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Nutritional and neuroprotective characterization of 'Tadanishiki' yuzu according to harvesting period or extraction condition

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Summary

The present study investigated the phenolic profile, antioxidant activity, and neuroprotective properties of 'Tadanishiki' yuzu (*Citrus junos*, a seedless variety of yuzu) according to harvesting period and extraction condition. High-performance liquid chromatography (HPLC) was used to identify the functional components. To evaluate the neuroprotective properties, scopolamine was used to induce cholinergic dysfunction in human neuroblastoma SH-SY5Y cells pretreated with yuzu extracts. Among the harvesting periods, September provided the optimum fruit weight of yuzu and relatively high amounts of total phenolics (3.67 mg/g DW), flavonoids (10.13 mg/g DW), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (29.10 μg Vit. C eq.). Of the functional compounds, hesperidin (13.57 mg/100 g DW) and naringin (5.84 mg/100 g DW) were the highest in 5% (w/v) yuzu extracted with 80% ethanol and this extract showed the highest DPPH (289.2 μg Vit. C eq.) scavenging activity. This same extract showed the highest cell viability and lowest cortisol or acetylcholinesterase content in scopolamine-treated SH-SY5Y cells. These results indicate that 'Tadanishiki' yuzu harvested in September should be extracted at 5% (w/v) yuzu with 80% EtOH, and this extract might be useful for application as a natural functional additive.

Keywords: 'Tadanishiki', Harvest, Extraction, Neuroprotection

Introduction

Yuzu (*Citrus junos*) is a type of citrus grown in Japan and China. In Korea, it is mainly grown in southern coastal regions, including Jeju Island, Goheung, Wando, and Geoje (Shin et al., 2008), and is commonly used as a raw material for beverages and herbal medicines due to its unique flavor and effectiveness against colds. Unlike other citrus fruits, the pulp, peel, and juice of yuzu are used in Japanese and Korean cuisine, so it is easy to consume active ingredients in the peel (Fukutome, 2020). Studies suggest that the yuzu fruit peel contains more nutritionally beneficial and biologically active components than the fruit pulp (Yoo and Moon, 2016). As one of the fruits in the *Citrus* genus, yuzu has attractive aroma and notable contents of vitamin C, carotenoids, flavonoids, and citric acid, among various nutritive and non-nutritive compounds (Ji et al., 2008).

It is well known that the polyphenols and flavonoids found in yuzu fruit have potent antioxidant properties and the capacity to scavenge free radicals (Vinson et al., 2001). Hesperidin, naringin, and neohesperidin, the main flavonoids in yuzu, have antioxidant properties and can penetrate the blood-brain barrier, indicating that they prevent neurodegeneration and improve mental performance (Hirata et al., 2005). By reducing free radicals and inflammation, oral hesperidin treatment lessens the severity of rat brain damage after stroke (Raza et al., 2011). Yuzu has higher concentrations of vitamin C and phenolics than other citrus fruits, and the peel and pulp of immature yuzu contain more vitamin C as well as more overall antioxidant activity than the mature fruit because of the decline in the quantities of hesperidin, naringin, and total phenolics with fruit ripening (Yoo et al., 2004).

The composition and biological activity of fruit extracts are affected by a number of variables, including the extraction solvent, the extraction time, and the extraction temperature (Lee et al., 2015). Research has demonstrated that the total polyphenol, tannin content, and radical scavenging capacity of the 70% EtOH extract of *C. junos* are higher than those of the water extract; however, the scavenging capability of both types of extracts was dependent on their concentration (Lee and Lee, 2017). Another study reported that the antioxidant and immunological activities of 80% EtOH extracts from *C. junos* peel increased with increasing phenolics concentration (Park et al., 2008). In both cell culture and mouse models, the 70% EtOH extract of yuzu peel exerted antidiabetic effects via AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-gamma (PPARg) signaling (Kim et al., 2013).

Most studies on yuzu have focused on its physiochemical or nutritional characteristics, little was known about the influences of harvesting period or extraction way on the nutritional characteristics of yuzu. Moreover, our group previously assessed the physiochemical and functional characteristics of three major yuzu cultivars in Korea (Nam et al., 2021). This study analyzed and compared the functional compounds by HPLC and neuroprotective function of 'Tadanishiki' yuzu using human neuroblastoma cells according to harvesting period or extraction condition.

Materials and methods

Reagents and materials

The 'Tadanishiki' yuzu fruits in this study were grown at the fruit research facility of Jeonnam Agricultural Research & Extension Services (Wando, Jeonnam Province, Korea). The yuzu fruits were harvested on the 10th of each month from July to November 2020. The yuzu fruits were cut into slices, freeze-dried (FD8512, Ilshin, Korea), and pulverized (FM681C, Hanil Electric, Korea). Yuzu samples were prepared at different yuzu powder contents (1~10%, w/v; S 1%, S 3%, S 5%, S 7.5%, and S 10%) or ethanol (EtOH) content (v/v; E 10%, E 30%, E 50%, E 80%, and E 100%) and extracted by Soxhlet extraction (60 °C for 3 h) and evaporated to dryness under vacuum. Ascorbic acid, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the Folin-Denis reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Naringin, narirutin, hesperidin, and neohesperidin were purchased from ChromaDex (Irvine, CA, USA). All analytical reagents used for testing had a purity level of at least 90%.

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Functional phenolics by high-performance liquid chromatography-diode array detection (HPLC-DAD)

The method reported by WANG et al. (2008) was modified to determine the phenolic contents in yuzu using an Agilent 1216 Infinite LC Series system (Agilent Technologies, Palo Alto, CA, USA). The ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5 mm; Agilent Technologies) was used for the analysis, with mobile phases of 0.1% formic acid (solvent A) in distilled water and methanol-acetonitrile (solvent B). The absorbance was measured at 280 nm. The gradient elution program of the two solvents was as follows: A:80, B:20 at 5-10 min; A:60, B:40 at 10.1-15 min; A:50, B:50 at 15.1-20 min; A:30, B:70 at 20.1-25 min; and A:0, B:100 at 25.1-30 min. The flow rate was 0.5 mL/min, and the column temperature was 35 °C.

Total phenolic and flavonoids contents

The FOLIN and DENIS (1912) method was used to determine the total phenolic content. Sample aliquots of 30 µL were diluted by adding 32.5 µL of distilled water, followed by the addition of 12.5 µL of the Folin-Denis reagent and 6 min of reaction time in total darkness. Afterward, 12.5 µL of 7% sodium carbonate and 250 µL of distilled water were added and mixed. After 1 h, the absorbance at 760 nm was measured using a microplate spectrophotometer (Synergy HTX, Biotek Epoch, Agilent, Santa Clara, CA, USA). The total phenolic contents were calculated from a calibration curve of gallic acid as the standard. Diethylene glycol (200 µL), 2N sodium hydroxide (20 µL), and 20 µL of sample were added and reacted at 37 °C for 30 min, followed by measurement of the absorbance at 420 nm using a microplate reader. Flavonoid content was measured using a standard curve of rutin.

DPPH radical scavenging activity

The modified BLOIS (1958) method was used to determine the DPPH radical scavenging capacity. After sample aliquots of 50 µL were continuously diluted, 250 µL of 1 mM DPPH was added. The mixture was incubated at room temperature for 10 min, followed by measurement of the absorbance at 517 nm using a microplate spectrophotometer (Biotek Epoch) with ascorbic acid as the reference compound. The following equation was used to compute the DPPH radical scavenging activity:

DPPH radical scavenging activity (%) = 1 – (sample absorbance / control absorbance) × 100.

Cell culture and sample treatment

SH-SY5Y human neuroblastoma cells were purchased from the Korean Cell Line Bank (Seoul, Korea). In RPMI 1640 medium (Gibco, Waltham, MA, USA), 10% fetal bovine serum and 1% antimycotics/antibiotics (Gibco) were added to refine SH-SY5Y cells. Cells were incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 until 70-80% confluency. Cells were seeded in a 96-well plate for 24 h, and yuzu samples (10, 100, and 500 µg/mL) from 1~10% (w/v) yuzu extract (S 1%, S 3%, S 5%, S 7.5%, and S 10%) or 10~100% (v/v) EtOH content (E 10%, E 30%, E 50%, E 80%, and E 100%) were pretreated for 24 h. Theanine (10 µg/mL) was used as the positive control. Scopolamine (5 mM) was added to the SH-SY5Y cells as the stimulant. The survival rate was compared and examined, with the percentage representing the difference between the absorbance of the sample and the control. For acetylcholinesterase (AChE) quantification, 100 µg/mL of yuzu sample was used in the assay.

Cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) and acetylcholinesterase (AChE) content by enzyme-linked immunosorbent assay (ELISA)

Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide kit (Sigma). Briefly, cells were seeded onto a 96-well plate at a density of 5×10^4 cells/well. Different concentrations of yuzu extract were treated for 24 h. MTT solution was added to reach a final 0.45 mg/mL and incubated at 37 °C for 4 h. MTT solution was removed, and dimethyl sulfoxide (DMSO) was added. The absorbance was measured at 570 nm using an ELISA reader. The cell viability (%) was calculated using the following equation:

Cell viability (%) = (mean absorbance of the sample / mean absorbance of the control) ×100 AChE was quantified using an AChE ELISA kit (Cusabio, Wuhan, China). Sample-treated cells were prepared at a 500-fold dilution, and then 100 µl of diluted sample and standard were dispensed into each well and incubated at 37 °C for 2 h. Then, the liquid was removed from the well and incubated with 100 µL of biotin-antibody at 37 °C for 1 h. Conversion to AChE concentration according to absorbance was converted to a formula according to standard value.

Statistical analysis

All values were represented as the mean ± standard deviation (SD). Significant differences were determined by one-way analysis of variance (ANOVA) and Duncan's multiple range test. Significant differences were identified at $p < 0.05$. The SPSS 23.0 for Windows program (SPSS, Inc., Chicago, IL, USA) was used to conduct statistical analyses.

Results and discussion

The appearance of yuzu fruits cultivated from July to November is shown in Fig. 1. The weight or size of 'Tadanishiki' yuzu fruit increased depending on the harvest time. It is commonly assumed in Korea that the general harvesting time is November when the yuzu fruit is fully mature, the external color of the fruit is fully yellow, and the fruit attains the characteristic flavor (PHI and SAWAMURA, 2008). Yuzu peel gradually changes from green to yellow as the fruit matures (Fig. 1) because of the degradation of chlorophyll and the increase in carotenoids. Fig. 1(b) shows that the extracts of the 'Tadanishiki' yuzu fruit harvested every month from July to November contain four major flavonoids, including naringin, narirutin, hesperidin, and neohesperidin.

The primary phenolic compounds in citrus fruits are flavonoids, which have been linked to numerous health benefits, such as antioxidant, anti-inflammatory, anti-carcinogenic, and antibacterial properties (YOO and MOON, 2016). The concentrations of the individual flavonoids in 'Tadanishiki' yuzu are shown in Tab. 1. Narirutin was present at the highest concentration, followed by hesperidin, neohesperidin, and naringin. All four flavonoids were detected at the highest concentrations in yuzu harvested in July, the immature period, and tended to decrease as the harvest time was delayed. This trend was consistent with the study by NAM et al. (2021), in which the contents of hesperidin and naringin were high in immature yuzu harvested in July and then decreased steadily. Among citrus flavonoids, naringin, hesperidin, and naringenin are the most commonly studied (ZOU et al., 2016).

Results of total phenolics, total flavonoids, and antioxidant activity according to harvest time of 'Tadanishiki' yuzu are shown in Tab. 1. Consistent with the total flavonoid content, the total phenolic content was highest in yuzu harvested in July, presenting 4.88 mg/g DW. The subsequent decrease in the total phenolic content may reflect the ini-

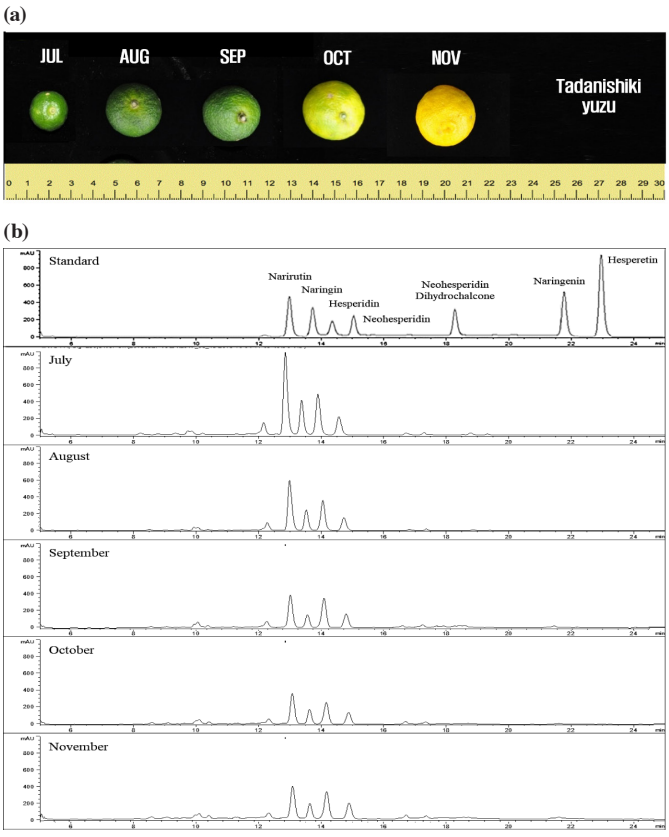

Fig. 1: (**a**) Photographs and (**b**) HPLC chromatograms of 'Tadanishiki' yuzu by harvesting period.

tiation of lignification of parenchyma cells around the secretory cavities of the peel (FREI, 2013). Color, flavor, and astringency are some other properties affected by the presence of phenolic compounds.

The flavonoid content of 'Tadanishiki' yuzu was investigated for each sample from immature in July to mature in November. The total flavonoid content in citrus fruits generally differs according to the variety, maturity period, and fruit area of the sample (DÍAZ-MULAB et al., 2009). The yuzu harvested in July showed a total flavonoid content about three times higher than the yuzu harvested in November. A rapid decline in the flavonoid content started in September. As a result, flavonoid content was found to be the highest in the immature fruit and tended to decrease as maturity progressed. Similarly, RHYU et al. (2002) reported that the flavonoid content of tangerines by harvest period generally decreased as the harvest period was delayed.

Yuzu had the highest content of narirutin from 352.00-992.90 mg/100 g (Tab. 1). This result was consistent with that of tangerines in that it showed the highest content of hesperidin and narirutin as the representative flavonoid components (YANG et al., 2019). GATTUSO et al. (2007) also reported that oranges, mandarins and lemons contained the most hesperidin but grapefruit showed the highest content of naringin among functional phenolics.

Antioxidant activity was measured using the DPPH assay. The results showed a comparable ranking of radical scavenging activity for the yuzu collected according to the harvest period from July to November (Tab. 1). The DPPH free radical scavenging activity was three-fold higher in 'Tadanishiki' yuzu harvested in July (69.09 μg Vit. C eq.) than in November (24.90 μg Vit. C eq.), rapidly decreasing from September to the final harvest (29.10 μg Vit. C eq.). Among the several antioxidant methods reported to date, the DPPH radical scavenging activity method is popular and straightforward (MATHEW and ABRAHAM, 2006). Among harvesting periods, yuzu fruit on September is optimal for practical application with fruit yield and sugar content(data not shown) with relatively high amounts of total phenolics, flavonoids, and antioxidant activity as shown in Tab. 1.

An extraction procedure is required to obtain the antioxidant components from citrus fruits (ALTEMIMI et al., 2017). Extraction is a process used to obtain plant secondary metabolites, such as alkaloids, phenolics, flavonoids, and glycosides, using selective solvents. Solvent selection is an important step in establishing the extraction procedure (AZWANIDA, 2015). EtOH is safe for human consumption when used as a solvent for natural compounds in food and natural medicine. Phenolic derivative antioxidant compounds have been effectively extracted from natural substances using absolute EtOH and aqueous EtOH (SULTANA et al., 2009). In this study, EtOH was used as the extraction solvent, and for the fruit harvested in September only, yuzu sample contents of 1-10% w/v (S 1%, S 3%, S 5%, S 7.5%, and S 10%) were analyzed for the functional components of each extract and antioxidant activity by the same methods used above. Additionally, cellular assays for cell viability and neuroprotective properties were also performed. Yuzu fruit harvested in September demonstrated higher fruit weight and size than those harvested in July and August (NAM et al., 2021). From the results of the functional content of each extract and antioxidant activity (Tab. 2), naringin and neohesperidin did not show any significant difference depending on the sample content but tended to decrease as the sample content reached 10%. Hesperidin and narirutin also showed the same tendency. The flavonoid contents were highest for S 5% and S 7.5%. The total phenolic content decreased as the sample content increased in the order of 7.10 mg/g (S 3%) > 6.82 mg/g (S 5%) > 5.18 mg/g (S 7.5%) > 4.72 mg/g (S 10%). The DPPH radical scavenging activity was contrary to the tendency of total phenols and flavonoids.

Following EtOH extraction, the functional components, total phenolics, and total flavonoids showed no significant difference between S 3% and S 5%; however, S 5% had a higher hesperidin concentration compared to S 1%, so S 5% was chosen as the sample content for the subsequent analyses. 'Tadanishiki' yuzu (S 5%) was extracted according to the EtOH content of 10-100% (E 10%, E 30%, E 50%,

Tab. 1: Functional components of yuzu extracts by harvesting period.

Harvesting period	**Total phenolics** (mg/g DW)	**Total flavonoids** (mg/g DW)	**DPPH radical** scavenging activity (μg Vit. C eq.)	**Functional compounds by HPLC** (mg/100 g DW)			
				Naringin	Narirutin	Hesperidin	Neohesperidin
July	4.88 ± 0.00[a]	26.33 ± 0.32[a]	69.09 ± 0.47[a]	298.60 ± 6.80[a]	992.90 ± 23.00[a]	690.20 ± 18.80[a]	305.00 ± 11.30[a]
August	4.02 ± 0.05[b]	16.29 ± 0.04[b]	53.83 ± 0.34[b]	194.40 ± 23.00[b]	674.30 ± 10.30[b]	430.10 ± 26.10[b]	191.80 ± 1.80[b]
September	3.67 ± 0.04[c]	10.13 ± 0.07[c]	29.10 ± 0.85[c]	111.00 ± 4.40[c]	354.40 ± 19.80[c]	273.00 ± 21.20[c]	111.40 ± 21.8[c]
October	3.22 ± 0.03[d]	9.71 ± 0.14[c]	26.40 ± 0.44[d]	88.20 ± 16.40[c]	341.80 ± 4.40[c]	261.00 ± 1.70[c]	110.60 ± 6.60[c]
November	3.05 ± 0.04[e]	9.11 ± 0.51[d]	24.90 ± 0.16[e]	96.90 ± 3.00[c]	352.00 ± 6.50[c]	250.50 ± 14.00[c]	108.70 ± 1.00[c]

[1)]Means with the same letter in each column are not significantly different by Duncan's multiple range test ($p < 0.05$). Values are represented as mean ± SD ($n = 3$).

Tab. 2: Functional components of yuzu extracts by yuzu powder content.

Yuzu powder content (w/v)	**Total phenolics** (mg/g DW)	**Total flavonoids** (mg/g DW)	**DPPH radical** scavenging activity (Vit. C eq. μg)	**Functional compounds by HPLC** (mg/100 g DW)			
				Naringin	Narirutin	Hesperidin	Neohesperidin
1.0%	8.62 ± 0.00^{a}	68.43 ± 2.21^{a}	35.61 ± 7.60^{e}	4.58 ± 0.27^{bc}	14.72 ± 0.02^{b}	10.81 ± 0.68^{b}	5.15 ± 0.09^{d}
2.5%	7.10 ± 0.13^{b}	68.49 ± 2.21^{a}	141.77 ± 5.07^{d}	5.40 ± 1.11^{ab}	16.68 ± 0.30^{b}	12.54 ± 0.32^{a}	6.02 ± 0.06^{c}
5.0%	6.82 ± 0.02^{c}	66.47 ± 0.35^{ab}	287.97 ± 4.53^{c}	5.64 ± 0.17^{a}	18.19 ± 0.08^{a}	13.27 ± 0.35^{a}	6.36 ± 0.15^{b}
7.5%	5.18 ± 0.00^{d}	64.06 ± 2.77^{b}	368.40 ± 20.10^{b}	5.84 ± 0.19^{a}	18.34 ± 0.39^{a}	13.57 ± 0.50^{a}	6.57 ± 0.08^{a}
10.0%	4.72 ± 0.02^{e}	68.91 ± 1.81^{a}	443.76 ± 24.45^{a}	3.88 ± 0.08^{c}	9.83 ± 0.15^{c}	9.38 ± 0.76^{c}	4.56 ± 0.03^{e}

$^{a-e}$ Mean with the same letter in each column are not significantly different by Duncan's multiple range test ($p < 0.05$). Values are represented as mean ± SD ($n = 3$).

Tab. 3: Functional components of yuzu extracts by extraction condition.

Extraction solvent	**Total phenolics** (mg/g DW)	**Total flavonoids** (mg/g DW)	**DPPH radical** scavenging activity Vit. C eq. μg	**Functional compounds by HPLC** (mg/100 g DW)			
				Naringin	Narirutin	Hesperidin	Neohesperidin
EtOH 10%	4.75 ± 0.05^{e}	50.02 ± 1.23^{d}	166.23 ± 9.24^{d}	4.58 ± 0.27^{bc}	14.72 ± 0.02^{b}	10.81 ± 0.68^{b}	5.15 ± 0.09^{d}
EtOH 30%	5.40 ± 0.05^{d}	58.69 ± 0.00^{c}	219.67 ± 0.36^{c}	5.40 ± 1.11^{ab}	16.68 ± 0.30^{b}	12.54 ± 0.32^{a}	6.02 ± 0.06^{c}
EtOH 50%	6.31 ± 0.01^{b}	63.29 ± 1.59^{b}	260.25 ± 10.14^{b}	5.64 ± 0.17^{a}	18.19 ± 0.08^{a}	13.27 ± 0.35^{a}	6.36 ± 0.15^{b}
EtOH 80%	6.62 ± 0.04^{a}	65.67 ± 0.26^{a}	289.23 ± 5.43^{a}	5.84 ± 0.19^{a}	18.34 ± 0.39^{a}	13.57 ± 0.50^{a}	6.57 ± 0.08^{a}
EtOH 100%	6.12 ± 0.06^{c}	57.54 ± 0.97^{c}	262.79 ± 4.71^{b}	3.88 ± 0.08^{c}	9.83 ± 0.15^{c}	9.38 ± 0.76^{c}	4.56 ± 0.03^{e}

$^{a-e}$ Mean with the same letter in each column are not significantly different by Duncan's multiple range test ($p < 0.05$). Values are represented as mean ± SD ($n = 3$). EtOH: ethanol concentration (% v/v).

E 80%, and E 100%) to investigate the functional components and antioxidant effect (Tab. 3). The naringin content was 3.88-5.84 mg/g, narirutin was 9.83-18.34 mg/g, hesperidin was 9.38-13.57 mg/g, and neohesperidin was 4.56-6.57 mg/g. Naringin and narirutin contents were highest for E 50% and E 80%, without a significant difference. Hesperidin content was highest for E 30%, E 50%, and E 80%, presenting 12.5, 13.2 and 13.5 mg/g, respectively. Neohesperidin was highest for E 80%, showing 6.57 mg/g. Phenolic compounds are mainly found in plants and are known to exhibit antioxidant power. The total phenolic content of the 'Tadanishiki' yuzu extract according to the extraction solvent concentration was 4.75-6.62 mg/g. The total flavonoid concentration ranged from 50.0 to 65.6 mg/g, with E 80% providing the highest contents of total flavonoids and total phenolics ($p < 0.05$). Flavonoids display different degrees of dissolution in water and EtOH depending on their chemical structure. Therefore, it is thought that the flavonoid content dissolved in the extraction solvent varies depending on the types of flavonoid compounds present in the sample.

It was confirmed that the DPPH radical scavenging activity of the 'Tadanishiki' yuzu extract was significantly affected by the concentration of EtOH as the extraction solvent, increasing as the EtOH content increased. According to Al-Dabbagh et al. (2018), the phenol content and the radical scavenging activity are proportional. Therefore, it is believed that the most efficient extraction method in this study would use 5% (w/v) immature yuzu harvested in September and 80% EtOH, taking into account the content of functional components and the antioxidant activity of yuzu. These results can be used as basic data for broadening the applications of yuzu in food formulations.

As a result of measuring cell viability to confirm the limit showing no toxicity in SH-SY5Y cells, toxicity was shown from the concentration of 500 μg/mL in all samples treated according to the sample concentration in the extraction (Fig. 2 and 3). However, in the S 1% and S 7.5% groups, the cell viability decreased significantly even at a concentration of 100 μg/mL, but it was 89.2% and 94.5% higher compared to the control, so concentrations up to 100 μg/mL were considered to be safe for the cell line tested. In addition, as a result of sample treatment according to the concentration of the extraction solvent, toxicity also appeared from the concentration of 500 μg/mL, and cell viability was significantly reduced even at the concentration of 100 μg/mL in E 50% and E 10%, presenting 89.75% and 92.07% compared to the control, so the experiment was conducted at a concentration of 100 μg/mL or less. Scopolamine, a non-selective muscarinic receptor, impairs short-term memory function and learning in both humans and animals (Marisco et al., 2013). Furthermore, scopolamine significantly increases the levels of AChE and malondialdehyde in the cortex and hippocampus (Tota et al., 2012a, b). Treatment of SH-SY5Y cells with scopolamine caused cell damage and death (Baral et al., 2015). The present study confirmed that cell viability was significantly reduced, and 5 mM scopolamine caused damage to nerve cells. Yuzu extract reversed scopolamine-induced neurotoxicity in SH-SY5Y cells in a concentration-dependent manner. Cell viability was most effectively increased in the group treated with S 5% and E 80% at a concentration of 100 μg/mL.

Acetylcholine is a neurotransmitter supplied to the brain by cholinergic neurons. Acetylcholine is decomposed into choline and acetate by AChE, thereby terminating neurotransmission (Shaikh et al., 2014). AChE is present in high concentrations in the synaptic cleft and generally helps to rapidly remove acetylcholine for the normal functioning of skeletal muscle (Kalamida et al., 2007). However, it has been reported that when AChE acts excessively, it suppresses the action of acetylcholine, causing nerve damage and inducing diseases, such as Alzheimer's (Kihara and Shimohama, 2004). Therefore, in this experiment, the effect of sample treatment on the content of AChE was confirmed to check the protective effect of yuzu extract in scopolamine-treated nerve cells. According to the sample and extrac-

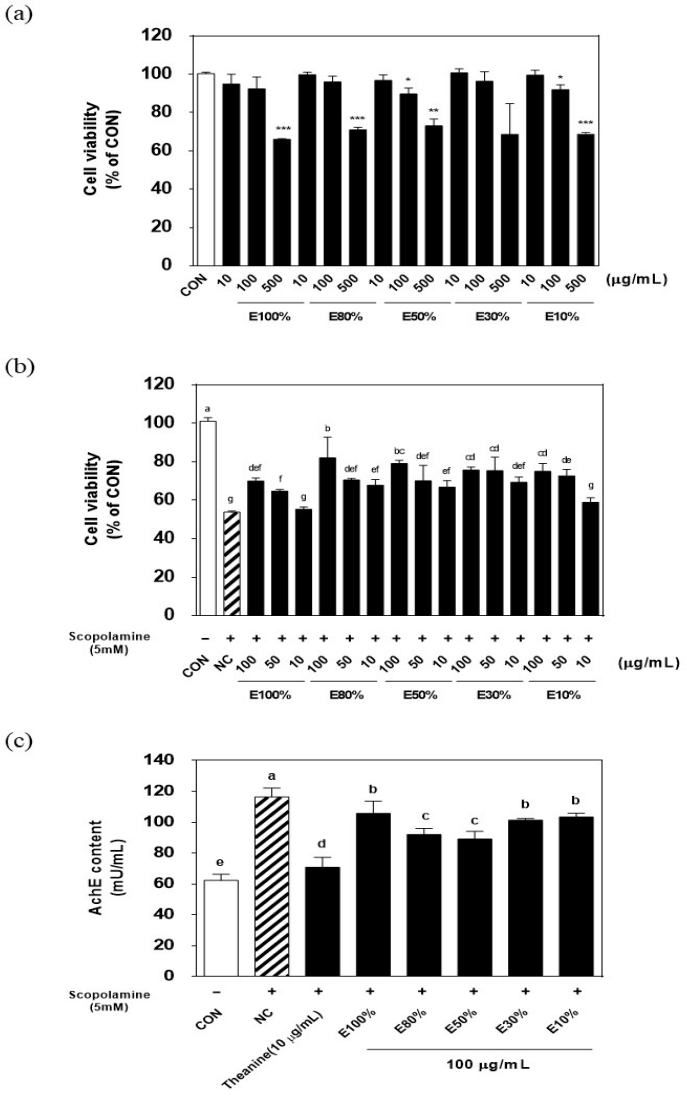

Fig. 2: Neuroprotective effects of yuzu extracts by yuzu powder contents on human neuroblastoma cells (SH-SY5Y). (**a**) Cell toxicity; (**b**) cell viability after scopolamine treatment; (**c**) acetylcholinesterase content (AChE) by ELISA. Data are shown as mean ± SD. Significance levels (*p < 0.05, **p < 0.01, ***p < 0.001) compared to respective parameter value of control group. Different letters above the bar represent a significant difference between groups by Duncan's multiple range test (p < 0.05). "S" denotes yuzu powder content (%) in extraction conditions with 80% EtOH solvent.

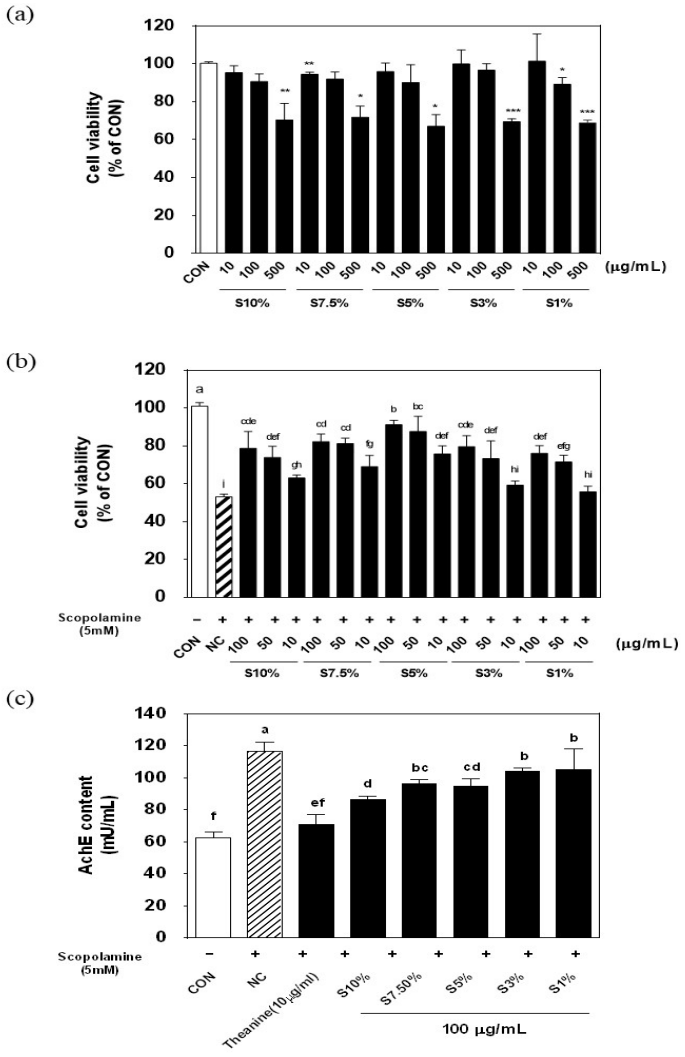

Fig. 3: Neuroprotective effects of yuzu extracts by extraction condition on human neuroblastoma cells (SH-SY5Y). (**a**) Cell toxicity; (**b**) cell viability after scopolamine treatment; (**c**) acetylcholinesterase content (AChE) by ELISA. Data are shown as mean ± SD. Significance levels (*p < 0.05, **p < 0.01, ***p < 0.001) compared to respective parameter value of control group. Different letters above the bar represent a significant difference between groups by Duncan's multiple range test (p < 0.05). "E" denotes ethanol concentration (% v/v) in extraction solvents with 5% yuzu powder.

tion solvent concentrations, the AChE content was most effectively reduced by yuzu extracted at S 5% with E 80%. The aforementioned information also revealed that this extract successfully protected the SH-SY5Y nerve cells.

Conclusions

This research revealed the phenolic compounds and functional activity (DPPH antioxidant activity and neuroprotective properties) of the extract of 'Tadanishiki' yuzu fruit according to harvest time or extraction conditions, including sample content (1-10%, w/v) and extraction solvent (EtOH 10%, 30%, 50%, 80%, and 100%, v/v). Although the yuzu harvested in July has higher total phenolics and total flavonoids, 'Tadanishiki' harvested in September was finally chosen in this study due to its reasonable fruit weight while still containing relatively high contents of functional compounds. In addition, it was confirmed that the SH-SY5Y nerve cell protection effect was most effective when the sample was extracted with 80% EtOH at 5% yuzu content. Therefore, it is suggested that this work will inform about the harvesting period of 'Tadanishiki' yuzu and its extraction condition for the highest neuroprotective effect as a functional material.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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