

Original article

Peel extract of water chestnut (*Trapa bispinosa Roxb.*) inhibits glycation, degrades α -dicarbonyl compound, and breaks advanced glycation end product crosslinks

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Abstract

Objective: We evaluated the anti-glycation and glycation product degrading activities of peel extract of the water chestnut (*Trapa bispinosa Roxb.*), and studied the potential of this extract as a food ingredient to combat glycation stress.

Method: Glycation product production inhibiting activity was evaluated in an in vitro test system using a glycated protein model established by incubating glucose and human serum albumin (HSA) for 30 h at 60°C. A mixture of glucose, HSA, and water chestnut peel extract (TBE) was reacted, the amount of produced glycated proteins was compared with that of a control system not supplemented with TBE, and the production inhibition rate was calculated. In evaluating the amount of produced glycated proteins, in addition to measuring fluorescence levels characteristic of advanced glycation end products (AGEs), the amount of pentosidine and carboxymethyllysine (CML) produced were measured by ELISA, and the amount of the produced intermediate 3-deoxyglucosone (3DG) was measured by HPLC. α -Dicarbonyl compound cleavage activity was evaluated using HPLC by measuring the amount of benzoic acid produced by the degradation of α -dicarbonyl model compound 1-phenyl-1,2-propanedione (PPD) after addition of TBE. For evaluation of the AGE crosslink cleavage activity, a model system was established wherein AGE crosslinks formed on a collagen-coated plate were broken by adding TBE, and the quantity of broken AGE crosslinks was measured by ELISA and evaluated.

Results: The TBE exhibited high anti-glycation activity, with an IC₅₀ value of 2.53 μ g/mL. This value is approximately 1/50th of that of aminoguanidine. TBE also exhibited a dose-dependent production inhibitory activity against pentosidine, CML, and 3DG. Furthermore, TBE degraded α -dicarbonyl compounds and AGE crosslinks in a dose-dependent manner, and the cleavage rates were higher than those of the positive control *N*-phenacylthiazolium bromide.

Conclusion: The results indicate high activity of TBE in inhibition of glycation product synthesis, degradation of α -dicarbonyl compounds, and breaking of AGE crosslinks. This suggests that TBE may inhibit production of AGEs and their intermediates and degrade α -dicarbonyl compounds and AGE crosslinks *in vivo*, and we believe that it may alleviate glycation stress.

KEY WORDS: glycative stress, anti-glycation, AGE breaker, water chestnut (*Trapa bispinosa*)

Introduction

In recent years, glycation in organisms has been positioned as a major risk factor for accelerated aging process, and the concept of glycation stress has gained attention¹⁾. The concept of glycation stress is that the reaction of reducing sugars, aldehydes, etc. with proteins and amino acids produces advanced glycation end products (AGEs), and the accumulation of these substances overloads cells and tissues and disrupts the functioning of functional proteins as well as causes a series of reactions such as induction of inflammation²⁾.

The glycation reaction at the core of glycation stress is

a reaction in which proteins and reducing sugars bond non-enzymatically to produce glycation products. Glycation products that have been identified include the AGEs pentosidine, *N*^ε-(carboxymethyl) lysine (CML), and pyrline, and their intermediates 3-deoxyglucosone (3DG), glyoxal (GO), and methylglyoxal (MGO)³⁾. Bioaccumulation of these glycation products is said to be involved in kidney disease and eye disorders associated with progression of diabetes⁴⁾, arteriosclerosis⁵⁾, osteoporosis⁶⁾, Alzheimer's disease⁷⁾, infertility⁸⁾, and sclerema⁹⁾. Based on these facts,

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we believe that inhibiting the production and accumulation of glycation products and stimulating the degradation and metabolism of previously produced glycation products will lead to an increased life expectancy, improved quality of life, and disease prevention.

The plant we focused on in this study, the water chestnut (*Trapa bispinosa* Roxb.), is an annual aquatic plant belonging to the family *Trapaceae*. The peel of the fruit has been used in medicinal teas and other herbal medicines. Dried peel extract of the water chestnut has been reported to have beneficial properties such as antioxidant¹⁰⁾ and antibacterial activity¹¹⁾. However, there are no reports concerning its anti-glycation activity or its degradation action against glycation products. Thus, we conducted a detailed study on its anti-glycation activity and degradative action against glycation products, which are expected to prevent diseases.

Materials and Methods

Preparation of Water chestnut (*T. bispinosa*) Peel Extract (TBE)

Water chestnut peel was dried, sterilized, and crushed, and then extracted using hot water (approximately six-fold the weight of the water chestnut peel). Dextrin was added to this solution at a ratio of 67:33 of dry weight, and the resulting solution was spray-dried to obtain TBE. Sample solutions for subsequent measurements were prepared by dissolving the TBE in distilled water.

Glycated Protein Sample Preparation

To synthesize glycated proteins, a glycation reaction system of glucose and human serum albumin (HSA) was used according to previously described method.¹²⁾ A glycated protein solution was obtained by mixing 500 μL of 0.1 mol/L phosphate buffered saline (PBS) (pH 7.4), 200 μL of 4.0 mg/mL HSA (Sigma-Aldrich, St. Louis, MO, USA), 100 μL of 2.0 mol/L glucose aqueous solution, and 180 μL of distilled water, to which 20 μL of sample solution of arbitrary concentration or distilled water was added, and the mixture was incubated for 30 h at 60°C. Additionally, aminoguanidine sulfate (AG) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) instead of sample solution was dissolved in distilled water and this was used as a positive control.

The glycated protein solution was used for the measurements of anti-glycation activity, pentosidine, CML, and 3DG.

Anti-glycation Activity Measurement

Anti-glycation activity of the test substance was measured as previously described¹²⁾ by incubating HSA in the presence (glucose[+]) or absence (glucose[-]) of glucose for 30 h at 60°C. The glucose(+) reaction solution was obtained by mixing 500 μL of 0.1 mol/L PBS (pH 7.4), 200 μL of 4.0 mg/mL HSA, 100 μL of 2.0 mol/L glucose aqueous solution, and 180 μL of distilled water. The glucose(-) reaction solution was obtained by mixing 500 μL of 0.1 mol/L PBS (pH 7.4), 200 μL of 4.0 mg/mL HSA, and 280 μL of distilled water. After adding 20 μL of sample solution of arbitrary concentration to glucose(+) and glucose(-) solutions, they were incubated for 30 h at 60°C. The post-reaction glucose(+) solution was used

as a sample system solution, and the post-reaction glucose(-) solution was used as sample blank. The solutions obtained by adding distilled water instead of the sample to glucose(+) and glucose(-) solutions were used as control and blank, respectively.

A total of 200 μL of post-reaction solution was loaded into a 96-well black assay plate (BD Biosciences, San Jose, CA, USA), and fluorescence was measured at an excitation wavelength of 370 nm and emission wavelength of 440 nm using a Tecan Infinite 200 microplate reader (Tecan Japan Co., Ltd., Kanagawa, Japan).

The inhibition rate of fluorescence production in samples was calculated by comparing the fluorescence with that of the control, and 50% inhibitory concentration (IC₅₀) was calculated as anti-glycation activity.

Anti-glycation activity (%) = $100 \times (1 - ([S] - [SB])/[C] - [B]))$, where [S], [SB], [C], and [B] indicate the fluorescence levels of the sample system solution, sample blank, control, and blank, respectively.

Pentosidine Measurement

Pentosidine was measured by enzyme-linked immunosorbent assay (ELISA) as previously described¹³⁾, using an FSK pentosidine ELISA kit (Fushimi Pharmaceutical Co., Ltd., Kagawa, Japan). Fifty-microliters of each glycated protein solution or 50 μL of isotonic saline for enzyme blank measurement was added to 100 μL of pronase solution included in the kit, and 150 μL of each of these mixtures was incubated for 90 min at 55°C, after which they were heated for 15 min in boiling water to inactivate the pronase. Fifty microliters of 0.2 M disodium edetate was added to each mixture as a stabilizer to produce samples for ELISA measurements.

A total of 50 μL of sample or pentosidine standard solution was added to 96-well microplate with a solid phase of pentosidine-keyhole limpet hemocyanin antigen included in the kit, and 50 μL of anti-pentosidine rabbit polyclonal antibody solution included in the kit was added as a primary antibody to each well and mixed. The plate was hermetically sealed, and after incubation for 60 min at 37°C, the reaction solution in the wells was discarded and the wells were washed thrice with 0.0025% Tween-20 solution. After washing, 100 μL of peroxidase-labeled anti-rabbit IgG goat polyclonal antibody solution included in the kit was added as a secondary antibody to each well, and after incubating for 60 min at room temperature, the wells were washed in the same manner as described above. After washing, 100 μL of a solution containing 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added as a color developer to each well. It was incubated for 10 min in the dark, and then 100 μL of a stop solution was added to stop color development. Obtained reaction solution was subjected to absorbance measurement at primary wavelength of 450 nm and reference wavelength of 630 nm using a Tecan Infinite 200 microplate reader. The pentosidine concentration in each of the measurement samples was calculated from a pentosidine standard calibration curve and used to calculate the production inhibition rate in the samples versus the control.

CML Measurement

CML was measured by ELISA using a CircuLex CML/*N*^ε-(Carboxymethyl)lysine ELISA Kit (CycLex Co., Ltd., Nagano, Japan) as previously described¹⁴⁾. The glycated

protein solution was diluted 4-fold using dilution buffer solution included in the kit, and the resulting solution was used as the sample.

Sixty microliters of anti-CML monoclonal antibody solution included in the kit was added to 60 μ L each of the included CML-HSA standard solution, blank solution, or sample and mixed well. Hundred microliters of each mixture was added to each well of the CML-bovine serum albumin (BSA) solid phase microplate included in the kit, and the plate was incubated while shaking for 1 h at room temperature. The solution was removed from the wells, and after washing each well four times with 200 μ L of washing buffer solution containing 0.2% Tween-20, 100 μ L of horseradish peroxidase (HRP)-labeled anti-mouse IgG polyclonal antibody included in the kit was added to each well as a secondary antibody and incubated while shaking for 1 h at room temperature. After the completion of the reaction, the wells were washed using the same procedure as described above. Solution (100 μ L) containing TMB included in the kit was added to each well, and incubated while shaking for 1 h at room temperature. The reaction was terminated by adding stop solution containing 1.0 N H₂SO₄ to each well and the absorbance was measured at a primary wavelength of 450 nm and reference wavelength of 540 nm using a Tecan Infinite 200 microplate reader. The CML concentration in each of the samples was calculated from a CML-HSA standard calibration curve and then used to calculate production inhibition rate of the samples versus the control.

3DG Measurement

3DG was measured by fluorescent high performance liquid chromatography (HPLC) as previously reported¹⁵. A volume of 12.5 μ L of 0.005% 2,3-butanedione (Wako) was added to 250 μ L of each glycated protein solutions and mixed by stirring. Then, 250 μ L of 6.0% perchloric acid (Wako) was added to remove proteins, the resulting solution was centrifuged for 20 min at 3,000 rpm, and 500 μ L of supernatant was recovered. The recovered supernatant was neutralized by adding 500 μ L of saturated sodium hydrogen carbonate and then, 25 μ L of 1.0 mg/mL 2,3-diaminonaphthalene (DAN) (Dojindo Laboratories, Kumamoto, Japan) was added to the solution. After stirring, the mixture was incubated overnight at 4°C in the dark and 1.0 mL of ethyl acetate was added to the solution and stirred well. The resulting solution was centrifuged for 10 min at 3,000 rpm, and 800 μ L of the supernatant was recovered, dried under vacuum, and the dried solid was redissolved in 50 μ L of 50% methanol. The resulting solution was filtered using a 0.45- μ m Millex-HV filter unit (Merck Millipore Ltd., Billerica, MA, USA) to produce an HPLC sample.

A TSKgel ODS-80T column, 150 × 6.0 mm I.D. (Tosoh Bioscience LLC, Tokyo, Japan) was used in the analysis. The eluent was prepared with 50 mM phosphoric acid, 100% acetonitrile, and 100% methanol in the ratio 14:3:3, the flow rate was 1.0 mL/min, and the UV detection wavelength was 268 nm.

A calibration curve was constructed using 3DG-DAN standard solution (Dojindo), which was then used to calculate the 3DG concentration in each sample and the production inhibition rate versus the control.

α -Dicarbonyl Bond Cleavage Activity Measurement

Dicarbonyl bond breaking activity was determined by

measuring benzoic acid concentration obtained by degradation of the α -dicarbonyl compound, 1-phenyl-1,2-propanedione (PPD), by using HPLC as previously described¹⁶.

A PPD solution was prepared by dissolving 4.2 μ L of 98% PPD solution (Sigma-Aldrich Japan, Tokyo, Japan) in 25 mL of 50 mM PBS (pH 7.4)/50% methanol to obtain a final concentration of 1.0 mM. Hundred microliters of TBE solution of arbitrary concentration was added to 900 μ L of the prepared PPD solution, mixed, and incubated for 4 h at 37°C while shaking. The reaction was terminated by adding 200 μ L of 2.0 N HCl and the resulting solution was filtered through a 0.45- μ m filter to produce an HPLC sample.

A TSKgel ODS-80T, 150 × 6.0 mm I.D. (Tosoh) column was used in the analysis. PBS (50 mM, pH 2.2) was used as the eluent, the flow rate was 1.0 mL/min, and the detection wavelength was 230 nm UV.

The benzoic acid concentration in each measurement sample was calculated from a calibration curve constructed using solutions of benzoic acid (Wako) of various concentrations, and based on the assumption that 1.0 mM benzoic acid is obtained from 1.0 mM PPD, dicarbonyl bond breaking activity in the PPD structure was determined using the formula below.

$$\text{Dicarbonyl bond breaking activity} = 100 \times \frac{\text{benzoic acid concentration (mM)}}{1 \text{ mM}}$$

N-phenacylthiazolium bromide (PTB) (Prime Organics, Inc., Woburn, MA, USA) was used as a positive control.

AGE Crosslink Cleavage Activity Measurement

For AGE crosslink breaking activity, crosslinks formed between type I collagen and AGE-BSA were measured by ELISA as previously reported¹⁶, and the crosslink breaking rate was calculated.

Hundred microliters of 10 μ g/mL AGE-BSA (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was added to a BD BioCoat Collagen I 96-well micro test plate (BD) and incubated for 4 h at 37°C. The plate was washed five times with 0.05% Tween-20/PBS(-), and 100 μ L of reagent of each concentration dissolved in PBS(-) was added and incubated for 20 h at 37°C. After the incubation period, the wells were washed thrice with 0.05% Tween-20/PBS(-), and 100 μ L of 1.0 μ g/mL anti-bovine serum albumin rabbit polyclonal antibody solution (Rockland Immunochemicals Inc., Limerick, PA, USA) was added as a primary antibody and the mixture was incubated for 30 min at room temperature. After incubation, the wells were washed five times with 0.05% Tween-20/PBS(-), and 100 μ L of 1.0 μ g/mL HRP-labeled anti-rabbit IgG antibody (Funakoshi Co., Ltd., Tokyo, Japan) solution was added to each well as a secondary antibody and incubated for 30 min at room temperature. The wells were washed thrice with 0.05% Tween-20/PBS(-), and after washing, 100 μ L of TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was added and the wells were incubated for 15 min at room temperature. The reaction was terminated by adding 100 μ L of 1N H₂SO₄ to each well and absorbance at 450 nm was measured using a Tecan Infinite 200 microplate reader. Using the measured values of the sample and control groups, the AGE-BSA crosslink breaking rate was calculated from the following formula.

$$\text{AGE-BSA crosslink breaking rate (\%)} = 100 \times (1 - \frac{\text{absorbance of sample group}}{\text{absorbance of control group}})$$

Comparative analysis was carried out using PTB as a positive control.

Statistical Analysis

Results were expressed as mean \pm standard deviation. The significance of the results was calculated using Student's t-test or Dunnett's test. The significance level was set at $p < 0.05$ (two tailed).

Results

Figure 1 shows the anti-glycation activity results for TBE, and **Figure 2** shows the rate of production inhibition of pentosidine, CML, and 3DG by the TBE samples. The IC₅₀ values of each parameter, calculated for comparing the potency, are shown in **Table 1**. According to these results, TBE exerted an anti-glycation activity with an IC₅₀ value of 2.53 $\mu\text{g}/\text{mL}$, which is lower than the IC₅₀ of AG (128.13 $\mu\text{g}/\text{mL}$). The TBE also inhibited the synthesis of pentosidine,

CML, and 3DG in a dose-dependent manner, at IC₅₀ values of 1.23 $\mu\text{g}/\text{mL}$, 12.76 $\mu\text{g}/\text{mL}$, and 14.70 $\mu\text{g}/\text{mL}$, respectively. AG inhibited the synthesis of pentosidine, CML, and 3DG at IC₅₀ values of 1.76 $\mu\text{g}/\text{mL}$, 115.70 $\mu\text{g}/\text{mL}$, and 338.31 $\mu\text{g}/\text{mL}$, respectively, which were all higher than those of the TBE.

Figure 3 shows the results of α -dicarbonyl compound cleavage activity. The α -dicarbonyl compound cleavage rates caused by the addition of the TBE were 7.13% (100 $\mu\text{g}/\text{mL}$, $p < 0.01$) and 32.49% (1,000 $\mu\text{g}/\text{mL}$, $p < 0.01$), increasing in a dose-dependent manner. The α -dicarbonyl compound cleavage rates due to addition of PTB were 4.88% (100 $\mu\text{g}/\text{mL}$, $p < 0.01$) and 28.37% (1,000 $\mu\text{g}/\text{mL}$, $p < 0.01$), which were lower than those of the TBE.

Figure 4 shows the results of the AGE crosslink cleavage rate. The AGE crosslink cleavage rates due to the TBE were 50.66% (10 $\mu\text{g}/\text{mL}$, $p < 0.01$) and 81.90% (100 $\mu\text{g}/\text{mL}$, $p < 0.01$), increasing in a dose-dependent manner. The inhibition rates after PTB addition were 18.63% at 8.5 $\mu\text{g}/\text{mL}$ and 21.35% at a concentration of 85 $\mu\text{g}/\text{mL}$, demonstrating that the AGE crosslink cleavage rate of the TBE was higher than that of PTB.

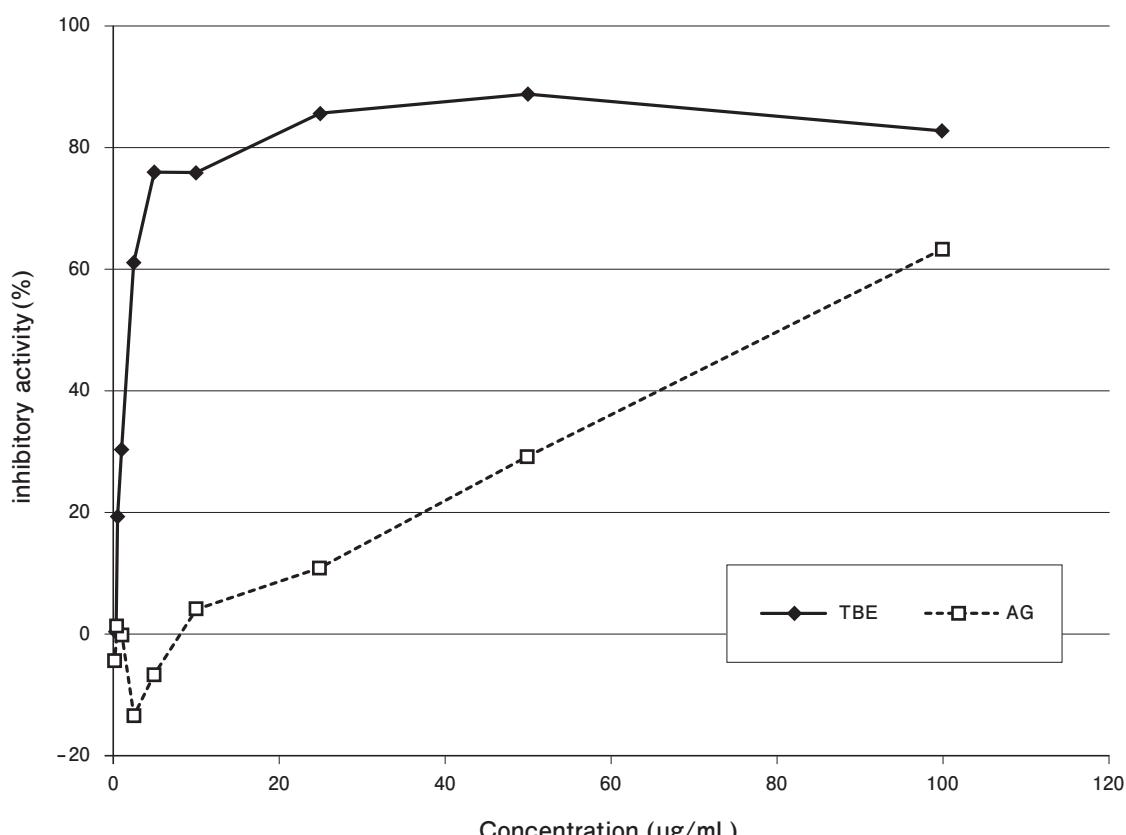
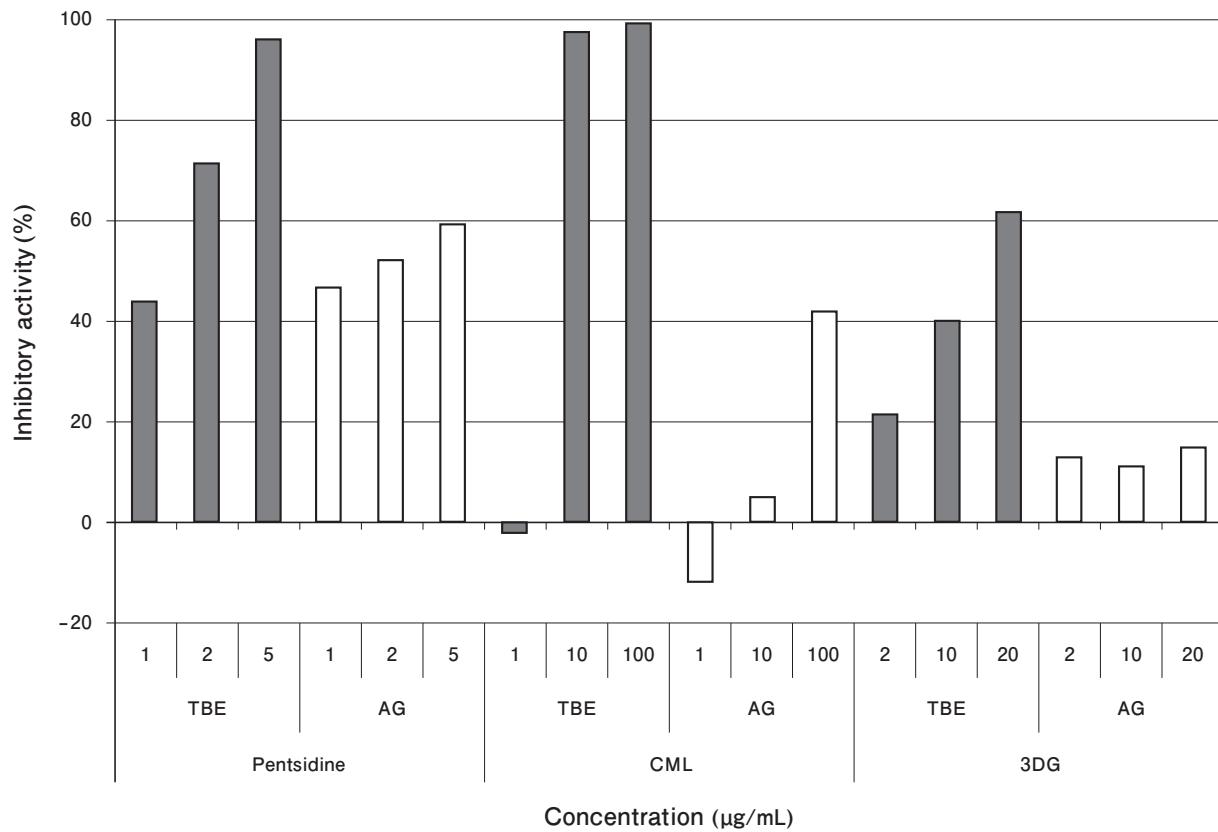


Fig 1. Inhibitory activity of TBE and AG. against fluorescent AGE formation.

AGE, advanced glycation end product; TBE, water chestnut (*T. bispinosa*) peel extract; AG, aminoguanidine.

**Fig 2. Inhibitory activity of TBE and AG against pentosidine, CML and 3DG formation.**TBE, water chestnut (*T. bispinosa*) peel extract; AG, aminoguanidine; CML, carboxymethyllysine; 3DG, 3-deoxyglucosone.**Table 1. Inhibitory activity against formation of fluorescent AGEs and each glycated products.**

Sample name	Fluorescent AGEs	Pentosidine	CML	3DG
IC ₅₀ of AG (µg/mL)	128.13	1.76	115.70	338.31
IC ₅₀ of TBE (µg/mL)	2.53	1.23	12.76	14.70

AGEs, advanced glycation end products; IC₅₀, 50% inhibitory concentration; CML, carboxymethyllysine; 3DG, 3-deoxyglucosone; AG, aminoguanidine; TBE, water chestnut (*T. bispinosa*) peel extract.

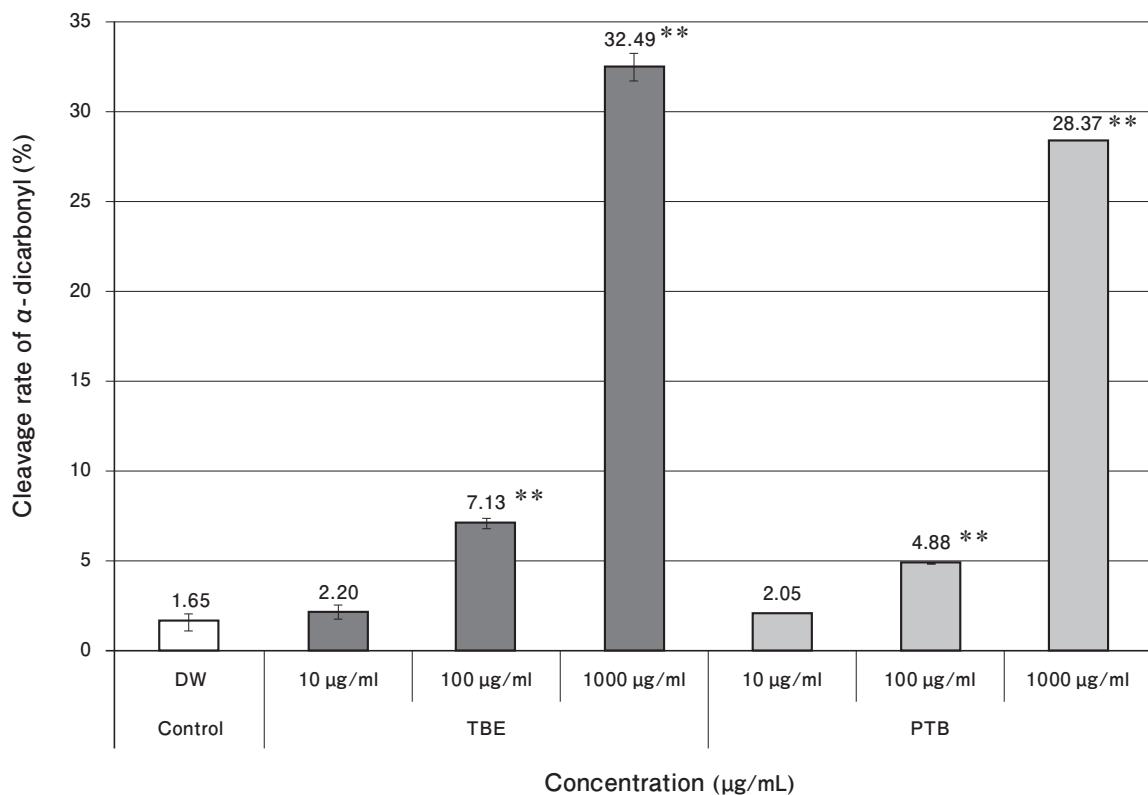


Fig 3. Cleavage activity of TBE and PTB against α -dicarbonyl structure in PPD.

Results are expressed as means \pm standard deviation. ** p < 0.01, vs. control, Student's t test. DW, distilled water; TBE, water chestnut (*T. bispinosa*) peel extract; PTB, *N*-phenacylthiazolium bromide; PPD, 1-phenyl-1,2-propanedione. PTB has been proposed the mechanism for cleaving C-C bond.

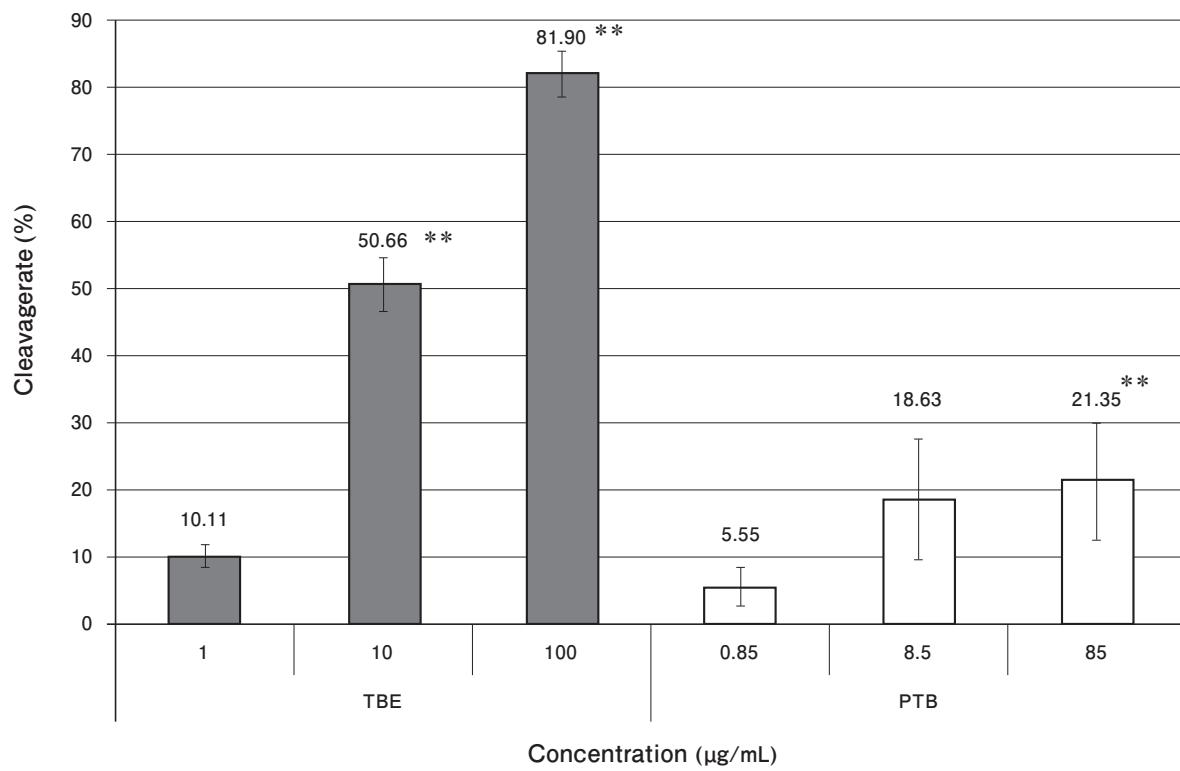


Fig 4. Cleavage rate of TBE and PTB against AGE-BSA-collagen crosslinks.

Results are expressed as means \pm standard deviation. ** p < 0.01, vs. control, Dunnett's test. TBE, water chestnut (*T. bispinosa*) peel extract; PTB, *N*-phenacylthiazolium bromide; AGE, advanced glycation end product; BSA, bovine serum albumin..

Discussion

The water chestnut fruit used as the subject of this study is a large fruit among plants in the *Trapaceae* family ¹⁷⁾. Its flesh is consumed in Thailand and China, while in Japan, the peel is dried to produce tea and is distributed as a specialty of Fukuoka and Saga Prefectures. Because there is a long history of consumption of this fruit in local diets, we expect that the fruit can be used as a safe plant-based ingredient. However, the efficacy of ingesting water chestnuts is mostly anecdotal, with only few scientific reports. Thus, we studied the inhibition of glycation product synthesis and the degradative activity against glycation product by the TBE using an *in vitro* test system, and observed that the TBE has a strong anti-glycation activity. In particular, by comparing the IC₅₀ values, we found that the anti-glycation activity of the TBE was approximately 50-fold higher than that of AG, and the activity of the TBE against production of pentosidine, CML, and 3DG were nearly equal, nine-fold, and 23-fold stronger, respectively, than those of AG. Based on these results, we believe that TBE is a potent anti-glycation ingredient, which acts at a much lower concentration than AG.

AG binds to the keto group of Amadori compounds and 3DG, which are intermediates of the glycation reaction, and inhibits further progress of the reaction ¹⁸⁾. In addition to a report concerning inhibition of glycation reaction *in vitro* ¹⁹⁾, it has also been suggested to have effects on kidney disease ^{20, 21)} and inhibit the onset or progression of diabetic, neurological, and vascular disorders ^{22, 23)} in diabetic rats. The results of this study show that TBE has a more potent anti-glycation action than AG, and we expect that the TBE will exhibit efficacy *in vivo* when ingested.

Next, we ascertained that the TBE has α -dicarbonyl compound cleavage action and AGE crosslink cleavage action, which is higher than that exerted by positive control PTB. The α -dicarbonyl structure is thought to be present in some AGEs as well as the highly reactive glycation intermediates 3DG, GO, and MGO ³⁾. It is also believed that the AGE crosslink structure with collagen influences the degree of diabetic complications ²⁴⁾. For this reason, degradation of previously produced α -dicarbonyl compounds and AGE crosslinks in the body leads to improvement of disease severity, and research on “AGE breakers” has progressed. In addition to PTB ²⁵⁾,

which was used as a positive control in this study, AGE crosslink cleavage activity has also been reported for phenyl-4,5-dimethylthiazolium chloride (ALT-711), which is a more stabilized version of PTB, and 3-benzyloxycarbonylmethyl-4-methyl-thiazol-3-iium bromide (C36), which has a different structure ^{26, 27)}. The possibility of ALT-711 breaking AGE crosslinks formed in α -crystallin derived from a human lens in an *in vitro* test system has been suggested ²⁸⁾, and in a human clinical study, a pulse pressure improvement effect was observed after consumption of 210 mg/day of ALT-711 for 8 weeks in men and women aged 50 years and older with cardiovascular sclerosis ²⁹⁾. Based on those studies, we believe that ingredients that are capable of degrading α -dicarbonyl compounds and breaking AGE crosslinks in an *in vitro* test system may also be involved in solubility and mobility of structural proteins *in vivo*. Additionally, since TBE has a carbonyl bond cleavage activity similar to PTB and a more potent AGE crosslink cleavage activity than PTB, we believe the TBE may act as an AGE breaker *in vivo*.

Other plants from the genus *Trapa* are also expected to be functional food ingredients. For example, peel extract of the related species *Trapa japonica* Flerov ³⁰⁾ is reported

to inhibit glucose metabolizing enzymes amylase and glucosidase as well as inhibit the postprandial hyperglycemia in rats ³¹⁾, whereas the extract of a related species, *Trapa natans*, is reported to reduce blood glucose concentration in streptozotocin-induced diabetic rats.

The glycation response inhibiting activity and glycation product degradation activity of the TBE confirm its production inhibitory effect against AGEs and its effect in reducing the amount of accumulated AGEs by degradation of previously produced AGEs. Further, since activities other than anti-glycation have been demonstrated, we expect TBE to have wide applications as an anti-aging ingredient. We plan to study its efficacy in humans in the future.

Conclusion

The study verified TBE to inhibit glycation product synthesis, degrade α -dicarbonyl compound, and break the AGE crosslinks. The above results suggest that TBE may inhibit production of AGEs and their intermediates and degrade existing α -dicarbonyl compounds and AGE crosslinks *in vivo*. We expect that TBE may be suitable to reduce glycation stress.

Conflicts of interest statement

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