of different *C. morifolium* cultivars. Sugawara *et al.* reported the concentrations of major flavonoids in 4 cultivars (Iwakaze, Kotobuki, Mottenohoka with pale purple petals, and Mottenohoka with yellow petals), and found that the flavonoid components differed greatly depending on the cultivar (Sugawara *et al.*, 2009). We investigated the phenolic compounds contents of the extracts of Aboukyu and Enmeiraku flowers, which are well known *Chrysanthemum* spp. cultivars in Japan. It has been suggested that cultivation conditions influence the various constituent components of plants (Jaakola and Hohtola, 2010). To avoid the influence of environmental factors on the phenolic profile, we obtained the flowers of two *Chrysanthemum* cultivars cultivated on the same farm and harvested in the same season. Total content of phenolic compounds in crude methanol extracts of the two cultivars was determined by the Folin-Denis method. Total phenolic compounds content was expressed as gallic

Table 1. Total phenolic compounds and flavonoids content of extracts from chrysanthemum petals of Aboukyu and Enmeiraku

	Aboukyu	Enmeiraku
(mg/g dry wt.)		
Total polyphenol	21.8 ± 1.07 ^a	11.4 ± 1.01 ^b
(μmol/g dry wt.)		
Apigenin	2.60 ± 0.32 ^a	0.88 ± 0.16 ^b
Luteolin	1.30 ± 0.34 ^a	3.24 ± 0.17 ^b
Acacetin	0.71 ± 0.13	0.78 ± 0.16

Total phenolics content was measured by Folin-Denis, using standard as gallic acid. Contents of flavonoid were determined by quantitative HPLC analysis. Values are mean ± SD of three determinations. Statistics were calculated by t-test. Difference at $p < 0.05$ are indicated by different letter (a and b)

Fig. 1. Chemical structures of some major flavonoids found in Chrysanthemum petal

acid equivalents (Table 1). Aboukyu contained a higher total phenolic compounds content compared to Enmeiraku (21.8 ± 1.07 vs. 11.4 ± 1.01 mg/g dry weight, respectively). Contents of neuritogenic flavonoids, luteolin and acacetin, in petals were assayed by quantitative HPLC. Apigenin was also assayed because it is one of the characteristic flavonoids in chrysanthemum flowers (Table 1). Fig. 1 presents the chemical structures of these compounds. Enmeiraku contained higher luteolin content (3.24 ± 0.17 vs. 1.30 ± 0.34 μmol/g dry weight, respectively) and lower apigenin content compared to Aboukyu (0.88 ± 0.16 vs. 2.60 ± 0.32 μmol/g dry weight, respectively). There was no difference in acacetin content between Aboukyu and Enmeiraku cultivars (0.71

vs. 0.78 ± 0.16 μmol/g dry weight, respectively).

HPLC-DAD characterization of major phenolic components of Chrysanthemum flowers To characterize the phenolic components in the petals of *Chrysanthemum* spp. cultivars, Aboukyu and Enmeiraku petal extracts were subjected to HPLC-DAD (Fig. 2). Twelve specific peaks (labeled peaks 1 – 12) in the HPLC chromatograms were characterized by typical UV absorptions obtained with the DAD, with maximum absorptions (λ_{max}) at 252, 265 – 268 and 332 – 348 nm for flavones and at 239, 300 and 326 nm for caffeoylquinic acid derivatives (Table 2). Identification of the compounds in Table 2 was made based on a comparison of the retention time and UV spectra for either authenticated standards or previously identified compounds (Wako and Tanikawa, 2004). We found that compounds corresponding to six peaks were identified as chlorogenic acid (peak 1), luteolin-7-O-glucoside (peak 2), 3,5-di-caffeoylquinic acid (peak 4), luteolin (peak 8), apigenin (peak 11), and acacetin (peak 12). The compounds corresponding to three peaks (5, 6 and 7) were purportedly luteolin glucosides (peak 6) and apigenin glucosides (peaks 5 and 7) by comparison of UV spectra between these peaks and their aglycone peaks. The compounds corresponding to the twelve specific peaks characterized by HPLC-DAD are common components of Aboukyu and Enmeiraku flowers, according to their retention times and UV spectra of peaks (Fig. 2, Table 2). However, the area under the curve (AUC) of the twelve peaks clearly differed between cultivars. Enmeiraku showed higher AUC for luteolin and luteolin-7-O-glucoside compared to Aboukyu. These results show that the composition of phenolic compounds differed greatly between Aboukyu and Enmeiraku, with respect to the content and composition of luteolin and apigenin, and their glycosides.

Antioxidant activity of Chrysanthemum flower extracts We measured the antioxidant activities of petal extracts from the two *Chrysanthemum* cultivars Aboukyu and Enmeiraku. It has been proposed that phenolic compounds are specific nonenzymatic scavengers of superoxides and can prevent biological damage by trapping free radicals such as SOD (Kim *et al.*, 2012). We

Fig. 2. Chromatograms (350 nm) of the chrysanthemum flowers extract from Aboukyu (A) and Enmeiraku (B). Peaks assignment listed in Table 2.

Table 2. Peaks data list from chromatograms (350 nm) of chrysanthemum flower

Peak. No	T _R (min)	UV λ _{max} (nm)			Aboukyu	Enmeiraku	identification
					Area %	Area %	
Flavonoids							
2	22.821 ± 0.125	252	265	347	4.48 ± 0.52 ^a	12.48 ± 2.35 ^b	Luteolin-7- <i>O</i> -glucoside [*]
5	26.244 ± 0.360		283	328	12.04 ± 5.23	3.24 ± 0.39	
6	27.018 ± 0.114	253	265	347	6.86 ± 2.68 ^a	21.97 ± 2.11 ^b	
7	29.732 ± 0.087		266	336	26.21 ± 5.54 ^a	4.30 ± 0.73 ^b	Luteolin [*]
8	31.476 ± 0.079	253	265	348	3.59 ± 0.82 ^a	9.91 ± 1.81 ^b	
9	32.701 ± 0.718		266	331	4.96 ± 2.28 ^a	1.43 ± 0.40 ^b	
10	34.313 ± 0.194		266	337	7.50 ± 0.42 ^a	3.12 ± 1.01 ^b	Apigenin [*]
11	34.724 ± 0.297		268	332	7.88 ± 2.35 ^a	1.94 ± 0.25 ^b	
12	40.713 ± 1.156		268	332	1.72 ± 0.46	2.32 ± 1.01	Acacetin [*]
Cafeoylquinic acids							
1	12.173 ± 0.199	239	300	326	2.47 ± 2.06	4.27 ± 1.34	Chlorogenic acid [*]
3	24.338 ± 0.128	239	300	326	2.40 ± 1.30	5.81 ± 3.83	3,5-Di-cafeoylquinic acid ^{**}
4	24.469 ± 0.128	239	300	326	3.77 ± 2.65	4.62 ± 3.77	

* Peak estimation was confirmed by comparison of their retention times with those standards.

** Previously identified from NMR and mass spectral data.

Values are mean $\pm$ SD of three determinations. Statistics were calculated by t-test. Difference at $p < 0.05$ are indicated by different letter (a and b)

Fig. 3. Reduction of the superoxide radical anion in the presence of *Chrysanthemum* petal extracts of Aboukyu and Enmeiraku. Inhibition (%) is shown in (A) as a function of *chrysanthemum* extracts concentration. Inhibition (%) is also expressed as a function of the polyphenol ingredient concentration in (B). Values are expressed as mean $\pm$ S.D. of three determination.

determined the SOD-like activity in order to estimate the antioxidant activities of Aboukyu and Enmeiraku extracts (Fig. 3). The SOD level of Aboukyu ($IC_{50} = 370 \mu\text{g/mL}$) was found to be more potent than Enmeiraku ($IC_{50} = 1060 \mu\text{g/mL}$; Fig. 3A). By contrast, when comparing the values of SOD-like activity by phenolic content weight of the extract, differences in activity levels between the two cultivars was diminished (Aboukyu, $IC_{50} = 15 \mu\text{g/mL}$; Enmeiraku, $IC_{50} = 26 \mu\text{g/mL}$; Fig. 3B). These data suggest that the SOD-like antioxidant activity of petals in these cultivars was dependent on the content as well as the composition of phenolic compounds.

Protective effects of *Chrysanthemum* flower extracts against oxidative stress in SH-SY5Y cells To compare the protective effect of Aboukyu and Enmeiraku cultivars against oxidative stress, we investigated the effect of the petal extracts on t-BOOH-induced cell death in human neuroblastoma SH-SY5Y cells. Cell viability was assayed using various extract concentrations $< 200 \mu\text{g/mL}$, levels that did not exhibit cytotoxicity. The cells were cultured for 16 h, and cell viability was determined using the CCK-8 assay.

The cell viability assay showed that the Enmeiraku extract prevented t-BOOH-induced cell death in a concentration-dependent manner from 12.5 to 100 $\mu\text{g/mL}$ (Fig. 4B). By contrast, the Aboukyu extract affected cell viability in a biphasic manner with increasing concentrations of the extract (Fig. 4A). At lower concentrations, cell viability was protected from injury, but at concentrations $> 25 \mu\text{g/mL}$, cell viability decreased. To investigate the differences in the neuroprotective effects between the extracts of Aboukyu and Enmeiraku, we treated SH-SY5Y cells with apigenin and luteolin, since the cultivar extracts contained different levels of those compounds. We also treated SH-SY5Y cells with increasing concentrations of flavonoid compounds (up to 40 μM) for 16 h and assayed cell viability. Luteolin lowered t-BOOH-induced cell death to control levels at concentrations ranging from

A. Aboukyu

B. Enmeiraku

Fig. 4. *Chrysanthemum* petal extracts of Aboukyu (A) and Enmeiraku (B) protect the viability of SH-SY5Y cells from oxidative stress induced by treatment with t-BOOH. SH-SY5Y cells were pre-treated with the extract at various concentrations for 2 h. Then t-BOOH was given to the medium to 0.13 mM and cultured for 16 h. The cell viability was measured by the CCK-8 assay and presented as percentage of cell viability relative to untreated control. Each bar presents the mean $\pm$ S.D. from two independent experiments. *, $p < 0.05$ versus t-BOOH treated control cells. †, $p < 0.05$ versus untreated control cells.

A. Apigenin

B. Luteolin

Fig. 5. Cytoprotective and cytotoxic effects of flavonoids on SH-SY5Y cell viability in response to t-BOOH-induced oxidative stress injury. SH-SY5Y cells were pre-treated with apigenin (A) or luteolin (B) at various concentrations for 2 h. Then t-BOOH was given to the medium to the 0.13 mM and cultured for 16 h. The cell viability was measured by the CCK-8 assay and presented as percentage of cell viability relative to untreated control. Each barre presents the mean $\pm$ S.D. from 3 determinations. *, $p < 0.05$ versus t-BOOH treated control cells. †, $p < 0.05$ versus untreated control cells.

2.5 to 40 μ M (Fig. 5B). In contrast, apigenin partially protected against cell death at concentrations $< 5 \mu$ M, but impaired cell viability at concentrations $> 5 \mu$ M (Fig. 5A). To investigate why apigenin and the Aboukyu extract did not protect SH-SY5Y cells from t-BOOH-induced cell death at higher concentrations, the neuroprotective effects of apigenin and the Aboukyu extract in combination with N-acetyl-cysteine (NAC) was assessed. SH-SY5Y cells were treated with 0.15 mM t-BOOH in combination with 10 μ M apigenin or 200 μ g/mL Aboukyu extract in the presence or absence of NAC (Fig. 6). NAC restored oxidative stress injury in a concentration-dependent manner (10 – 100 μ M) in treated cells.

Several flavonoids such as luteolin, myricetin, and icariin confer natural neuroprotection. Luteolin has been recently shown to elicit various biological effects owing to its antioxidant and anti-apoptotic properties (López-Lázaro, 2009). Law *et al.* (2014) showed that orientin (luteolin-8-C-glucoside) protected SH-SY5Y

A. Aboukyu (200 μ g/mL) B. Apigenin (10 μ M)

Fig. 6. Effect of N-acetyl-cysteine on t-BOOH-induced oxidative stress injury in SH-SY5Y. SH-SY5Y cells were treated by t-BOOH in combination with 200 μ g/mL of Aboukyu extracts (A) or 10 μ M of apigenin (B) in the presence or absence of NAC for 18 h. The cell viability was measured by the CCK-8 assay and presented as percentage of cell viability relative to untreated control (Cont 1). Each barre presents the mean $\pm$ S.D. from two independent experiments. *, $p < 0.05$ versus NAC absent cell treated with t-BOOH in combination with apigenin.

cells from H_2O_2 -induced cytotoxicity by decreasing apoptotic cell numbers and inhibiting caspase 3/7 activities. In the present study, the neuroprotective effect of petal extracts from both Aboukyu and Enmeiraku cultivars against oxidative stress was evaluated using SH-SY5Y cells. The Enmeiraku extract (containing 0.08 – 1 μ M of luteolin) showed a significant neuroprotective effect. This may be attributable to decreased neuronal apoptosis due to flavonoids such as luteolin, which is in accordance with the above-mentioned studies reporting similar effects of luteolin glucoside (Fig. 5B). The differences in the neuroprotective effects of Aboukyu and Enmeiraku extracts might be attributable to the anti-apoptotic capacity of their phenolic compounds as well as compositional differences. Some flavonoids induce apoptosis in cancer cells, and it has been suggested that apigenin-induced apoptosis in 22Rv1 cells is initiated by a ROS-dependent disruption of the mitochondrial membrane potential through the p53 pathway (Shukla and Gupta, 2008). In the context of our results, we

hypothesized that apigenin induced apoptosis in SH-SY5Y neuroblastoma cells in combination with t-BOOH and caused cell injury at higher concentrations (Fig. 5A). This hypothesis was further supported by the fact that treating the cells with NAC restored oxidative stress injury (Fig. 6). The impaired neuroprotective effects conferred by the Aboukyu extract at concentrations $> 50 \mu\text{g/mL}$ (containing $0.26 \mu\text{M}$ apigenin) would therefore also be attributable to apoptosis (Fig. 4A). This suggests that the levels of flavonoids such as apigenin that easily induce apoptosis were higher in the Aboukyu extract than in the Enmeiraku extract. Thus, the characteristics of the neuroprotective capacity across *Chrysanthemum* cultivars differed markedly according to the flavonoid composition.

Chrysanthemum flower extracts modulate neurite outgrowth in PC12 cells Neurotrophic factors such as nerve growth factor (NGF) play an important role in regulating differentiation, survival, and functional maintenance of nerve cells (Bothwell *et al.*, 1995). Moreover, neurotrophic factors regulate microtubule-dependent extension and maintenance. Neurite loss is one of the cardinal features of neuronal injury (Enciu *et al.*, 2011). Reorganization of lost neuronal networks in the injured brain is necessary for restoration of normal physiological functions (Pesavento *et al.*, 2002). Because of these properties, neuritogenic substances are anticipated as promising therapeutic tools for the treatment of neuronal injuries. Studies suggest that specific compounds derived from natural sources induce neurite growth (More *et al.*, 2012). Nishina *et al.* found that extracts of Enmeiraku significantly induced neurite outgrowth in PC12 cells, and the components associated with the neurite outgrowth effects were 3',5,5',7-tetrahydroxyflavanone, luteolin, and acacetin (Nishina *et al.*, 2013). Quercetin is also one of the major flavonoids found in *Chrysanthemum* flowers (Kathy *et al.*, 1993) and exhibits neuritogenic effects (Sagara *et al.*, 2004).

In the present study, we therefore evaluated the neuritogenic capacity of the petal extracts of Aboukyu and Enmeiraku cultivars. Incubation of PC12 cells with the two extracts for 24 h showed subtle but significant progressive elongation of neurite-like processes (Fig. 7B, C). The maximum percentages of neurite-induced cells attributable to the two extracts were $25.3 \pm 2.5\%$ (Aboukyu) and $26.3 \pm 4.0\%$ (Enmeiraku). These percentages were lower than the cells treated with 50 ng/mL NGF ($91.7 \pm 2.8\%$) as an endogenous neuritogenic molecule. Luteolin, a typical flavonoid found in *Chrysanthemum* flowers, exhibits significant neurotrophic activity (Fig. 7E); whereas, apigenin, another typical flavonoid, shows poor neurotrophic activity (Fig. 7D). The Aboukyu extract showed lower concentrations of luteolin than the Enmeiraku extract; however, there were no significant differences in neurite outgrowth induced by the two extracts. A strong correlation between the concentration of neurite growth-inducing flavonoids and neurotrophic activity was previously suggested (Sagara *et al.*, 2004). To further evaluate the effect of the two extracts on neurite

outgrowth, it is necessary to examine other flavonoids that induce neurite outgrowth, such as 3',5,5',7-tetrahydroxyflavanone and quercetin, in addition to luteolin. Previous studies have confirmed the role of natural products in enhancing the neurite outgrowth activity of NGF (Li *et al.*, 2002). Extracts of *Chrysanthemum* cultivars only slightly induced neurite outgrowth in our present study; however, the extracts acted synergistically to induce neurite outgrowth in combination with NGF (Fig. 7G, H). Aboukyu and Enmeiraku extracts, along with 1 ng/mL NGF, synergistically increased the percentages of neurite-induced cells ($69.0 \pm 6.2\%$ and $63.7 \pm 6.7\%$, respectively). The most effective extract concentration for neurite outgrowth was $100 - 150 \mu\text{g/mL}$. Neurite length also increased by several degrees in cells treated with a combination of *Chrysanthemum* extracts and NGF compared with those treated with NGF alone (Fig. 7F, G, H). We did not, however, observe any evident differences in the synergistic effects of Aboukyu and Enmeiraku extracts on neurite outgrowth activity in combination with NGF.

The MAPK family is a group of serine/threonine protein kinases, including ERKs and p38MAPK, known to be involved in various cellular events such as survival/death, differentiation, and migration (Cobb and Goldsmith, 1995). Lin *et al.* suggested that luteolin promotes neurite outgrowth through activation of ERK and PKA signaling pathways in PC12 cells (Lin *et al.*, 2011). In addition Nishina *et al.* suggested that luteolin-stimulated neurite extension was augmented by the inhibition of p38MAPK activity (Nishina *et al.*, 2013). To evaluate the signal transmission pathway for neurite outgrowth during *Chrysanthemum* extract stimulation, we examined the effects of a specific inhibitor of ERK, U0126, and an inhibitor of p38, SB203580, on neurite outgrowth induced by two *Chrysanthemum* extracts in PC12 cells. U0126 treatment significantly inhibited neurite outgrowth induced by *Chrysanthemum* extracts (Fig. 7J, K). SB203580 treatment did not augment neurite extension, although it caused a slight inhibition of neurite outgrowth in the presence of extracts (Fig. 7M, N). These results show that ERK is an essential factor in neurite outgrowth during *chrysanthemum* extract stimulation, whereas p38 is not clearly implicated in PC12 neurogenesis in our experiments. There was no difference in response to these inhibitors in terms of PC12 neurogenesis between Aboukyu and Enmeiraku cultivars.

Activation of ERK and p38MAPK by induction of neurite outgrowth in PC12 cells treated with Chrysanthemum extracts We also compared the effects of extracts from Aboukyu and Enmeiraku on levels of phosphorylated ERK and p38MAPK in PC12 cells by immunoblot analysis. Cells were stimulated with extracts ($100, 200, 400 \mu\text{g/mL}$) of Aboukyu and Enmeiraku for 30 min. Both Aboukyu and Enmeiraku extracts significantly increased levels of pERK 1/2 (Fig. 8A). The ratio of pERK1/2 was higher in cells treated with Aboukyu compared to Enmeiraku. However, as described above, there were no significant differences in neurite outgrowth activity between cells treated with either extract.

Fig. 7. Morphology of PC12 cells treated with the extracts of chrysanthemum petals, flavonoid specimens, NGF in combination with the extracts, and inhibitors. Cells were seeded on collagen coated plates and serum starved by low serum medium for 16 h and treated with no addition (A), with 200 μ g/mL of Aboukyu extract (B), with 300 μ g/mL Enmeiraku extract (C), with 20 μ M of apigenin (D), and with 20 μ M of luteolin (E). PC12 cells were treated by combination of NGF and extracts, treated with 1 ng/mL of NGF alone (F), in combination with 100 μ g/mL of Aboukyu extract (G), and with 150 μ g/mL Enmeiraku extract (H). Effects of inhibitor U0126 or SB203580 on neurite outgrowth induced by chrysanthemum extracts in PC12 cells. Cells were preincubated with U0126 (10 μ M) or SB203580 (5 μ M) for 1 h before addition of extracts, and treated with no addition in the presence of U0126 (I) or SB203580 (L), with 200 μ g/mL of Aboukyu extract (J) or 300 μ g/mL of Enmeiraku extract (K) in the presence of U0126, and treated with 200 μ g/mL of Aboukyu extract (M) or 300 μ g/mL of Enmeiraku extract (N) in the presence of SB203580. Photographs were taken after 24 h cultured under phase-contrast observation at a magnification of 200 $\times$.

Watanabe *et al.* (2012) suggested that phosphorylation of ERK, as an upstream signal, is necessary for neurite outgrowth; however, there was no correlation between the strength of ERK activity and the length of neurite outgrowth. Levels of p-p38MAPK did not change in cells treated with either *Chrysanthemum* extract (Fig. 8 B). Flavonoids have been shown to prevent apoptosis via suppression of H₂O₂ induced p38 activation (Ishikawa and Kitamura, 2000; Choi *et al.*, 2005). Moreover, studies suggested that phosphorylation of p38MAPK is necessary for neural differentiation (Morooka and Nishida, 1998; Yung *et al.*, 2008), whereas others suggested that phosphorylation of p38MAPK inhibits neurite outgrowth (Nishina *et al.*, 2013). Our results did

not indicate that the p38MAPK pathway is involved in neurite outgrowth in cells treated with *Chrysanthemum* extracts.

Conclusion

The recent literature suggests that some natural products capable of eliciting neurotogenic activity may be useful for the treatment of neurological disorders and the maintenance of brain health (More *et al.*, 2012). Many compounds derived from natural sources have demonstrated neurotrophic and neuroprotective abilities. Nishina *et al.* found that components of *Chrysanthemum* flowers induced significant neurite outgrowth in PC12 cells (Nishina *et al.*, 2013). In the present study, to elucidate differences

Fig. 8. Chrysanthemum petal extracts of Aboukyu and Enmeiraku induced MAPK phosphorylation in PC12 cells. Cells were cultured with extracts of Aboukyu and Enmeiraku (100 – 400 µg/mL) for 30 min. Cell lysates were prepared from these cells and subjected to Western immunoblotting using anti-phospho-Erk 1/2 and anti-total Erk 1/2 antibody (A), or anti-phospho-p38 and anti-total p38 antibody (B). Data are represented as mean ratios of phospho-Erk 1/2 to total Erk 1/2 (A), or anti-phospho-p38 and anti-total p38 (B) $\pm$ S.D. from densitometric analyses. Each barre presents the mean $\pm$ S.D. from two independent experiments. *p, < 0.1 versus untreated control cells

in the neurotrophic-like effect between *Chrysanthemum* cultivars, the neuroprotective and neurite outgrowth activities of Aboukyu and Enmeiraku flower extracts were comparatively assessed. We demonstrated that differences in the neuroprotective effect of Aboukyu and Enmeiraku extracts are attributable to differences in the composition of their phenolic compounds. Petal extracts of Aboukyu and Enmeiraku cultivars synergistically enhanced the neurite outgrowth activity of NGF; however, significant differences in the synergistic effects of cultivars were not found.

The use of functional foods and components for potential health benefits has attracted attention. Recently, differences in the health benefits of nutrients from different cultivars or cultivation regions have also garnered interest. To evaluate the food functionality of *Chrysanthemum* cultivars, the relationship between chemical composition and functionality needs to be validated.

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