Evaluation to the antioxidant activity of total flavonoids extract from persimmon (*Diospyros kaki* L.) leaves

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ABSTRACT

Persimmon leaves are commonly consumed as beverages, but are also used as a popular folk medicine in China. The purpose of this work is to assess the antioxidant activity of an extract of total flavonoids from persimmon leaves (TFPL). The effect of TFPL on total antioxidant activity, reducing power, 1,1-diphenyl-2-picrylhydrazyl (DPPH$\cdot$) radical scavenging, superoxide anion ($\cdot O_2^-$) radical scavenging, hydroxyl (OH$\cdot$) radical scavenging and metal chelating activities was examined. We found that TFPL possesses considerable amounts of flavonoids (192 μg catechin equivalent/g of extract). The effect of this extract in total antioxidant activity, scavenging activity of superoxide anion and hydroxyl radical, reducing power and iron chelating activity was significantly better than that of rutin. However, the effect of TFPL in free radical scavenging of DPPH$\cdot$ was significantly not as good as than rutin. In addition, TFPL significantly decreased the level of reactive oxygen species (ROS) and malondialdehyde (MDA), while increasing the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in MC3T3-E1 cells in a dose-dependent manner. In conclusion, TFPL possess potent antioxidant and free radical scavenging activities. These antioxidant activities could contribute, at least in part, to the traditionally claimed therapeutic benefits of persimmon leaves.

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1. Introduction

Oxidative stress is a result of imbalance between the antioxidant defence system and the formation of reactive oxygen species (ROS). It is believed to damage cell membranes and DNA, as well as membrane lipid peroxidation with subsequent decreases in membrane fluidity (Finkel and Holbrook, 2000; Melov et al., 2000). Oxidative damage may cause cell injury, death and exacerbate the development of several age-related chronic diseases including cancer, Alzheimer's disease, Parkinson's disease and heart disease (Raouf et al., 2000). Therefore, antioxidants are considered to be important nutraceuticals with many health benefits. Antioxidants are widely used in the food industry as potential inhibitors of lipid peroxidation (Scherer and Godoy, 2009). However, many synthetic antioxidants used in foods, such as butylated hydroxyanisole and butylated hydroxytoluene, may accumulate in the body, resulting in liver damage and carcinogenesis (Valentao et al., 2002; Luo and Fang, 2008). For this reason, more attention has been paid to natural non-toxic antioxidants in an effort to protect the human body from free radicals and retard the progress of many chronic diseases.

Persimmon (*Diospyros kaki* L.) is a kind of plant native to China, used traditionally for many medicinal purposes, including the treatment of paralysis, frostbite, and burns, and to stop bleeding (Matsuo and Ito, 1978). Flavonoid oligomers, tannins, phenols, organic acids, chlorophyll, vitamin C, and caffeine are found in persimmon leaves (Matsuo and Ito, 1978; Jo et al., 2003). The leaves are commonly used for tea in Asia. Previous studies have shown that persimmon leaves have beneficial effects on haemostasis, constipation, hypertension, apoplexy and atherosclerosis (Kotani et al., 2000; Matsumoto et al., 2002; Tanaka et al., 2003; Sakanaka et al., 2005). In particular, flavonoid aglycones in persimmon leaves, such as catechin, kaempferol, and quercetin, reportedly possess strong antioxidant activities by acting as oxygen radical scavengers and metal chelators (Morel et al., 1993; Birt et al., 2001). Chemically, flavonoids and isoflavonoids are one-electron donors. They are derivatives of conjugated ring structures and hydroxyl groups that have the potential function as antioxidants in cell culture *in vitro*, or in cell free systems. The previous studies suggest that the flavonoids present in persimmon leaves could contribute to the health benefits attributed to persimmon (Bei et al., 2005, 2009; Lee et al., 2006).

The balance between osteoclastic bone resorption and osteoblastic bone formation maintains bone mass at a homeostatic steady state. The imbalance that occurs when bone resorption is greater than bone formation can lead to skeletal diseases, including

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osteoporosis. Oestrogen deficiency causes osteoporosis via increased generation of ROS. Several reports have demonstrated that osteoblast differentiation can be inhibited by oxidative stress or induced by exogenous stimuli such as hydrogen peroxide or xanthine/xanthine oxidase (Mody et al., 2001; Bai et al., 2004). These findings suggest that ROS may represent a critical target for the treatment and/or prevention of osteoporosis. For this reason, antioxidants may prove to be effective therapeutic candidates for osteoporosis (Riggs et al., 2002). If a total flavonoid extract of persimmon leaves (TFPL) is able to prevent free radical damage in osteoblast-like cells *in vitro*, it should be able to prevent cell death. The prevention of bone loss by the inhibition of oxidative stress and the prevention of osteoblast death *in vitro* could be considered as groundwork for the therapeutic benefit of TFPL. The clinical experiment is still needed to investigate the therapeutic effects of TFPL administration to humans, especially those at highest risk of osteoporosis, i.e., elderly women.

To our knowledge, few investigations have been made of the antioxidant properties of persimmon leaves, although this medicinal plant is widely used by Chinese traditional healers. The aims of this study were to determine the total flavonoid content of persimmon leaves and to evaluate the properties of TFPL using widely accepted antioxidative and free radical-scavenging model systems. Rutin, a lipid-soluble analogue of flavonoids, was used as a reference antioxidant compound (Yang et al., 2008). In addition, the levels of ROS and antioxidant enzymes such as superoxide-dismutase (SOD) and glutathione (GSH-Px) were also determined in MC3T3-E1 cells after treatment with TFPL. The results suggest that TFPL could be useful as a potential natural antioxidant in the functional food and pharmaceutical industries.

2. Materials and methods

2.1. Materials

Persimmon (*D. kaki* L. *folium*) leaves were harvested in Shaanxi Province, China in October 2010, and identified according to the identification standard of the Pharmacopeia of the People's Republic of China. Persimmon (*D. kaki* L. *folium*) leaves were dried in the shade for a week and powdered and passed through 60 mesh sieves. The raw material was stored at −20 °C before extraction. The reagents 1, 1-diphenyl-2-picrylhydrazyl (DPPH·), ferrozine, Dimethylsulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma Chemicals (St Louis, MO, USA). Assay kits for superoxide anion radical (·O$_2^-$), hydroxyl radical (OH·), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), protein, and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). All other reagents were of analytical grade and made in China.

2.2. Preparation of extracts of persimmon leaves

Persimmon leaves (50 g) were cut into pieces approximately 2 cm in width and dried. Leaves were soaked in a 70% ethanol solvent (1:10, w/v) for 2.5 h and then placed in an ultrasonic bath and sonicated at 200 kHz at 55 °C for 45 min. Samples were then filtered through a 0.45 μm microporous membrane (Shanghai Wanzi Shi-ye Co. Ltd., Shanghai, China). The filtrate was collected, and the solid was extracted two more times with the same volume of fresh solvent. Solutions were combined and concentrated to dryness under reduced pressure in a rotary evaporator to yield dried crude total extracts. The extract was added to distilled water and defatted with petroleum ether and ethyl acetate.

2.3. Determination of total flavonoid content

The total flavonoid content of the extract was determined by the method described in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2005). The 500 μl extract was diluted appropriately and mixed with 1 mL NaNO$_2$ (5%). After standing for 6 min, 1 mL of 10% AlCl$_3$ and 10 mL of NaOH (1 M) were added to the mixture. The mixture was adjusted to 25 mL with 70% ethanol and allowed to rest for 15 min. The absorbance (A) was measured at 510 nm, with 70% ethanol as a blank control. Rutin was used as a reference standard and the total flavonoid content was expressed as rutin equivalents (RE, μg/mg extract). All determinations were performed in triplicate.

2.4. Antioxidant activity assays

2.4.1. Total antioxidant capacity (TAOC)

Suitable working standards (0.24, 1.0, 5.0, and 10 mg/mL) were prepared by dissolving the TFPL in distilled water. Aliquots (0.30 mL) were mixed with 3 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped with aluminium foil and incubated at 95 °C for 90 min (Umamaheswari and Chatterjee, 2008). The tubes were cooled to room temperature and absorbance was measured at 695 nm against a blank. Ascorbic acid was used as a standard. Total antioxidant capacity was expressed as equivalents of ascorbic acid (Raghavan et al., 2003).

2.4.2. Radical scavenging activity

2.4.2.1. DPPH· radical scavenging activity.
The ability of the TFPL to scavenge the DPPH· free radical was assayed according to the method of Shimada et al. (1992). Briefly, a 0.1 mM solution of DPPH· in 100% MeOH was prepared. To 1 ml of this solution was added 4 ml of sample solution in 40% MeOH at different concentrations (1–160 μg/mL). The mixture was shaken vigorously and incubated for 15 min in the dark at room temperature until stable absorption values were obtained. The reduction of the DPPH· radical was measured by continuously monitoring the decrease in absorption at 517 nm. In the control, 40% MeOH was substituted for samples. Lower absorbances of the reaction mixture indicated higher free radical scavenging activity. The DPPH· radical scavenging activity was calculated by the following equation:

$$\text{Scavenging effect } (\%) = (1 - A_{\text{sample517}}/A_{\text{control517}}) \times 100$$

The EC$_{50}$ value is the concentration of the sample required to scavenge 50% of the DPPH free radical.

2.4.2.2. Superoxide anion radical scavenging activity.
The measurement of superoxide anion scavenging activity was based on the method described by Nishikimi et al. (1972) with slight modifications. Superoxide radicals were generated in the PMS–NADH system containing 3 mL Tris–HCl buffer (16 mM, pH 8.0), 338 μM NADH (adenine dinucleotide), 72 μM NBT (nitroblue tetrazolium), and 30 μM PMS (phenazine methosulphate). Varying concentrations of samples ranging from 25 to 400 μg/mL were added to the PMS–NADH system for free radical scavenging. These reaction mixtures were incubated at room temperature for 5 min before the absorbance was read at 560 nm against the blank. In the control, the sample was substituted with Tris–HCl buffer. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The ability of TFPL to scavenge superoxide radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = (1 - A_{\text{sample560}}/A_{\text{control560}}) \times 100$$

EC$_{50}$ value (mg/ml) is the concentration at which the scavenging activity is 50%.

2.4.2.3. Hydroxyl radical scavenging activity.
The hydroxyl radical scavenging activity of samples of TFPL was measured using a modified Smirnoff and Cumbes' (1989) method. Hydroxyl radicals were generated in a solution of 2 mM EDTA–Fe (0.5 mL), 3% H$_2$O$_2$ (1 mL), and 360 μg/mL crocus in 4.5 mL sodium phosphate buffer (150 mM, pH 7.4). Samples at concentrations ranging from 25 to 400 μg/mL were incubated for 30 min at 37 °C and hydroxyl radicals were detected by monitoring absorbance at 520 nm. In the control, the sample was substituted with distilled water and sodium phosphate buffer replaced H$_2$O$_2$. The capability of hydroxyl radical scavenging was calculated using the following equation:

$$\text{Scavenging effect } (\%) = (1 - A_{\text{sample520}}/A_{\text{control520}}) \times 100$$

EC$_{50}$ value (mg/ml) is the concentration at which the scavenging activity is 50%.

2.4.3. Reducing power

The reducing power of TFPL was determined using the method of Yen and Chen (1995). Briefly, 0.13 mL of different concentrations of samples (25–400 μg/mL) suspended in phosphate buffer (0.2 M, pH 6.6) were mixed with 0.125 mL of potassium ferricyanide (1%, w/v) and incubated at 50 °C. At 20 min, 0.125 mL of trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. The solution was mixed with 1.5 ml of ferric chloride (0.1%, w/v), and the absorbance was measured at 700 nm. An increased absorbance of the reaction mixture indicated increased reducing power.

2.4.4. Chelating activity on ferrous ions

The chelating activities of test compounds on Fe^{2+} were estimated based on the decrease in the maximal absorbance of the iron (Fe^{2+})–ferrozine complex assayed following previously reported methods (Dinis et al., 1994), with some modifications. Briefly, 1.0 ml of test compounds dissolved in ethanol were incubated with 0.5 ml of FeCl$_2$·4H$_2$O (1.0 mmol/L). The reaction was initiated by the addition of 1 ml of ferrozine (5.0 mmol/L), and the total reaction volume was adjusted to 4 ml with ethanol. After the mixture had reached equilibrium (10 min), the