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Antioxidant potential in non-extractable fractions of dried persimmon (*Diospyros kaki* Thunb.)

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ABSTRACT

Dried fruits of persimmon (*Diospyros kaki* Thunb.) are a traditional food in Japan and contain large quantities of tannins. In this study, we investigated the *in vitro* and *in vivo* antioxidant potentials of non-extractable fractions from dried persimmons. Hydrolysed non-extractable fractions showed the highest antioxidant activities *in vitro*. In subsequent experiments, the plasma oxygen radical absorbance capacity (ORAC) values in rats supplemented with a 5% non-extractable fraction were approximately 1.5 times higher than those in control rats after 1 week *in vivo*. Furthermore, using an *in vitro* model of the gastrointestinal tract, the ORAC values of the non-extractable fraction were significantly increased with colonic fermentation in the large bowel stage. These data indicate that non-extractable fractions may possess significant antioxidant potential *in vitro* and *in vivo*.

1. Introduction

Fruits and vegetables are important sources of minerals, vitamins and dietary fibre, and contain natural antioxidants that may decrease disease risks (Basu, Newman, Bryant, Lyons, & Betts, 2013; Seifried, Anderson, Fisher, & Milner, 2007). Thus, fruits and vegetables are considered valuable contributors to human health (Michels et al., 2000; Nöthlings et al., 2008). Persimmon fruits (*Diospyros kaki* Thunb.) contain beneficial compounds, such as condensed tannin and carotenoids. Soluble tannins in astringent persimmon fruits are converted into insoluble tannins via a dehydration processes, and dried persimmons lose their bitterness and present a sweet taste. Moreover, condensed tannins are reportedly polymerized flavanols and are non-hydrolysable, but have a high antioxidant activity (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009). The astringent persimmon fruits contain large quantities of tannin, and various studies demonstrate their hypolipidemic actions (Gorinstein, Bartnikowska, Kulasek, Zems, & Trakhtenberg, 1998; Gorinstein et al., 2000, 2011; Matsumoto, Yokoyama, & Gato, 2010; Matsumoto et al., 2011; Uchida et al., 1990), condensed tannin compositions (Akagi et al., 2010) and antioxidant activities (Chen, Fan, Yue, Wu, & Li, 2008). Dietary antioxidants may reduce oxidative stress to DNA, proteins and lipids; however, data demonstrating that these antioxidants act as efficiently *in vivo* as they do *in vitro* is insufficient. Saura-Calixto (2012) showed that non-extractable polyphenols exhibit physiological and biological activities and have health-related properties. In addition, soluble extracts and insoluble macromolecular substances were previously evaluated to elucidate dietary antioxidant capacity (Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2015). In most previous studies, the antioxidant activities of fruits and vegetables have been evaluated pertaining to their soluble extracts and antioxidant constituents of non-extractable fractions have received little attention.

In this study, we investigated the antioxidant activities of non-extractable fractions from dried persimmon fruits *in vitro* and *in vivo*. In addition, non-extractable fractions were digested in an *in vitro* model of the gastrointestinal tract and antioxidant activities were investigated at each digestive stage.

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2. Materials and methods

2.1. Plant material

Astringent persimmons (*D. kaki* Thunb., cv. 'Hohrenbo') harvested in Yoshino-gun, Nara, Japan, were peeled and sulfurated to prevent discoloration and oxidation; furthermore, they were processed into dried persimmons by air drying for 1 month. Dried persimmons were provided by Ishii-Bussan, Inc. (Nara, Japan).

2.2. Chemicals and apparatus

Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and fluorescein sodium salt were obtained from Sigma–Aldrich Japan Co., (Tokyo, Japan). The 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and catechin mixture from green tea were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). All reagents were of analytical grade and were commercially available.

Column chromatography was performed using a Diaion HP-20 column (Mitsubishi Chemical Co., Tokyo, Japan) and ORAC values were determined using a PerkinElmer 2030 Multilabel Microplate Reader ARVO™ X4 (PerkinElmer Japan Co., Ltd., Kanagawa, Japan). Absorbance measurements of total phenol contents and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities were performed using a Shimadzu UV mini-1240 spectrophotometer (Shimadzu Co., Kyoto, Japan).

2.3. Sample preparation

Dried persimmon (1.72 kg) was pitted (1.49 kg of edible portion), cut into small pieces and homogenized in 10 l of 90% aqueous ethanol (EtOH), and the extract was filtered from the homogenate. Another 10 l of 90% aqueous EtOH was added to the residue. These extracting and filtering procedures were repeated three times and the combined extract was evaporated *in vacuo* to remove EtOH. The extract was dissolved in water, hexane was added, and then partitioned to obtain water- and hexane-soluble fractions (4.1 g). The water-soluble fraction was separated by column chromatography using a DIAION HP-20 column with H_2O as an eluting solution followed by elution with MeOH. All solutions were evaporated *in vacuo* to give H_2O (1.28 kg) and MeOH (3.4 g) eluates. Furthermore, the extracting residue was dehydrated to obtain the non-extractable fraction (352 g).

2.4. Preparation of hydrolysed non-extracted fractions

Portions (100 mg) of non-extracted fractions were heated at 90 °C for 3 h with 5 ml of a 1.2 N HCl–50% MeOH solution in screw-capped tubes and then centrifuged at 1750*g* for 15 min at room temperature to obtain the supernatant. Subsequently, 5 ml of a 1.2 N HCl–50% MeOH solution was added to the precipitates; this solution was then heated and centrifuged twice. Combined supernatants were diluted to 25 ml using MeOH.

2.5. Analysis of total phenol contents

Phenol contents of each fraction were measured using the modified Folin–Ciocalteau method (Singleton & Rossi, 1965). Measurements were normalized using a gallic acid standard curve, and data were expressed as percentages of gallic acid equivalents in the edible portion of dried persimmons.

2.6. DPPH radical-scavenging activity

Antioxidant activities of each fraction were estimated based on scavenging activities of the stable DPPH free radical using the method previously described by Blois (1958) with slight modifications. Briefly, sample absorbance was measured at 517 nm using EtOH as the blank and ascorbic acid as the standard. Radical scavenging activities of samples are expressed as ascorbic acid equivalents.

2.7. ORAC assay

ORAC values of each fraction were measured according to a previously described method (Cao, Verdon, Wu, Wang, & Prior, 1995; Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002) with slight modifications. This assay was performed based on the principle that antioxidant compounds delay decreases in fluorescein following the addition of the peroxyl radical generator AAPH. ORAC assays were performed using an ARVO™ X4 microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

The fluorescence of each microplate well was read every 2 min over a 90 min period at 37 °C. The area under the fluorescence curve was calculated, and the ORAC values for each sample were expressed as units for 1 μmol equivalents of Trolox. Each sample was measured in triplicate, and data were expressed as means ± standard deviations. The statistical analyses of ORAC values were performed using *t*-tests.

2.8. Animal studies

2.8.1. Animal and feeding procedures

Eight-week-old male rats (Wistar strain) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Rats were randomly divided into three groups of eight animals, and each animal was individually housed. Rats on the control diet group were fed an AIN-93G-modifeied basal diet (CLEA Japan Inc., Tokyo, Japan) and those of the positive control group were fed tea catechin supplemented basal diet (TC diet). A group of rats were fed a basal diet supplemented with a 5% non-extracted persimmon fraction instead of cellulose (NEP diet). Catechin contents in diets were calculated according to equivalent ORAC values in the NEP diet and β-cyclodextrin was added to reduce the bitterness of tea catechins. Study diet compositions are presented in Table 1.

The animals were fed *ad libitum*, and the food intake and body weights were monitored daily for 3 weeks. Blood was collected from tail veins weekly and plasma was isolated and stored at −80 °C. All animal procedures were performed according to Kio University's guidelines for the care and use of laboratory animals, which are in compliance with the Japanese Law for the Humane Treatment and Management of Animals.

2.8.2. Preparation of plasma samples for ORAC assays

Plasma ORAC values were determined according to the modified method of Leite et al. (2011). Briefly, plasma samples were removed from storage at −80 °C and were slowly thawed and shaken using a vortex. Plasma aliquots (50 μl) were then transferred into microtubes and 100 μl of EtOH and 50 μl of H_2O were added. Solutions were shaken for 30 s using a vortex, and 200 μl of 0.75 M metaphosphoric acid was then added. Subsequently, mixtures were shaken using a vortex and then centrifuged at 210*g* for 5 min at 10 °C. Prior to ORAC analyses, 80 μl of supernatant was diluted using 420 μl of 75 mM phosphate buffer (pH 7.4) to obtain the plasma solution. Further dilution (2–8 times) of the plasma solution was performed using 75 mM phosphate buffer (pH 7.4),