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Short communication

High molecular weight persimmon tannin is a potent antioxidant both *ex vivo* and *in vivo*

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ABSTRACT

The antioxidant activities of high molecular weight persimmon condensed tannin (HMWPT) were evaluated in an *ex vivo* tissue system and *in vivo*. Addition of HMWPT to mouse liver homogenate protected the samples against auto-peroxidation or H_2O_2- or Fe^{2+}/ascorbic acid-induced lipid peroxidation. The IC_{50} values for HMWPT were 4.32 ± 0.20 μg/mL (auto-oxidation), 1.36 ± 0.40 μg/mL (H_2O_2-induced peroxidation) or 0.20 ± 0.09 mg/mL (Fe^{2+}/ascorbic acid-induced peroxidation). Mice were oxidatively stressed with bromobenzene to test the antioxidant activity of HMWPT *in vivo*. An oral dose of HMWPT at 200 or 400 mg/kg HMWPT significantly ($P<0.01$) prevented the bromobenzene-induced decrease in serum and liver superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, and decreased liver malondialdehyde levels in bromobenzene-treated mice ($P<0.01$). The results suggest that dietary HMWPT may provide protection from oxidative damage both *ex vivo* and *in vivo*.

1. Introduction

Persimmon (*Diospyros kaki L.*) is cultivated widely in China, Japan and Korea, with China accounting for 70% of the world's production in 2008 (FAOSTAT, 2010). Persimmon has been traditionally used to treat conditions such as coughs, hypertension, dyspnoea, paralysis, frostbite, burns and bleeding (Matsuo & Ito, 1978; Mowat, 1990). Foods containing condensed tannin, such as persimmon fruit, have been associated with antioxidant activity, anti-inflammatory activity and atherosclerosis prevention. Gorinstein et al. (2000) demonstrated that a persimmon-supplemented diet significantly lowered plasma total cholesterol, triglycerides and LDL-cholesterol in rats fed a high cholesterol diet. Recent studies showed that the persimmon also reverses antitumor and multidrug resistance (Kawase et al., 2003), is hypocholesterolemic and is an antioxidant (Del Bubba et al., 2009; Dembitsky et al., 2011; Ercisli, Akbulut, Ozdemir, Sengul, & Orhani, 2008; Fukai et al., 2009; Gorinstein et al., 1998; Kondo, Yoshikawa, & Katayama, 2004; Park, Jung, Kang, & Efren, 2006), is antidiabetic (Lee, Chung, & Lee, 2006), and protects DNA against oxidative damage (Jang et al., 2009). However, most of these studies were conducted with crude persimmon extracts or persimmon peel and pulp as test materials. The crude plant extract is a complex mixture containing vitamins, *p*-coumaric acid, gallic acid, catechin, flavonoids, carotenoids and condensed tannin (Mallavadhani, Panda, & Rao, 1998). It is not clear which of these compounds is associated with the beneficial effects of consuming persimmon or persimmon extracts.

We have previously reported that high molecular weight persimmon condensed tannin (HMWPT) is the main antioxidant in persimmon pulp (Gu et al., 2008). HMWPT was more effective than grape seed proanthocyanidin (GSP) in scavenging hydroxyl or superoxide anion radicals, and in inhibiting linoleic acid peroxidation, suggesting that persimmon may be a source of therapeutically useful polyphenolics. However, our previous work was carried out exclusively with *in vitro* models. The antioxidant potential of persimmon high molecular weight tannin has not been directly tested *in vivo*. The importance of testing antioxidant capacity of a compound in tissue systems and *in vivo* is that the antioxidant capacity of a plant derivative in simple model systems may not necessary reflect its activity in more complex biological systems, including tissues and animals. The antioxidant capacity of condensed tannins may be confounded by biomolecules such as proteins in the alimentary canal or blood (Riedl & Hagerman, 2001), and by bioavailability and biotransformation. Therefore, we proposed to evaluate the antioxidant capacity of persimmon high molecular weight tannin in both an *ex vivo* tissue system and *in vivo*.

2. Materials and methods

2.1. Chemicals

1,1,3,3-tetraethoxypropane (TEP) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ascorbic Acid, H_2O_2, and bromobenzene were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Commercial kits used for determination of protein,

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malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). GSP was obtained from FuZhou Corona Science & Technology Development Co., Ltd. (FuJian, China). All solvents and reagents were analytical grade.

2.2. Plant material and sample preparation

Mature and fully colored fruits of the astringent persimmon (*D. kaki Niuxin shi)* were harvested in late October from an orchard in Shan'xi (China). After harvest, fruits were held at 100 °C in water for about 5 min to inactivate polyphenol oxidase, and were then stored deep frozen at −20 °C. The high molecular weight persimmon tannin (HMWPT) was prepared according to the method of Gu et al. (2008). The content of total polyphenols in HMWPT was 98.7% on a mass basis by the Folin–Denis method (Gahler, Otto, & Bohm, 2003) using gallic acid as a standard and the condensed tannins content was 93.4% on a mass basis by the acid butanol assay (Porter, Hrstich, & Chan, 1986) with apple procyanidin dimers as a standard (Li, Trombley, Schmidt, & Hagerman, 2010).

The HMWPT was characterized by MALDI-TOF, thiolysis-HPLC-ESI-MS and NMR (Li, Leverence, et al., 2010). The mean degree of polymerization of HMWPT was estimated to be 26 by thiolysis. The extension units were epicatechin, epigallocatechin, (epi) gallocatechin-3-*O*-gallate, and (epi) catechin-3-*O*-gallate with the relative moles of 2.78, 3.95, 11.0 and 7.58, respectively, and the terminal units were catechin, (epi) gallocatechin-3-*O*-gallate, and myricetin with the relative moles of 0.29, 0.26, and 0.45. GSP that was reported to have a total phenol content of 99.0% and oligomer content above 70% (manufacturer's literature) was used as comparison. All animal studies were approved by the Animal Ethics Committee of Huazhong Agricultural University.

2.3. Ex vivo determination of lipid peroxidation in mice liver homogenate

Kunming mice weighing 18–20 g were purchased from Hubei Provincial Center of Disease Prevention and Control (Wuhan, China). Equal numbers of male and female mice were sacrificed by cervical dislocation and livers were removed. Each individual mouse was a replicate. The livers were homogenized in cold normal saline (1 g tissue in 9 mL normal saline) and the homogenates were stored at 4 °C for the determination of thiobarbituric acid reactive substances (TBARS). Lipid peroxidation was assessed by measuring TBARS in the liver homogenates (Susanta, Goutam, Gupta, & Mazumder, 2006) after auto-peroxidation or induction by Fe^{2+}/ascorbic acid or H_2O_2.

Auto-peroxidation was conducted with mixtures of 1.0 mL of liver homogenate and 1.0 mL of HMWPT (0–1 mg/mL). H_2O_2- or Fe^{2+}/ascorbic acid-induced lipid peroxidation was carried out using samples containing 1.0 mL of liver homogenate and 1.0 mL of HMWPT (0–1 mg/mL)., with either 0.05 mL of 1 mM ascorbic acid and 0.05 mL of 0.1 mM $FeSO_4$, or 0.1 mL of 0.1 M H_2O_2 added to each sample before incubating the mixtures at 37 °C for 1.5 h. Normal saline replaced the tannin in the blanks, and the positive controls were prepared with 1 mL of 1 mg/mL GSP instead of sample solution HMWPT. The reactions were stopped by the addition of 2 mL of 10% (w/v) trichloroacetic acid followed by addition of 1 ml of 0.67% (w/v) thiobarbituric acid (TBA). The mixtures were placed in a boiling water bath for 15 min, cooled in ice-cold water and centrifuged at 5000×*g* for 15 min before reading the absorbances of the supernatants at 532 nm. A standard curve was obtained by using TEP as a standard to estimate the concentration of TBARS as MDA equivalents, which were expressed as nmol per mg of protein. The IC_{50} value was obtained by regression analysis from the dose–response curve plotted with MDA equivalents against the log of sample concentration.

2.4. Animal treatment

Male Kunming mice weighing 18–22 g were purchased from the Experimental Animal Center of Disease Prevention and Control of Hubei province (Wuhan, China). The mice were housed in a temperature and humidity controlled room with a 12 h light–dark cycle and free access to a standard pellet diet and drinking water. After an adaptation period of one week, the mice were randomly divided into six groups, each consisting of 10 mice. The six groups were designed as follows: an untreated control group that received only the vehicle (no treatment/no bromobenzene); a bromobenzene-stressed group that received bromobenzene but no treatment; a GSP-treated group that was pretreated with 400 mg/kgBW GSP intragastrically (*i.g.*) daily for 30 days before bromobenzene stress; and low, medium and high HMWPT-treated groups that were pretreated with 100 mg/kgBW, 200 mg/kgBW or 400 mg/kgBW HMWPT *i.g.* daily for 30 days before bromobenzene stress. During the 30 days of pre-treatment, the untreated control and bromobenzene/no treatment mice received 0.2 mL of saline *i.g.* daily. After the pre-treatment, animals in all groups except the untreated control were challenged with bromobenzene dispersed in olive oil at a dose of 160 mg/kgBW *i.g.,* while mice in the untreated group were treated with olive oil only. The animals were sacrificed by decapitation 24 h after the challenge and blood and liver were collected for the determination of GSH-Px, SOD, MDA and protein.

2.5. Measurement of SOD, GSH-Px activity

After mice were sacrificed, blood was collected and livers were removed rapidly and homogenized in 9 volumes of ice cold saline followed by centrifugation at 5000×*g* for 15 min. Serum was obtained by centrifuging the whole blood at 4000×*g* at 4 °C for 10 min. The activities of SOD and GSH-Px were determined using commercial kits. The assay for SOD was based on its ability to inhibit of the oxidation of oxyamine by the xanthine–xanthine oxidase system. One unit (U) of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50% after reacting for 10 min, and data were expressed as U/mg protein (Oyanagui, 1984). The activity of GSH-Px was determined by quantifying the rate of enzyme-catalysed oxidation of GSH per minute (Yang et al., 2010). One unit (U) of GSH-Px was defined as a decrease of 1.0 μM GSH per 5 min at 37 °C, and data were expressed as U/mg protein for liver and U/mL for serum.

2.6. Protein assay

Protein concentration was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Bovine serum albumin was used as the standard.

2.7. Statistics

The data are presented as means ± standard deviation (SD). Statistical Product and Service Solutions (SPSS 13.0) or Prism Graph Pad (4.0) were used to calculate IC_{50} values and analyze the variance (ANOVA). P-values <0.05 were regarded as significant.

3. Results and discussion

3.1. The inhibition of lipid peroxidation in liver homogenates by HMWPT

As indicated in our previous work (Gu et al., 2008), HMWPT is a potent antioxidant which scavenges hydroxyl or superoxide anion radicals and inhibits linoleic acid peroxidation. Testing HMWPT in tissue or animal models is critical to evaluating its activity, so here we describe the results of using either tissues or live animals to evaluate HMWPT.