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Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha)

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Abstract

Water and methanol extracts of persimmon leaf tea were studied for antioxidant and radical-scavenging activities. Antioxidant activity was measured using a β-carotene bleaching method. The activity of water extract was very strong and 0.125% higher than that of 10 mM ascorbic acid. The scavenging activity against superoxide anion radicals by methanol extract was stronger than that of the water extract, while 0.05% concentration of methanol extract showed about 50% inhibition. DPPH radical-scavenging activity of water and methanol extracts was strong, and 0.1% water extract showed more than 90% inhibition. The hydroxyl radical-scavenging activity of the extracts, at 1%, was nearly equal to that of 1 mM ascorbic acid. These results show that persimmon leaf tea could be considered as a natural antioxidant source.

Keywords: Persimmon leaf tea; Antioxidant activity; Radical-scavenging activity; Total phenolics; Flavonoids

1. Introduction

Epidemiological data, as well as in vitro studies, strongly suggest that foods containing phytochemicals with antioxidant potential have strong protective effects against major disease risks including cancer, diabetes, cardiovascular diseases and Alzheimer's disease (Ames, Shigenga, & Hagen, 1993; Knekt et al., 1997; Willett, 2002). Consumption of fruits, vegetables, and teas has been strongly linked to reduced risk of those diseases (Kanekt, Jarvinen, Reunanen, & Maatela, 1996; Le-Marchand, Murphy, Hankin, Wilkens, & Kolonel, 2000; Xing, Chen, Mitchell, & Young, 2001). The protective action of those foods has been attributed to the presence of antioxidants, especially polyphenolic compounds and antioxidant vitamins, including ascorbic acid, tocopherol and β-carotene (Kalt & Kushad, 2000). Among various foods, green tea has been the most often reported to possess numerous beneficial effects (Mitscher et al., 1997). The pharmacological effects of green tea have been reviewed, including antioxidant activity, antimutagenic activity and anticancer activity. Several studies have conclusively shown that most of antioxidant activity is produced from polyphenolic compounds rather than from ascorbic acid, tocopherol or β-carotene (Eberhardt, Lee, & Liu, 2000; Hanasaki, Ogawa, & Fukui, 1994; Kähkönen et al., 1999; Wang, Cao, & Prior, 1996). In Japan, green tea is drunk almost every day and kakinoha-cha (Japanese persimmon leaf tea) is also a well-known indigenous tea, which has become increasingly popular as a health beverage.

Persimmon (*Diospyros kaki*) grows in the countries of East Asia, such as Japan, China and Korea. The fruit of persimmon is eaten as fresh or dry fruit and the leaves of this tree are infused with hot (rather than boiling) water and drunk as kakinoha-cha in the same way as green tea. It has traditionally been drunk in localities with many persimmon trees, in the mountainous areas of Japan. The fruits of the persimmon are known to contain persimmon tannin (Matsuo & Ito, 1978), which has been traditionally used for the treatment of hypertensive diseases (Kameda et al., 1987). Two flavonol glucosides, isolated from persimmon, were shown to have a hypotensive action in rats (Funayama & Hikino, 1979). The polyphenols, including condensed tannin of

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persimmon, are related to the various physiological actions. Leaf of persimmon is also considered to have persimmon tannin, but there are still some unclear points with regard to the content and physiological effects of persimmon leaf tannin.

There are very few studies of reactive oxygen species in relation to water and methanol extracts of persimmon leaf tea against autooxidation and free radicals, such as superoxide anion radical, DPPH radical and hydroxyl radical. In this study, the antioxidant activities of water and methanol extracts of persimmon leaf tea were evaluated by using widely accepted anti-autooxidation and free radical-scavenging model systems.

2. Materials and methods

2.1. Materials and chemicals

Japanese persimmon leaf tea, cut into pieces of about 2 cm in width and dried, was purchased from a local store in Hiroshima Prefecture. Folin–Ciocalteu reagent was obtained from Kanto Chemical Co. (Tokyo, Japan). (+)-Catechin was obtained from Sigma–Aldrich Co. (MO, USA). Gallic acid, sodium carbonate, L-ascorbic acid, 2,4-dinitrophenyl hydrazine, sodium nitrite, aluminium chloride, 2,2′-azobis (2-amidinopropane) dihydrochloride, nitroblue tetrazolium salt, xanthine, 1,1-diphenyl-2-picrylhydrazyl, 2-deoxy-D-ribose, 2-thiobarbituric acid and xanthine oxidase (from butter milk, 0.049 U/ml) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were of analytical grade.

2.2. Preparation of extracts of persimmon leaf tea

Water extract of persimmon leaf tea was obtained as follows. In brief, 50 g of persimmon leaf tea was suspended and extracted with 10 volumes of distilled water with shaking at 80 °C for 1 h. The extracts were filtered through a filter paper and the supernatants were pooled. The residue was re-extracted under the same conditions. Pooled supernatants were condensed with a rotary evaporator under reduced pressure at 50 °C and then condensed supernatants were lyophilized.

Methanol extract of persimmon leaf tea was obtained by the same procedure as water extract with the exception of the extraction temperature and period. Persimmon leaf tea was extracted with methanol at room temperature for 15 h. Pooled extracts were condensed (and methanol removed) with a rotary evaporator at 50 °C.

2.3. Determination of total phenolic content

Samples were analyzed spectrophotometrically for contents of total phenolics by a modified Folin–Ciocalteu colorimetric method (Singleton, Orthofer, & Lamuela-Raventós, 1999; Wolfe, Wu, & Liu, 2003). Volume of 0.5 ml of deionized water and 0.125 ml of a known dilution of the extract were added to a test tube, followed by addition of 0.125 ml of Folin–Ciocalteu reagent. They were mixed well and then allowed to stand 6 min before 1.25 ml of a 7% sodium carbonate solution was added. The mixture was diluted to 3 ml with deionized water. The colour was developed for 90 min at room temperature and the absorbance was measured at 760 nm using a spectrophotometer (UVIDEC-50, JASCO Corporation, Tokyo, Japan). The measurement was compared to a standard curve of prepared gallic acid solutions and expressed as means (±SD) mg of gallic acid equivalents per gramme for the triplicate extracts.

2.4. Determination of L-ascorbic acid content

Total L-ascorbic acid content was determined using a 2, 4-dinitrophenyl hydrazine method adapted from the Handbook of Food Analysis (Tadokoro, Kawada, Nakashima, & Tsujimura, 2000). All values were expressed as means (±SD) μg of ascorbic acid equivalent per gramme of dried leaf for the triplicate extracts.

2.5. Determination of total flavonoid content

Total flavonoid content was determined by using a colorimetric method described previously (Dewanto, Wu, Adom, & Liu, 2002; Zhishen, Mengcheng, & Jianming, 1999). Briefly, 0.25 ml of the extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water in a test tube, followed by addition of 75 μl of a 5% sodium nitrite solution. After 6 min, 150 μl of a 10% aluminium chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 ml of 1 M sodium hydroxide was added. The mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer (UVIDEC-50, JASCO Corporation, Tokyo, Japan). The results were expressed as means (±SD) mg of (+)-catechin equivalents per gramme gram for the triplicate extracts.

2.6. Determination of anti-autooxidant activity

The anti-autooxidant activity was assayed by using the β-carotene bleaching method (Kauer & Kapper, 2002; Miller, Rice-Evans, Davies, Gopinathan, & Miller, 1993; Wanasundara, Amarowicz, & Shahidi, 1994). β-Carotene (2 mg) was dissolved in 20 ml of chloroform. A 4 ml aliquot of the solution was added to a conical flask with 40 mg linoleic acid and 400 mg Tween 40. Chloroform was removed with a rotary evaporator at 50 °C. Distilled water (100 ml) was added to the β-carotene emulsion, mixed, and aliquots (3 ml) of the β-carotene emulsion and 0.2 ml of extracts were

placed in capped culture tubes and mixed well. The tubes were immediately placed in a water-bath and incubated at 50 °C. Oxidation of the β-carotene emulsion was monitored taking absorbance at 20 min intervals at 470 nm for 100 min. A control consisted of 0.2 ml distilled water instead of extract.

2.7. Superoxide anion-scavenging activity

The superoxide radicals were generated in vitro by the xanthine oxidase. The scavenging activity of the extract was determined by the nitro-blue tetrazolium (NBT) reduction method. In this method, O_2^- reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the purple NBT formation (Cos et al., 1998; Parejo et al., 2002). The capacity of the extracts to scavenge the superoxide radical was assayed as follows: the reaction mixture contained 0.5 ml of 0.8 mM xanthine in 0.1 mM phosphate buffer (pH 8.0), 0.48 mM NBT in 0.1 mM phosphate buffer (pH 8.0) and 0.1 ml of extract solution. After heating to 37 °C for 5 min, the reaction was started by adding 1.0 ml of XOD (0.049 U/ml) and carried out at 37 °C for 20 min; the reaction was stopped by adding 2.0 ml of 69 mM SDS. The absorbance of the reaction mixture was measured at 560 nm.

The results were expressed as the percentage inhibition of the NBT reduction with respect to the reaction mixture without any sample.

$$\%\text{inhibition} = [\{(C - CB) - (S - SB)\}/(C - CB)] \times 100,$$

where S, SB, C and CB are the absorbance of the sample, the blank sample, the control and the blank control, respectively.

2.8. DPPH radical-scavenging activity

The assay mixture contained 0.3 ml of 1.0 mM DPPH radical solution, 2.4 ml of ethanol and 0.3 ml of extract solution. The solution was rapidly mixed and after standing for 30 min, the absorbance of the mixture was measured at 517 nm (Nagai, Inoue, Inoue, & Suzuki, 2003).

$$\%\text{inhibition} = [\{(C - CB) - (S - SB)\}/(C - CB)] \times 100,$$

where S, SB, C and CB are the absorbance of the sample, the blank sample, the control and the blank control, respectively.

2.9. Hydroxyl radical-scavenging activity

The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method (Chung, Osawa, & Kawakishi, 1997). 2-Deoxyribose is oxidized by hydroxyl radical that is formed by the Fenton reaction and degraded to malondialdehyde (Gutteridge, 1984; Gutteridge, 1987). The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM $FeSO_4$–EDTA, 0.15 ml of 10 mM hydrogen peroxide, 0.525 ml of distilled water and 0.075 ml of extract solution in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37 °C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1.0% of thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice and then measured at 520 nm. Hydroxyl radical-scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical.

3. Results and discussion

3.1. Ascorbic acid, total phenolics and total flavonoid contents of persimmon leaf tea extracts

Water and methanol extracts were prepared from persimmon leaf tea (50 g) and their yields were 10.3 and 9.4 g, respectively. Table 1 shows the contents of ascorbic acid, total phenolics and total flavonoids of persimmon leaf tea extracts. Fresh leaf of persimmon is known to have a relatively high content of ascorbic acid (Matsuura, Asano, Ohba, & Mizuno, 1971), for example the content of ascorbic acid in fresh leaf was 22.1 mg/g in our analytical data, but the water extract in this experiment contained only 3.6 mg/g. This shows that the water-soluble ascorbic acid was lost during the preparation of the extract of persimmon leaf tea. Ascorbic acid is a heat-unstable vitamin and several studies shown the decline in ascorbic acid during the heat preparation and cooking of vegetables (Dewanto et al., 2002; Giovanelli et al., 2001). Thus, heating for the extraction of persimmon leaf tea probably led to a loss of ascorbic acid. The content of ascorbic acid in the extract might be too low to act as an antioxidant. On the other hand, the water extract contained a total of 112 and 58.4 mg/g of polyphenols and flavonoids, respectively.

3.2. Anti-autooxidation activity

We evaluated the antioxidant activity of persimmon leaf tea extracts by the β-carotene bleaching assay,

Table 1
Contents of ascorbic acid, total phenolics and total flavonoids in water and methanol extracts of persimmon leaf tea[a]

	Ascorbic acid (mg/g)	Total phenolics (mg/g)	Total flavonoids (mg/g)
Water extract	3.6 ± 0.3	112 ± 4.5	58.4 ± 2.8
Methanol extract	0	59.3 ± 2.1	29.2 ± 1.2

[a] Values are the means of three replicates ± standard deviation.

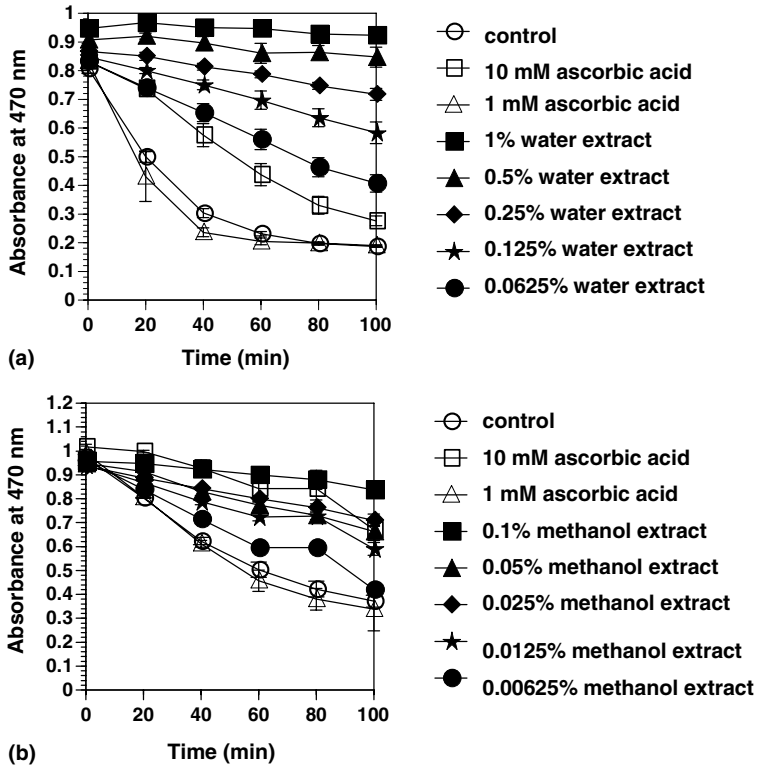

Fig. 1. Antioxidant activities of water and methanol extracts of persimmon leaf tea measured by bleaching of linoleic acid–carotene emulsion. Ascorbic acid (asc) was used as positive control. (a) Water extract of persimmon leaf tea; (b) methanol extract of persimmon leaf tea. Values are means ± SD ($n = 3$).

because β-carotene shows strong biological activity and is physiologically important compound (Kumazawa et al., 2002; Sarkar, Bishayee, & Chatterjee, 1995). Furthermore, β-carotene is used as a colouring agent for the beverages and its discolouration would markedly reduce the quality of those products.

Fig. 1 shows the decrease in absorbance of β-carotene in the presence of different concentrations of extracts with the oxidation of β-carotene and linoleic acid. Persimmon leaf tea extracts suppressed discolouration of β-carotene strongly as compared with 1 mM ascorbic acid and the control. The absorbance of the control dropped at a faster rate, 0.20–0.3 after 40 min, whereas, at 0.125% water extract of persimmon leaf tea, this rate was slower, maintaining 0.75 after 40 min and 0.70 after 60 min, respectively. The water extract (0.125%) and methanol extract (0.1%) of persimmon leaf tea showed much stronger antioxidant effects than did 10 mM ascorbic acid. Thus, it is apparent that persimmon leaf tea extracts have strong effects against the discolouration of β-carotene.

3.3. Superoxide anion-scavenging activity

Superoxide radicals have been observed to kill cells, inactivate enzymes and degrade DNA, cell membranes and polysaccharides (Fridovich, 1978). The radicals may also play an important role during the peroxidation of unsaturated fatty acids and possibly other susceptible substances (Nice & Robinson, 1992). Therefore, the study of the scavenging effects of persimmon leaf tea extracts on superoxide is one of the most important ways of illustrating the mechanism of antioxidant activity.

Superoxide-scavenging activities of water extract and methanol extract of persimmon leaf tea were measured using the xanthine–xanthine oxidase system and the results indicate the inhibition rate of superoxide activity. As shown in Fig. 2, each persimmon leaf tea sample exhibited superoxide-scavenging activity and these activities showed dose-dependence. Methanol extract of persimmon leaf tea had higher superoxide-scavenging activity than did water extract of persimmon leaf tea. These results show that persimmon leaf tea extracts have strong scavenging effects on superoxide radicals.

3.4. DPPH radical-scavenging activity

DPPH is a free radical compound and has been widely used to test the free radical-scavenging ability of various samples (Hatano, Takagi, Ito, & Yoshida, 1997; Shimoji et al., 2002). To evaluate the scavenging effects of DPPH of water extract and methanol extract of persimmon leaf tea, DPPH inhibition was investigated

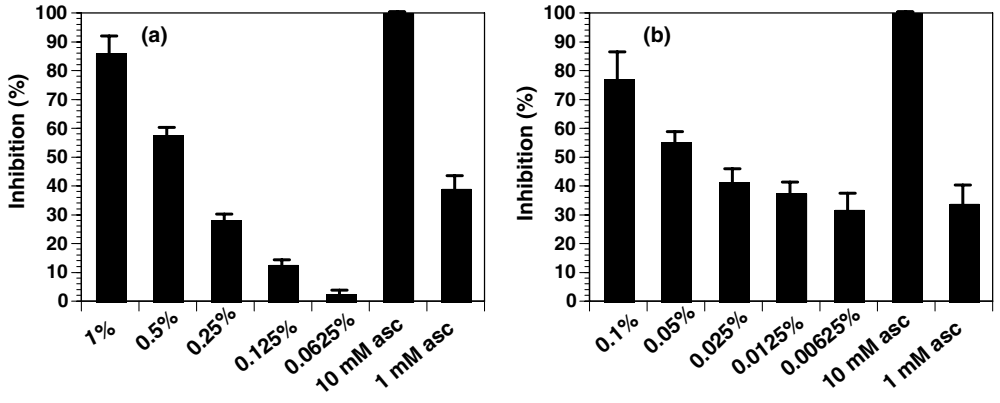

Fig. 2. Superoxide anion radical-scavenging activities of water and methanol extracts of persimmon leaf tea. Ascorbic acid (asc) was used as positive control. (a) Water extract; (b) methanol extract. Values are means ± SD ($n = 3$).

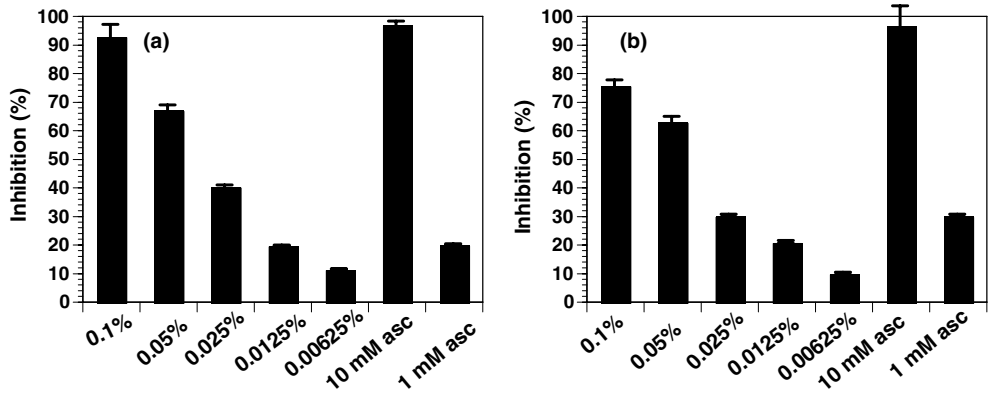

Fig. 3. DPPH radical-scavenging activities of water and methanol extracts of persimmon leaf tea. Ascorbic acid (asc) was used as positive control. (a) Water extract; (b) methanol extract. Values are means ± SD ($n = 3$).

and these results are shown as relative activities against the control (Fig. 3). The activity of 10 mM ascorbic acid was high, followed by persimmon leaf tea extracts. The water extract activity was a little higher than that of methanol extract. The activities of both extracts of persimmon leaf tea were dose-dependent.

3.5. Hydroxyl radical-scavenging activity

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules. The scavenging effect against hydroxyl radical was investigated by using the Fenton reaction. Fig. 4 shows the hydroxyl radical-scavenging effects by the 2-deoxyribose oxidation method. The results were indicated as the inhibition rate. Each extract showed hydroxyl radical-scavenging activity and its activity was increased with increasing concentration of the extract sample. The activity of 1% concentration of both extracts was nearly equal to that of 1 mM ascorbic acid.

The antioxidant activity was assayed by using several test systems. Recent investigations showed differences between the test systems for the determination of antioxidant activity (Gahler, Otto, & Böhm, 2003; Schlesier, Harwat, Böhm, & Bitsch, 2002). It was recommended to use at least two methods. In this study, we used several methods showing different sensitivities and using different systems. Persimmon leaf tea extracts showed antioxidant activity in all of the tested methods. This fact suggests that persimmon leaf tea is a good source of natural antioxidant.

On the other hand we are constantly exposed to a variety of oxidizing agents and continuous production of oxidants causes oxidation stress, which is associated with chronic diseases (Sun, Chu, Wu, & Liu, 2002). Therefore, consumption of foods containing various antioxidants has been recommended to prevent or slow the oxidative stress caused by free radicals. Many constituents of foods have been examined for their effectiveness against oxidative stress. Persimmon leaf tea has been drunk in Japan, but the antioxidant activity and total phenolic contents of the tea have not been clear. This paper reveals that persimmon leaf tea is rich in phenolics and has a strong antioxidant activity and a radical-scavenging action. These results show that drinking persimmon leaf tea may have health benefits

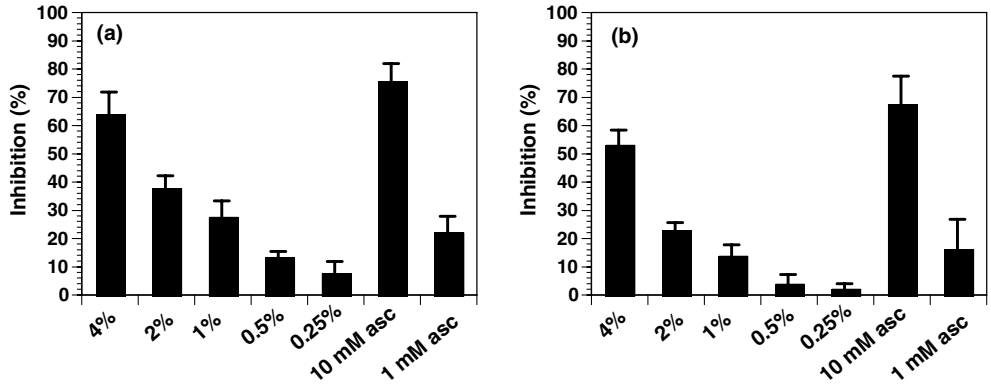

Fig. 4. Hydroxyl radical-scavenging activities of water and methanol extracts of persimmon leaf tea. Ascorbic acid (asc) was used as positive control. (a) Water extract; (b) methanol extract. Values are means ± SD ($n = 3$).

for consumers. We believe the tea shows potential as a functional food or value-added ingredient. The tea may assist in the prevention of chronic diseases. Further works on the characterization of antioxidant compounds in the extracts are in progress to establish the connection between antioxidant activity and chemical composition.

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