Title: Inhibitory effects of polyphenols from water chestnut (*Trapa japonica*) husk on glycolytic enzymes and postprandial blood glucose elevation in mice

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Running title: Water-chestnut husk Inhibitory inhibits effect of blood- glucose elevation by water chestnut husk

Abstract

Water chestnut is an annual aquatic plant that grows in Asia and Europe. Although water chestnut has been used as food and herbal medicine, its physiological functions and active ingredients are unknown. Here, we extracted polyphenols from the husk of the Japanese water chestnut (*Trapa japonica*) and assessed their effects on blood glucose levels. Three hydrolysable polyphenolics (WCPs), eugenin, 1,2,3,6-tetra-*O*-galloyl- β -D-glucopyranose, and trapain, were predominant with dry-weight contents of 2.3 ± 0.0 , 2.7 ± 0.1 , and 1.2 ± 0.1 g/100 g, respectively. These WCPs exhibited inhibitory activity against α -amylase and α -glucosidase. Whereas (-)-epigallocatechin gallate does not inhibit α -amylase, WCPs exhibited high inhibitory activity ($> 80\%$ at 0.15 mg/mL). In mice, administration of WCPs (40 mg/kg) significantly reduced blood glucose and serum insulin levels as assessed by the carbohydrate tolerance test.

Keywords: *Trapa japonica*; water chestnut; polyphenol; α -amylase; α -glucosidase; blood glucose

1. Introduction

Diabetes is a lifestyle-related disease that frequently affects the middle-aged and the young, and its incidence rises each year. Heredity and environment are considered primary factors in the development of diabetes. In particular, environmental factors are related to excessive sugar intake (Symonds, 2009). Ingested starch and sugar are degraded by α -amylase and α -glucosidase to monosaccharides, which are absorbed from the small intestine and then used for energy metabolism. Diabetes and pre-diabetes result in high blood glucose levels and can cause clinical complications, such as retinopathy, nephropathy, and neuropathy (Forbes & Cooper, 2013). Thus, the blood glucose levels must be regulated in patients with diabetes and other lifestyle-related diseases. Accordingly, many studies have investigated foods or natural products that help regulate blood glucose levels. For instance, catechin in green tea (Matsumoto, Ishigaki, Ishigaki, Iwashina, & Hara, 1993), polyphenol in leaves of persimmon (Kawakami, Aketa, Nakanami, Iizuka, & Hirayama, 2010), apple-leaf extract (Shirosaki, Koyama, & Yazawa, 2012) and chestnut astringent skin extract (Tsujita & Takaku, 2008) have been reported to inhibit increased blood glucose levels.

Water chestnut is an annual aquatic plant that grows wild in rivers, ponds, marshes, and creeks in Asia and Europe. The thorny seed overwinters by attaching to the bottom of the water body, sprouts in spring, and bears fruit in autumn. Water chestnut preserves the water environment, purifies water, and suppresses the elution of nutritional salts, such as nitrogen and phosphorus. In Asia, the fruit of water chestnut is steamed or boiled and then eaten, and its taste is similar to that of a chestnut. There are many types of water chestnut, including the Japanese water chestnut (*Trapa japonica*), Chinese water chestnut (*T. bicornis*), devil water chestnut (*T. natans*), princess water chestnut (*T. incisa*), and varieties that crossbreed naturally (Suriyagoda, Arima, Suzuki, & Hoque,

2007). The fruits of water chestnuts contain high starch levels (Hizukuri et al., 1988) and are most often eaten after boiling or are used as sources of alcohol. Moreover, the husk of water chestnut contains many dietary fibers (Wang, Ciou, & Chiang, 2009) and polyphenols (Parr, Waldron, Ng, & Parker, 1996; You et al., 2007). Although water chestnut has long been used in Chinese medicine, little is known about its physiological functions and active ingredients. In the present study, we utilized the husk of water chestnut, which is typically discarded. There were two advantages of using the husk. First, its use would reduce the waste load to the environment. Second, this would enhance the economic value of water chestnuts, which could stimulate the local economy. We investigated the effects of polyphenols from the husk of Japanese water chestnut (*T. japonica*) on glycolytic enzymes in vitro and on postprandial blood glucose elevation in vivo using mice.

2. Materials and methods

2.1. Chemicals

We used Japanese water chestnuts (*T. japonica*) picked from a creek in Saga, Japan in October 2011. The voucher specimen (NHN 0103-11/10) was deposited in the Herbarium, Faculty of Health and Nutrition Sciences, Nishikyushu University. Green tea (Sencha) was obtained from the Saga Prefectural Tea Experiment Station (Saga, Japan). Since water chestnuts sink to the bottom of creeks as soon as they fully ripen, we used them just before they were fully ripe. The water chestnuts were 1.5–2 cm in length and 2–3 cm in width. Their color was green. The water chestnuts were washed with tap water and dried under sunlight. Then, the fruit and husk were separated by cutting them with scissors. Furthermore, the husk was dried with a microwave dryer at room temperature and crushed to a fine powder (water content, 8.99 ± 0.25 g/100 g).

Gallic acid monohydrate, phenol reagent, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were of analytical grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trolox was purchased from Calbiochem (Darmstadt, Germany). Fluorescein sodium salt, α -amylase from human saliva (type IX-A), and intestinal acetone powders from rat were obtained from Sigma-Aldrich (St. Louis, MO). (-)-Epigallocatechin gallate (EGCG) and eugenin were purchased from Nagara Science Co. Ltd. (Gifu, Japan). Trapain was isolated as previously described (Nonaka, Matsumoto, & Nishioka, 1981). Polyphenols were dissolved in methanol and diluted more than 100 times with water.

2.2. Analysis of total polyphenol

The total polyphenol content in water chestnut and green tea as a comparator were determined by using the modified Folin-Ciocalteu method (Sun, Tang, & Powers, 2005). Two grams of fine powder of water chestnut was extracted with 10 mL of 60% acetone for 1 h by using an ultrasonic cleaner (3510 Branson Ultrasonics, Emerson Japan, Ltd., Kanagawa, Japan). After the solution was centrifuged at 3,000 rpm for 10 min, the supernatant was filtered through a 0.45- μ m membrane.

Each sample solution (400 μ L) and phenol reagent were diluted twice with water, mixed, and allowed to stand at room temperature for 3 min. Then, 400 μ L of sodium carbonate solution (10%, w/v) was added, and the mixture was vortexed and allowed to stand at room temperature for 1 h. The solutions were centrifuged, and their absorbance was measured at 750 nm with a V-560 UV/Vis spectrophotometer (JASCO Corp.,

Tokyo, Japan). The total polyphenol content in water chestnut husk was reported as gallic acid equivalent (GAE) per 100 g of dry weight (GAE g/100 g).

2.3. Isolation of polyphenols from water-chestnut husk

Acetone (60%, 1.5 L) was added to the crushed water chestnut husk (250 g); the mixture was incubated overnight and then filtered. The filtrate was concentrated with an evaporator to remove the acetone. The condensate was centrifuged and filtered under reduced pressure. The filtrate was further dried with an evaporator, and the concentrate was eluted using a gel-filtration column (column size; 4 cm I.D. × 40 cm) comprised of Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Step-gradient elution (300 mL for 11 steps) was performed using a water/methanol mixture of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% methanol sequentially, and the eluate was fractionated with a fraction collector (fraction volume, 30 mL). The fractions were analyzed by thin-layer chromatography (TLC; developing solution, benzene/ethyl formate/formic acid, 1:7:1 v/v), and the polyphenolic fractions were confirmed after spraying with 1% ferric chloride ethanol solution. After the polyphenol fractions were collected (fraction numbers 76–110), they were concentrated by using an evaporator. In order to further purify the polyphenol components, the polyphenolic fractions were eluted by gel-filtration chromatography (Sephadex LH-20, column size; 4 cm I. D. × 40 cm) with 80% methanol (1.8 L) as the isocratic mobile phase. The polyphenolic fractions were collected with a fraction collector (fraction volume, 15 mL; collected fraction numbers, 76–120) and were concentrated with an evaporator. The dry concentrate was termed crude WCPs.

Crude WCPs were further purified under the following conditions: HPLC equipment, Prominence LC-20AT (Shimadzu Corporation, Kyoto, Japan); column, CAPCELL

PAK C18 UG120 (250 mm × 10 mm, 5 μm, Shiseido, Tokyo, Japan); mobile phase, 0.1% formic acid/acetonitrile from 95:5% (v/v) to 70:30% (v/v) (30-min linear gradient); column temperature, 40°C; flow rate, 5 mL/min; detection, UV (280 nm). LC-MS analysis was performed using an LCMS-2020 system (Shimadzu Corporation, Kyoto, Japan) equipped with a CAPCELL PAK C18 UG120 column (150 mm × 2 mm, 3 μm; Shiseido, Tokyo, Japan). The mobile phase was the same as that used for HPLC purification. The column temperature, injection volume, flow rate, and ion polarity of MS were 40°C, 1 μL, 0.2 mL/min, and negative mode, respectively. In order to confirm the concordance between sample and the standard (eugenin or trapain), the standard was co-injected into the sample. ¹H- and ¹³C-NMR spectra were measured in acetone-*d*₆ on a JEOL JNM-AL400 (400 MHz for ¹H- and 100 MHz for ¹³C-NMR) spectrometer (JEOL Ltd., Tokyo, Japan).

Polyphenols were analyzed by HPLC under the following conditions: column, CAPCELL PAK C18 UG120 (250 mm × 4.6 mm, 5 μm, Shiseido, Tokyo, Japan); mobile phase, 0.1% formic acid/acetonitrile from 95:5% (v/v) to 70:30% (v/v) (30-min linear gradient); column temperature, 40°C; flow rate, 1 mL/min; injection volume, 10 μL; detection, UV (280 nm). Polyphenols were extracted from the water-chestnut husk as described above (2.2. analysis of total polyphenol).

2.4. Measurement of antioxidant activity

The antioxidant properties of water-chestnut husk were examined using DPPH, ABTS, and the oxygen radical absorbance capacity (ORAC) methods.

The DPPH radical-scavenging activity was evaluated using the method described by Yoo et al. (2012) with minor modifications. Briefly, 300 μL of sample solution and 900 μL of a DPPH mixture (400 μM DPPH/buffer [0.1 M MES-NaOH, pH 6]/20% ethanol,

1:1:1) were added to a microtube and stirred. After incubation at room temperature (25°C) for 20 min, the absorbance at 520 nm was measured using a V-560 spectrometer (Jasco Corp., Tokyo, Japan). The DPPH radical-scavenging activity was expressed in terms of EC₅₀.

The antioxidant properties of ABTS were analyzed using the method of Re et al. (1999). The sample solution (50 µL) and ABTS radical solution (100 µL) were added to a microplate, and the mixture was incubated at 25°C for 10 min. Then, the absorbance at 734 nm was measured using a microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT). The ABTS radical-scavenging activity was estimated in terms of Trolox equivalents (µmol TE/g).

The ORAC method was performed according to the method of Watanabe et al. (2012). Briefly, the sample solutions were diluted with the assay buffer (75 mM phosphate buffer, pH 7.0) as required. Trolox solutions at various concentrations (50, 25, 12.5, and 6.25 µmol/L in assay buffer) were prepared to obtain a standard curve. Samples solutions and the Trolox calibration solution or assay buffer (blank) (20 µL) were added to a 96-well plate. Fluorescein (200 µL, 94.4 nM) and AAPH (50 µL, 31.7 mM) were added to the 96-well plate and incubated at 37°C. The fluorescence intensity (excitation at 485 nm and emission at 520 nm) was monitored every 2 min for 90 min by using a microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT). The area under the curve (AUC) of the fluorescence decay from 8 to 90 min after the addition of AAPH was calculated for each well according to the following formula (1):

$$\text{AUC} = [(0.5 \times f_{8\text{min}}) + f_{10\text{min}} + f_{12\text{min}} + f_{14\text{min}} + \dots + f_{88\text{min}} + (0.5 \times f_{90\text{min}})] / (f_{0\text{min}} \times 2)$$

(1)

where f represents the fluorescence intensity at each time point.

Net AUC was calculated by subtracting the AUC of the blank from that of the sample or Trolox. A regression equation ($y = ax^2 + bx + c$) was computed from the graph in which the net AUC of each Trolox solution was plotted onto the x -axis, and the concentration of each Trolox solution was plotted onto the y -axis. From this regression equation, the ORAC value was calculated as (2):

$$\text{ORAC } (\mu\text{mol TE/g}) = \{[a \times (\text{netAUC}_{\text{sample}})^2] + [b \times (\text{netAUC}_{\text{sample}})] + c\} \times V \times d / W$$

(2)

where a , b , c are the coefficients of the regression equation, and V , d , W represent the volume (mL), dilution magnification, and weight of sample (mg), respectively.

Antioxidant activities were measured in triplicate.

2.5. Measurement of α -amylase and α -glucosidase inhibition

Amylase inhibition was measured using previously published methods (Miller, 1959; Deguchi, Osada, & Watanuki, 2003). Briefly, 50 μL of sample solution and 300 μL of 0.4% soluble starch solution including 5 mM calcium chloride in acetic acid/sodium acetate buffer solution (pH 6.0) were mixed in a microtube. Enzyme solution (150 μL α -amylase from human saliva, 2 U/mL in the above buffer solution) was added and incubated at 37°C for 15 min. The enzymatic reaction was stopped by heating in boiling water for 10 min. After cooling, 100 μL of 2 M sodium hydroxide and 200 μL of 0.5% dinitrosalicylic acid were added, and the solution was heated in boiling water for 10 min. After cooling, the absorbance at 540 nm was measured using a microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT).

The inhibitory activity of α -glucosidase was measured using the method by Ogawa et al. (2009) with slight modifications. This method was used to quantify glucose produced from the enzymatic breakdown of maltose or sucrose. One hundred

microliters of the substrate solution (74 mM maltose or 74 mM sucrose in 50 mM maleic acid buffer, pH 6.0) was added to 25 μ L of the sample solution; then, 25 μ L of 0.1 M maleic acid buffer (pH 6) was added, and the solution was heated at 37°C for 3 min. To prepare the sucrase enzyme solution, 1 g of rat small-intestine acetone powder was dissolved in 9 mL of 50 mM maleic acid buffer solution (pH 6.0) and stirred with an ultrasonic cleaning machine. The supernatant containing sucrase was centrifuged at 10,000 rpm for 10 min at 4°C. To obtain the maltase enzyme solution, the supernatant was diluted three times with 50 mM maleic acid buffer solution (pH 6.0). The enzyme solution (50 μ L) or 50 mM buffer solution (50 μ L) was mixed, and the reaction was allowed to proceed for 30 min at 37°C. After adding 800 μ L of distilled water, the solution was heated in boiling water for 2 min to stop the enzymatic reaction. After cooling, 50 μ L of the reaction mixture was placed in a microtube; 750 μ L of the color reagent of the glucose C-II test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added, and the solution was heated at 37 °C for 30 min (Miwa et al., 1972). Subsequently, the absorbance at 505 nm was measured using a microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT).

This procedure was repeated using distilled water as the control (AC) instead of the sample (AT). The blank of the sample (ABT) or the control (ABC) was analyzed without addition of the enzyme. The enzyme inhibitory activity was calculated using the following formula (3):

$$\text{Inhibition (\%)} = 100 - (\text{AT} - \text{ABT}) / (\text{AC} - \text{ABC}) \times 100 \quad (3)$$

We performed the same experiment to assess the inhibition of carbohydrate-catabolism enzymes. To do so, we added bovine serum albumin (BSA, Wako Pure Chemical Industries, Ltd., Osaka, Japan) to investigate whether it specifically or non-specifically inhibited these enzymes. BSA was added at the same concentration as the

carbohydrate-degrading enzymes at sample concentration of 0.13 mg/ml. The enzyme inhibitory activities were measured in triplicate.

2.6. Toxicity of WCPs

The experimental protocol was approved by the Animal Study Committee of the Tama Institute of Japan Food Research Laboratories. The toxicity of crude WCPs was examined based on acute oral toxicity in rats using the OECD Guideline for Testing of Chemicals 420 (OECD, 2001). Five-week-old female Wistar rats (BrlHan:WIST@Jcl) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Rats were housed in an air-conditioned room at $23 \pm 2^{\circ}\text{C}$ under a 12-h light/dark cycle. Food (pellet form, Labo MR Stock, Nosan Corporation, Kanagawa, Japan) and drinking water (tap water) were given *ad libitum*. After acclimatization for 1 week, the rats were divided into three groups ($n = 5$). The test group was administered crude WCPs (2,000 mg/kg), and the control group was administered only water; each group consisted of five female rats (6 weeks of age). After fasting for 17 h, rats were weighed, and WCPs solution or water was administered in a single dose with a feeding needle. The observation period was 14 days. Rats were observed several times on the day of administration and once daily thereafter. Rats were weighed seven and 14 days after administration, and all animals were autopsied at the end of the observation period.

2.7. Oral carbohydrate tolerance test in mice

Six-week-old male ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were housed in an air-conditioned room at $23 \pm 2^{\circ}\text{C}$ under a 12-h light/dark cycle. Food (pellet form, Labo MR Stock, Nosan Corporation, Kanagawa, Japan) and drinking water (tap water) were given *ad libitum*. After acclimatization for 1 week, the

mice were divided into three groups ($n = 6$). The control group was fed only starch, while the test groups were administered starch supplemented with 20 or 40 mg/kg (body weight [b.w.]) of crude WCPs. Crude WCPs were suspended in soluble potato starch solution (100 mg/mL), and test solutions (1 or 2 mg/mL) were prepared. After fasting for approximately 18 h, the blood glucose level was measured to ensure comparable blood glucose levels among the groups. The mice were administered a single 20 mL/kg b.w. dose of each starch solution by a feeding needle. Blood samples were collected from the tail vein at 0, 30, 60, 90, and 120 min after administration. Blood glucose levels and serum insulin levels were measured using Accu-Chek Aviva (Roche Diagnostics Japan, Tokyo, Japan) and mouse insulin kits (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan), respectively. The experimental protocol was approved by the Animal Study Committee of the Tama Institute of Japan Food Research Laboratories.

2.8. Statistical analysis

Data on antioxidant activities and glycolytic enzymes were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison test. Data on oral carbohydrate tolerance test in mice were analyzed for statistically significant differences between control and tested mice by two-way ANOVA followed by Dunnett's post-hoc tests. Differences were considered to be statistically significant when P was < 0.05 .

3. Results and discussion

3.1. Polyphenol content in water-chestnut husk

The total polyphenol content in the water-chestnut husk was 11.7 ± 0.2 g/100 g (dry weight), which was similar to the catechin content in green tea (12.0 ± 0.4 g/100 g). To

further investigate the polyphenols present in the water chestnut husk, they were isolated and purified. Although a large peak was observed at a retention time of approximately 5 min in the chromatogram (Fig. 1A) from the 60% acetone extract of water-chestnut husk, it likely corresponded to impurities, such as proteins, amino acids, or small polyphenols. In addition, three peaks at 21.8 (peak 1), 22.7 (peak 2), and 25.3 (peak 3) min were detected in the chromatogram. The percent areas of the large peak (5 min), peaks 1, 2, and 3 were 74, 10, 9, and 5%, respectively. Peaks 1, 2, and 3 were predominant in the crude WCPs extract after removal of the impurities by gel-filtration chromatography (Fig. 1B); these polyphenols were separated and fractionated by HPLC and were named WCP-1 (1), WCP-2 (2), and WCP-3 (3), respectively (Fig. 1C, D, and E). WCP-1 and 2 were white crystals, and WCP-3 was a yellow crystal

(Fig. 1).

LC/MS analysis of the polyphenols provided m/z values. The highest relative intensities were 937, 787, and 476 corresponding to WCP-1, WCP-2, and WCP-3, respectively. The elution time and m/z value of WCP-1 were fitted to eugenin (molecular weight = 938). The structure of eugenin has three gallic acids and one hexahydroxydiphenoyl (HHDP) group combined with glucose (Fig. 2, WCP-1; Nonaka, Harada, & Nishioka, 1980). The elution time and MS spectrum of WCP-3 were consistent with those of trapain (molecular weight = 954, Fig. 2, WCP-3; Nonaka, Matsumoto, & Nishioka, 1981). Moreover, the m/z value (476) of the largest fragment of WCP-3 indicated that the three galloyl groups (positions 1, 2, and 3) of trapain were desorbed and that one OH group and one hydrogen (6' position) were also removed. Therefore, WCP-3 was identified as a quinone-type molecule. One feature of trapain is that the 5' and 6'' positions in the HHDP group of eugenin are connected by an oxygen

atom. In the LC/MS co-injection experiment, the retention times and mass spectra did not differ between WCP-1 or WCP-3 and those of the standard (eugenin or trapain).

(Fig. 2)

In addition, the structures of WCP-1 and WCP-3 were confirmed by comparing the ¹H-NMR spectra with those of authentic samples previously isolated from the same plant source by our co-authors (Nonaka, Matsumoto, & Nishioka, 1981). The ¹H-NMR spectrum of WCP-2 showed signals at δ 4.13 (m, 2 H, H-4 and H-5), 4.59 (m, 2 H, H-6), 5.46 (t, 1 H, *J* = 8 Hz, H-2), 5.66 (t, 1 H, *J* = 9 Hz, H-3), 6.16 (d, 1 H, *J* = 8 Hz, H-1), and 6.99, 7.07, 7.08, and 7.17 (s, each 2 H, galloyl H), suggesting that this compound is a tetragalloyl glucose. This was further supported by the [M-H]⁻peak at *m/z* 787 of the ¹³C-NMR spectrum [glucose moiety: 93.4, 76.0, 75.9, 71.8, 69.3, 63.6; ester carbons: 166.6, 166.2, 165.8, 165.0]. The locations of the galloyl groups on the glucopyranose core were determined to be C-1, 2, 3, and 6 because of the low-field shift of the protons at these positions. Based on these spectroscopic analyses and by comparison of the data with those of the authentic sample, WCP-2 was identified as 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (TGG, Fig. 2, WCP-2; Tanaka, Nonaka, & Nishioka, 1983). Thus, the polyphenols present in water-chestnut husk are hydrolyzed polyphenols that have many gallic acid groups.

Furthermore, the total content of the three polyphenols in water-chestnut husk was 6.3 ± 0.1 g/100 g (dry weight). The contents of eugenin, TGG, and trapain were 2.3 ± 0.0, 2.7 ± 0.1, and 1.2 ± 0.1 g/100 g (dry weight), respectively. The total content of three polyphenols in the crude WCPs was 64.4 ± 1.0 g/100 g. In order of increasing concentration, their contents were: trapain (13.9 ± 0.2 g/100 g) < eugenin (23.7 ± 0.2 g/100 g) < TGG (26.7 ± 0.8 g/100 g). “WCPs” were defined as three polyphenols

(eugenin, TGG, and trapain), and “crude WCPs” were defined as crude polyphenols containing all three polyphenols.

3.2. Antioxidant activity

(Table 1)

Kim et al. (2014) also reported the antioxidant activity and protective effects of water-chestnut (*T. japonica*) husk extracts against tert-butylhydroperoxide-induced oxidative damage in Chang cells. However, this group did not fractionate or identify individual polyphenols in water-chestnut husk. Therefore, we examined the antioxidant activities of crude WCPs and the three purified polyphenols and compared them with those of EGCG in green tea (Table 1). The EC₅₀ values (μM) of eugenin, TGG, and trapain for DPPH radical-scavenging activity were significantly ($P < 0.01$) higher than that of EGCG. However, crude WCPs showed nearly identical radical-scavenging activity as EGCG. Moreover, in the ABTS radical method, the antioxidant activities of eugenin and trapain were significantly ($P < 0.05$ and 0.01 , respectively) higher than that of EGCG. However, the antioxidant activity of crude WCPs was significantly ($P < 0.05$) lower than that of EGCG. The results from DPPH were similar to those from ABTS. The DPPH and ABTS methods assess radical-scavenging activity based on a single electron transfer (SET). It was previously reported that these two assays behave similarly (Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005). The ORAC method uses a hydrogen atom transfer (HAT) to directly measure the antioxidant activity of a chain cleaved form. The ORAC values for crude WCPs, eugenin, and TGG were significantly ($P < 0.01$) lower than that of EGCG. The ORAC value of trapain was similar to that of EGCG. Based on comparison with EGCG, the antioxidant activity measured by the ORAC method was lower than that measured by the DPPH

and ABTS methods. Therefore, we concluded that WCPs acted through an SET mechanism rather than an HAT mechanism. The three polyphenols derived from water-chestnut husk had strong antioxidant activities (TGG < eugenin < trapain) in all assays. This is because antioxidant activity depends on the number of the polyphenolic hydroxyl groups (Kumamoto, Sonda, Nagayama, & Tabata, 2001). Since eugenin and trapain differed, it is unlikely that the 5' and 6'' positions and the quinone group at the 4' position in the hexahydroxydiphenoyl group of trapain influenced the antioxidant activity.

3.3. Inhibitory activity of carbohydrate-catabolic enzymes

All three WCPs strongly inhibited α -amylase activity [Fig. 3(a)]. In contrast, EGCG had negligible inhibitory activity. The three WCPs equally inhibited maltase [Fig. 3(b)], while their inhibitory activity against sucrase [Fig. 3(c)] was lower than that against α -amylase and maltase. The concentrations of crude WCPs, pure WCPs, and EGCG that reduced the activity of α -amylase and α -glucosidase to 50% (IC_{50}) are summarized in Table 2. The IC_{50} values of WCPs for α -amylase and maltase were considerably lower than those for sucrase. For example, the IC_{50} values of eugenin for α -amylase and maltase were one-eighth and one-fifth of those for sucrase, respectively. Moreover, the IC_{50} values of eugenin and trapain for maltase were significantly lower than that of EGCG ($P < 0.01$).

(Fig. 3)

(Table 2)

Polyphenols, such as green-tea catechins, inhibit α -glucosidase activity (Matsui et al., 2007). Although WCPs are useful in health foods, the α -amylase inhibition activity of polyphenols is not well known. It was previously reported that eugenin inhibits α -

glucosidase in Caco-2 cells, a model of small-intestinal epithelium (Toda, Kawabata, & Kasai, 2000). Since WCPs inhibited carbohydrate-catabolism enzymes, they were expected to inhibit blood-glucose elevation. Therefore, we measured the inhibition of carbohydrate-catabolism enzymes after the addition of BSA. BSA interfered with the inhibition of maltase or sucrose by crude WCPs, pure WCPs, and EGCG. On the other hand, BSA reduced the α -amylase inhibitory activities of crude WCPs, eugenin, TGG, trapain, and EGCG to 98 ± 2 , 75 ± 1 , 99 ± 0 , 97 ± 4 , and $48 \pm 8\%$, respectively. Crude WCPs, pure WCPs, and EGCG acted as non-specific inhibitors of maltase and sucrose. Eugenin and EGCG also acted as non-specific inhibitors, however, crude WCPs, TGG, and trapain acted as specific inhibitors of α -amylase.

3.4. Toxicity of WCPs

In the toxicity test, neither group displayed behavioral abnormalities or death during the observation period. In the test group, there was no significant difference in body weight between the test and control groups on days seven or 14 after administration. In addition, autopsy revealed no abnormalities in any test animal at the end of the test period. Based on these results, the LD₅₀ value (the dose causing 50% mortality) of WCPs was determined to be >2,000 mg/kg for a single oral administration in rats. Therefore, WCPs are safe in vivo.

3.5. Inhibitory effect of WCPs on blood-glucose elevation in the carbohydrate-tolerance test for mice

We investigated the effect of crude WCPs on blood-glucose elevation in mice. In the test and control groups, blood-glucose level was elevated 30 min after administration of starch solution and gradually decreased after 60 min [Fig. 4 (a)]. Thirty minutes after

administration of 40 mg/kg crude WCPs, the blood-glucose level was significantly lower than that of the control group ($P < 0.05$). Moreover, administration of 20 mg/kg WCPs reduced blood-glucose levels as compared with the control group; however, this difference was not statistically significant. The serum insulin concentration of mice treated with 40 mg/kg crude WCPs was significantly lower than in the control group after 30, 60, and 90 min ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively) [Fig. 4 (b)]. Similar results were obtained for blood-glucose levels. Thus, WCPs effectively lowered blood-glucose levels while inhibiting insulin secretion. The dose of WCPs administered to mice is likely to be non-toxic in humans; therefore, it is possible to develop a food or drink containing WCPs for the prevention of diabetes and its complications.

(Fig. 4)

Plant polyphenols, such as green-tea catechins (Matsumoto, Ishigaki, Ishigaki, Iwashina, & Hara, 1993), persimmon-leaf polyphenols (Kawakami, Aketa, Nakanami, Iizuka, & Hirayama, 2010), and apple-leaf extract (Shirosaki, Koyama, & Yazawa, 2012), inhibit increases in blood-glucose levels. This phenomenon has been attributed to the promotion of insulin secretion (Anderson & Polansky, 2002). As such, it is surprising WCPs inhibited insulin secretion in the present study. There are two possible explanations. First, since WCPs have α -amylase- and α -glucosidase-inhibitory activities, they may have reduce the production of glucose and thus prevent the need for insulin secretion. Second, WCPs may have promoted glucose uptake into cells. Indeed, a previous study showed that EGCG promoted the translocation of the insulin-sensitive glucose transporter GLUT4 to the cell membrane in muscle cells and promoted the uptake of glucose in an insulin-dependent manner (Ueda et al., 2008). Moreover, EGCG promoted the translocation of GLUT4 to the cell membrane of skeletal muscle and increased glucose uptake through the activation of AMP-activated protein kinase

(Murase, Misawa, Haramizu, & Hase, 2009; Zhang et al., 2010). Furthermore, 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (PGG), which has a structure similar to that of TGG, stimulated insulin-like glucose transport (Klein et al., 2007). Although TGG, eugenin, and trapain are also thought to activate insulin receptors and induce transport of glucose, future experiments are necessary to test this hypothesis.

This effect of polyphenols was assessed at the cellular level, and the *in vivo* metabolism of WCPs (e.g., absorption in the small intestine) remains unknown. Therefore, future studies must characterize the metabolism and absorption of WCPs. Several studies have already investigated the *in vivo* metabolism of other polyphenols. For instance, it has been reported that catechin exists in different forms *in vivo*, but most exists as a glucuronic-acid conjugate, a methylated conjugate, and a sulfate conjugate. Catechin is metabolized in the body and is eventually excreted in the feces or urine (Lee et al., 1995). In addition, the metabolism of ellagitannin, a hydrolysis-type polyphenol similar to WCPs, has also been investigated (Seeram et al., 2007; Landete, 2011). After hydrolysis, ellagitannin releases ellagic acid, which is converted into urolithin derivatives (free, methylated, sulfate, and glucuronide types) and then absorbed in the small intestine. The urolithin derivatives are transported through the blood to various organs, but most are excreted. Moreover, one study demonstrated that EGCG is hydrolyzed into low-molecular-weight compounds by tannase, and these have antioxidant activities (Hong et al., 2013). Because WCPs are high-molecular-weight compounds, they are not absorbed well *in vivo*. However, esterase is thought to break down WCPs into water-soluble polyphenols that have enhanced physiological functions (Hong et al., 2013). Nevertheless, future studies must elucidate the absorption and physiological functions of WCPs *in vivo*.

Eugeniin has been reported to have antiviral effects (Kurokawa et al., 1998; Kurokawa & Shiraki, 2005), and TGG has been reported to inhibit angiotensin conversion enzyme (Liu et al., 2003). Because the antioxidant activity of trapain was slightly higher than that of eugeniin, which has a similar configuration, it is thought that the steric structure of the diphenol group is functionally important. To date, no studies have investigated the physiological functions of trapain.

4. Conclusion

Three WCPs from the husk of water chestnut (*T. japonica*) were identified as eugeniin (2.3 ± 0.0 g/100 g), TGG (2.7 ± 0.1 g/100 g), and trapain (1.2 ± 0.1 g/100 g). WCPs exhibited high antioxidant activities and inhibited the activities of glycolytic enzymes (α -amylase and α -glucosidase). In particular, WCPs had high inhibitory activity against α -amylase, while EGCG had no such inhibitory activity. In mice, administration of crude WCPs (40 mg/kg) significantly lowered blood glucose and serum insulin levels as assessed in the carbohydrate tolerance test. Reduced insulin secretion is a unique feature that is not seen with green-tea catechins. Because WCPs inhibited blood-glucose elevation and reduced insulin secretion, they may be effective food additives for the management of type 2 diabetes mellitus.

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6. References

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Figure captions

Fig. 1. Chromatogram of (A) the 60% acetone extraction solution, (B) crude WCPs, (C), (D), and (E) purified polyphenols of the water-chestnut husk. Compounds 1–3 are WCP-1 (1), WCP-2 (2), and WCP-3 (3), respectively. The polyphenols were analyzed under the following HPLC conditions: column, CAPCELL PAK C18 UG120 (250 mm × 4.6 mm, 5 μm, Shiseido, Tokyo, Japan), mobile phase, 0.1% formic acid/acetonitrile from 95:5% (v/v) to 70:30% (v/v) (30-min linear gradient), column temperature, 40°C, flow rate, 1 mL/min, detection, UV (280 nm).

Fig. 2. Structure of the polyphenols of water-chestnut husk. WCP-1, WCP-2, and WCP-3 (3) are eugenin, 1,2,3,6-tetra-*O*-galloyl-beta-D-glucopyranose (TGG), and trapain, respectively.

Fig. 3. Inhibitory activities of the polyphenols from water-chestnut husk and EGCG on (a) α-amylase, (b) maltase, and (c) sucrase. The symbols represent crude WCPs (open square), eugenin (open circle), TGG (open triangle), trapain (filled circle), and EGCG (filled triangle). The half-maximal (50%) inhibitory concentrations (IC₅₀) of each compound are summarized in Table 2. Each point represents the mean, and each error bar shows the standard deviation (SD, n = 3). The molecular weight of crude WCPs was based on the value calculated from the contents and the molecular weights of three polyphenols (eugenin, TGG, and trapain).

Fig.4. Effects of polyphenols from water-chestnut husk on blood glucose (a) and serum insulin (b) concentrations in mice. Mice fasted for 18 h, and then 20 (open circles) or 40 (open triangles) mg/kg b.w. of crude WCPs with starch (2 g/kg b.w.) were administered.

607 As a control, mice were given only starch (filled circles). The results are expressed as
608 the mean $\pm$ standard error (SE, n = 6). * P < 0.05 and ** P < 0.01 vs. control.

Table 1

Antioxidative activities of the crude and purified polyphenols of the water-chestnut husk, and EGCG.

sample	DPPH ^a (EC ₅₀ μM)			ABTS ^b (mol TE/mol)			ORAC ^b (mol TE/mol)		
crude WCPs ^c	11.83	±	1.13	3.48	±	0.16 *	1.97	±	0.01 **
eugeniiin	6.44	±	0.02 **	6.05	±	0.34 *	2.21	±	0.13 **
TGG	8.84	±	0.38 **	4.74	±	0.18	1.53	±	0.10 **
trapain	5.83	±	0.13 **	8.94	±	0.66 **	3.20	±	0.49
EGCG	11.55	±	0.40	4.76	±	0.05	3.74	±	0.17

^aThe antioxidative activities of the samples are expressed by the molar concentration that showed inhibition of 50% of DPPH radical.

^bThe antioxidative activities of the samples are estimated in terms of Trolox equivalents (TE).

The data are presented as mean ± standard deviation (SD, *n* = 3). Statistical evaluation was performed according to the Dunnett’s method. **P* < 0.05 and ***P* < 0.01 vs. EGCG.

^cThe molecular weight of crude WCPs is used the value calculated from the contents and the molecular weights of three polyphenols (eugeniiin, TGG and trapain).

Table 2

IC₅₀ of the polyphenols of the water-chestnut husk and EGCG on carbohydrate-decomposition enzyme^a.

sample	α -amylase (μ M)			α -glucosidase (μ M)			
				maltase		sucrase	
crude WCPs ^b	53	±	9	159	±	4 **	654 ± 30 **
eugenin	42	±	5	69	±	5 **	333 ± 11 *
TGG	58	±	3	109	±	1	260 ± 13
trapain	53	±	1	83	±	2 **	259 ± 12
EGCG	-			107	±	2	268 ± 15

^aIC₅₀ means half-maximal (50%) inhibitory concentration for the activity of α -amylase or α -glucosidase. The IC₅₀ values were calculated from the data in Fig. 3.

The data are presented as mean ± standard deviation (SD, n = 3). Statistical evaluation was performed according to the Dunnett's method. **P* < 0.05 and ***P* < 0.01 vs. EGCG.

^bThe molecular weight of crude WCPs is used the value calculated from the contents and the molecular weights of three polyphenols (eugenin, TGG and trapain).

Figure(1)

Fig. 1
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Figure(2)

Fig. 2
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Figure(3)

Fig. 3
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Figure(4)

Fig. 4
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