Antioxidant activities of ethanolic and acidic ethanolic extracts of astringent persimmon in H₂O₂-stimulated Caco-2 human colonic epithelial cells

Leeseon Kim¹ · Yunyoung Kim¹ · Oran Kwon^{1,2} · Ji Yeon Kim¹

Received: 26 December 2016 / Revised: 19 April 2017 / Accepted: 3 May 2017 / Published online: 18 August 2017
© The Korean Society of Food Science and Technology and Springer Science+Business Media B.V. 2017

Abstract The aim of this study was to investigate the protective effects of ethanol (EAP) and acidic ethanol extracts (AEAP) of astringent persimmon fruits (*Diospyros kaki*) against hydrogen peroxide (H₂O₂)-induced oxidative stress in Caco-2 epithelial cells. Levels of reactive oxygen species (ROS) and reduced glutathione (GSH), activities of superoxide dismutase (SOD) and catalase (CAT), as well as protein and DNA damage were measured. Treatment with H₂O₂ induced oxidative stress in Caco-2 cells but had no effect on SOD activity. Both EAP and AEAP protected the cells against oxidative damage and exhibited similar effects on cellular ROS scavenging activity and protein carbonyl levels. However, AEAP was more potent than EAP in normalizing CAT activity and cytosolic GSH level, and in protecting against DNA damage. These results demonstrated that astringent persimmon fruit extracts were biologically active, and that the tannin-rich fraction (AEAP) possesses stronger protective activity against H₂O₂-induced oxidative stress in Caco-2 cells.

Keywords Antioxidant · Astringent persimmon · Caco-2 cells · Hydrogen peroxide · Tannin

Introduction

The intestinal epithelium, which is the interface between an organism and its luminal environment, is prone to oxidative stress [1]. Among various oxidants, the hydrophobic bile acid, deoxycholic acid, has been reported to induce disturbances in cell membranes, consequently, increasing oxidative stress [2]. Because tannins and polyphenols contained in fruits show very limited absorption rates [3], they can be expected to persist in the gut and act as good physiological antioxidants.

Persimmon (*Diospyros kaki*) is one of the most popular fruits consumed in Korea and is known to contain sugars, vitamin C, carotenoids, and anthocyanins as its major constituents [4–7]. Among the various bioactive compounds found in astringent persimmon, tannins in particular show excellent capacity to bind to bile salts in the gut [8], and have been reported to exhibit potent antioxidant effects [9]. Tannins contained in persimmons are present as highly condensed forms and comprise flavan-3-ols, such as catechin, catechin-3-O-gallate, gallic catechin, and gallic catechin-3-O-gallate [10]. These highly condensed persimmon tannins barely dissolve in water and organic solvents [11], but can be partially solubilized in 1% hydrochloric acid (HCl) in methanol [12–14]. Thus, in order to extract both soluble and insoluble tannins from astringent persimmon, acidic methanol has been utilized as an extraction agent; however, methanol is not an appropriate solvent for use with food ingredients as it is toxic to humans [15]. In our previous study, ethanol was used as a substitute for methanol in the extraction of tannins from astringent persimmon, and the acidic ethanol extract of astringent persimmon was confirmed to contain higher levels of tannins with a high capacity to bind bile salts than that of ethanol extract [16]. In that study, acidic ethanol

✉ Ji Yeon Kim
jjiyeonk@seoultech.ac.kr

¹ Department of Food Science and Technology, Seoul National University of Science and Technology, Seoul 01811, South Korea

² Department of Nutritional Science and Food Management, Ewha Womans University, Seoul 03760, South Korea

extract of astringent persimmon significantly inhibited nitrite production in Caco-2 cells stimulated with deoxycholic acid and prevented significant reduction of trans-epithelial electrical resistance, suggesting that the acidic ethanol extract of astringent persimmon could act as an antioxidant in the gut. Astringent persimmon may contain other polyphenolic compounds or soluble tannins having a high antioxidant capacity in addition to insoluble tannins [13, 17]. Therefore, in the present study, a tannin-rich fraction and a non-specific polyphenol fraction were extracted using acidic ethanol and ethanol, respectively; both are suitable for food manufacturing. Levels of reactive oxygen species (ROS) and glutathione (GSH), activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), as well as protein and DNA damage were measured in Caco-2 cells under hydrogen peroxide (H_2O_2)-induced oxidative stress. The antioxidant activities of the acidic ethanol and ethanol fractions were compared using this model of human colonic epithelial cell oxidative stress.

Materials and methods

Reagents

Hydrogen peroxide was obtained from Daejung (Goryeong-gun, Korea). Thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and 2,7-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were procured from Biowest (Nuaille, Cholet, France). Penicillin–streptomycin and non-essential amino acids (NEAA) were obtained from Gibco (Rockville, MD, USA).

Preparation of astringent persimmon extracts

Astringent persimmon powder was provided by the Korean Rural Development Administration (Jeonju, Jeonranamdo, Korea). Briefly, 2 g of spray-dried astringent persimmon (Cheongdobanshi) powder was added to 20 mL of solvent (either 70% ethanol or 70% ethanol with 1% HCl) and the mixture was shaken for 24 h. After the impurities were removed, each extract was concentrated and solvent was removed by vacuum evaporation (EYELA, Tokyo, Japan) at 60 °C in a water bath for 30 min. For the acidic ethanol extract, after evaporation, the residues were washed with distilled water to remove water-soluble constituents. Finally, each extract (ethanol extract, EAP; acidic ethanol extract, AEAP) was freeze-dried at −83 °C and 5–8 mTorr vacuum for 36 h (Ilshinbiobase, Kyunggido, Korea). From

2 g dried astringent persimmon fruit powder, the yield of EAP and AEAP was 1.13 and 1.51 g, respectively.

Caco-2 cell culture

The Caco-2 cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). For routine maintenance, cells were grown in DMEM supplemented with 10% FBS, penicillin–streptomycin, and NEAA at 37 °C in an atmosphere of 5% CO_2 /95% air and saturating humidity. The cells were passaged once a week (1:3 split ratio) with 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution (Biowest). For the experiments, Caco-2 cells (1×10^5 cells/mL) were seeded into a 6-well plate and cultivated for 5 days. The cells were then incubated with the sample (EAP or AEAP) in FBS-free media for 24 h at various concentrations (0 and 500 $\mu\text{g/mL}$ for ROS detection; 0, 62.5, 250, and 500 $\mu\text{g/mL}$ for other experiments). Control cells were treated with vehicle (DMSO). Then, cells were treated with H_2O_2 (50 μM) for 3 h. For analysis, culture supernatant and cell pellets were collected and frozen at −80 °C. The supernatant was used for quantification of ROS level, while the cell pellets were used for the other analyses.

MTT assay

The viability of Caco-2 cells was determined using an MTT assay. Briefly, the cells (1×10^5 cells/mL) were seeded into a 96-well plate and cultivated for 6 days at 37 °C in humidified air containing 5% CO_2 . Cells were treated with EAP or AEAP (500 $\mu\text{g/mL}$) for 24 h. Then, the MTT reagent (20 μL , 0.5 mg/mL) was added and cells were incubated for 3 h. The yellow MTT was reduced to purple formazan by viable cells. The formazan crystals were dissolved with 100 μL DMSO, and absorbance was measured at 560 nm using a microplate reader (Biotek, Winooski, VT, USA). The administered concentration of the extracts was not cytotoxic to Caco-2 cells (data not shown).

Measurement of intracellular oxidative stress

Oxidative stress was quantified in cells using 2,7-dichlorofluorescein (DCF) assay, as described previously with slight modifications [8]. After treatment of samples for 24 h with FBS-free medium, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and then incubated with 1 mM DCF-DA. Cells were treated with H_2O_2 (50 μM) for 3 h and washed with DPBS. The fluorescence signal ($\lambda_{\text{excitation}} = 480 \text{ nm}$, $\lambda_{\text{emission}} = 530 \text{ nm}$) was measured in the dark using a fluorescence spectrophotometer (PerkinElmer, Waltham, MA, USA) and

also observed with a fluorescence microscope (Motic, British Columbia, Canada). Results were expressed as relative fluorescence levels compared to the control (% of control).

Activities of SOD and CAT

Catalase activity was measured according to a previously reported method with slight modifications [18, 19]. Cells were lysed using 10 mM Tris, 1 mM EDTA (pH 7.4). Formaldehyde (standard) and the lysate (sample) were then added to a mixture of phosphate buffer, methanol, and H_2O_2 . After vortexing, potassium hydroxide and Purpald[®] were mixed with the solution for 10 min at 20 °C. After the addition of potassium periodate, the solution was analyzed with a microplate reader (Biotek). Levels of SOD were measured using an assay kit according to manufacturer's instruction (Biovision, Milpitas, CA, USA).

Total GSH level

For analysis of total cellular GSH content, cells were lysed with 5% sulfosalicylic acid. After centrifugation, total GSH content in the lysate was measured. The supernatant and a standard GSH solution were mixed with a working solution made of 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, glutathione reductase, and 1.5 mg/mL dithionitrobenzoic acid. Then, NADPH (0.16 mg/mL) was added, and the absorbance was measured at 412 nm for 5 min.

Protein carbonyl levels

The level of protein damage was measured using the OxiSelect[™] protein carbonyl ELISA kit (Cell Biolabs Inc, San Diego, CA, USA) according to the manufacturer's protocol. Protein damage was assessed by measuring the absorbance of the sample at 450 nm using ELISA.

Comet assay

After treatment with the samples and H_2O_2 , Caco-2 cells were collected in cold PBS. The cell suspension and 1% low gelling temperature agarose were mixed and rapidly spread onto a slide to avoid producing bubbles. After the agarose had gelled, slides were submerged in a dish containing lysis solution for 1 h at 4 °C. After lysis, the slides were washed with distilled water three times and submerged in an alkaline solution for 20 min. Then, slides were placed in the electrophoresis solution; electrophoresis was performed for 30 min at a voltage of 31 V (Bio-Rad, Hercules, CA, USA). The slides were then washed twice with distilled water and once with 70% ethanol, before

drying at room temperature overnight. For determination of comet formation, the DNA was stained with 50 μL of ethidium bromide. Slides were scored blindly; for each experiment, two slides were prepared and 50 cells were analyzed in each slide with a computer-aided image analysis system (Comet Assay IV; Perceptive Instruments Ltd, Haverhill, UK).

Statistical analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple range test. A value of $P < 0.05$ was considered statistically significant versus the H_2O_2 -treated group.

Results and discussion

Effect of EAP and AEAP on cell viability and ROS level in Caco-2 cells stimulated with H_2O_2

The cytotoxicity of EAP and AEAP on Caco-2 cells was first assessed to determine the appropriate treatment concentration for subsequent experiments. In this test, treatment with EAP and AEAP up to 1000 $\mu\text{g}/\text{mL}$ exhibited no cytotoxicity (data not shown), despite AEAP being highly acidic ($\sim\text{pH}$ 2.5). Therefore, subsequent analyses of antioxidant effects were performed at 500 $\mu\text{g}/\text{mL}$ for both extracts. When considering the extraction yield and volume of human intestine (approximately 105 mL), the treated concentrations (500 $\mu\text{g}/\text{mL}$) for EAP and AEAP were equivalent to 93.20 and 69.46 mg, respectively [20]. Considering that the spray-drying yield for astringent persimmon fruit was 16.13%, the treated concentrations were equivalent to 577.81 and 430.63 mg of persimmon fruit, respectively. Therefore, considering that persimmon tannins are rarely absorbed, the treated concentrations were within normal levels in the gut environment, and likely lower than the levels expected from whole fruit.

Intracellular oxidant formation was determined by measuring ROS levels in H_2O_2 -treated Caco-2 cells using the DCF-DA staining method. Caco-2 cells were incubated with 500 $\mu\text{g}/\text{mL}$ EAP or AEAP for 24 h, and then exposed to 50 μM H_2O_2 for 3 h. Intracellular ROS level in Caco-2 cells treated with H_2O_2 increased significantly up to three times that of control cells (Fig. 1A). Both EAP and AEAP at 500 $\mu\text{g}/\text{mL}$ significantly suppressed intracellular oxidative stress induced by H_2O_2 ($P < 0.05$, vs. H_2O_2 -treated group). When comparing EAP and AEAP, the inhibitory activity of AEAP was greater than that of EAP at the same treatment concentration. At the lowest

Fig. 1 Effect of EAP and AEAP treatment on reactive oxygen species (ROS) in Caco-2 cells stimulated with H₂O₂. The level of cellular ROS production was quantified (A) and visualized by fluorescence imaging (B). Bars represent the mean $\pm$ SD ($n = 3$). * $P < 0.05$, versus H₂O₂-treated cells as analyzed by ANOVA followed by Dunnett's test. CON: treated with vehicle only, H₂O₂:

treated with vehicle and H₂O₂, EAP: treated with 70% ethanol extract of astringent persimmon (at the indicated concentrations) and H₂O₂, AEAP: treated with 70% ethanol with 1% HCl extract of astringent persimmon (at the indicated concentrations) and H₂O₂. DCF, 2,7-dichlorofluorescein

concentration (62.5 µg/mL), EAP did not show a suppressive effect, but AEAP significantly inhibited ROS production. The level of intracellular oxidative stress was also visualized using fluorescence cell imaging (Fig. 1B). Astringent persimmon is rich in condensed tannins, which have high antioxidant capacity [21], and contains various polyphenols such as anthocyanins [22]. In this experiment, AEAP, which was the tannin-rich fraction, as well as EAP showed similar ROS scavenging activity. Our result suggests that both AEAP and EAP are effective in scavenging free radicals and enhancing the cellular antioxidant defense system.

Effect of EAP and AEAP on antioxidant enzyme activities as well as GSH and protein carbonyl levels in Caco-2 cells stimulated with H₂O₂

Cellular antioxidant capacity is determined by both ROS-scavenging ability and endogenous cellular macromolecules, which decompose ROS [23]. Endogenous cellular defense systems are composed of GSH and antioxidant enzymes such as SOD, CAT, and glutathione peroxidase (GPx) [24]. Both SOD and GPx catalyze the conversion of superoxide anion (O₂⁻) to molecular oxygen (O₂) and hydrogen peroxide (H₂O₂). Then, H₂O₂ is converted to water and oxygen by CAT [25]. Additionally, GPx converts reduced GSH to glutathione disulfide (GSSG), which can be recycled back to GSH via the activity of NADPH-dependent glutathione reductase [25].

Accordingly, SOD and CAT activities and GSH levels in Caco-2 cells challenged with H₂O₂ were measured in order to investigate the effect of EAP and AEAP on the antioxidant capacity of these cells. Prior to H₂O₂ challenge, Caco-2 cells were incubated with EAP or AEAP for 24 h. The activity of SOD was not affected by H₂O₂ treatment

(Fig. 2A). On the contrary, H₂O₂ challenge significantly increased CAT activity by approximately 1.3-fold, while EAP and AEAP treatments reduced CAT activity even at low doses (62.5, 250, and 500 µg/mL; Fig. 2B). The effect of AEAP in lowering CAT activity was superior to that of EAP. The response of antioxidant enzyme activities to H₂O₂ has been shown to differ. At different H₂O₂ concentrations, catalase activity remained elevated; however, SOD activity varied in Caco-2 cells. SOD activity peaked with 50–100 µM H₂O₂ and decreased above 100 µM H₂O₂ with 30 min of treatment [26]. In the present study, H₂O₂ treatment was 50 µM for 3 h. Therefore, the response of SOD to H₂O₂ might vary in Caco-2 cells according to time and concentration of treatment.

Cytosolic GSH levels in Caco-2 cells decreased significantly by approximately 40% after H₂O₂ treatment, while EAP and AEAP increased the GSH level (Fig. 2C). Interestingly, the GSH level after AEAP treatment at the highest dose (500 µg/mL) was higher than that of the control group. These results show that the tannin-rich AEAP fraction possessed higher cellular antioxidant capacity than EAP. Elevated levels of GSH could indicate that EAP and AEAP can stimulate transcription of antioxidant response element (ARE)-related genes, including heme oxygenase 1, GSH S-transferase, γ -glutamylcysteine synthetase, and NAD(P)H:quinone oxidoreductase [27]. However, in this study, we only measured antioxidant activities, not mRNA or protein expression levels, which is a study limitation. In this present work, we aimed to find differences in antioxidant activities, started with housekeeping proteins that were more refractory to changes, and found differences that explained the cell physiology observation. Therefore, whether EAP and AEAP affect the expression of ARE-related genes and the mechanisms involved should be investigated further.

Fig. 2 Antioxidant enzyme activities (SOD; **A**, Catalase; **B**) and GSH levels (**C**) in Caco-2 cells stimulated with H₂O₂. Bars represent the mean $\pm$ SD ($n = 3$). * $P < 0.05$, versus H₂O₂-treated cells as analyzed by ANOVA followed by Dunnett's test. CON: treated with vehicle only, H₂O₂: treated with vehicle and H₂O₂, EAP: treated with

70% ethanol extract of astringent persimmon (at the indicated concentrations) and H₂O₂, AEAP: treated with 70% ethanol with 1% HCl extract of astringent persimmon (at the indicated concentrations) and H₂O₂. SOD superoxide dismutase, GSH reduced glutathione

Effect of EAP and AEAP on DNA and protein damage in Caco-2 cells stimulated with H₂O₂

Oxidative stress induced by ROS can cause damage to lipids, proteins, and DNA. However, consumption of dietary antioxidants may ameliorate such damage [28]. The most commonly used marker for assessing protein oxidation resulting from ROS attack is the formation of protein-bound carbonyls [29]. Protein carbonyls are formed early during oxidative stress conditions and are not a result of one specific oxidant; thus, they are markers of overall

protein oxidation [29]. In the present study, H₂O₂ challenge of Caco-2 cells significantly increased protein carbonyl levels by approximately 4.5-fold, whereas EAP and AEAP dose-dependently attenuated the increase in protein carbonyl levels to similar extents (Fig. 3A).

However, in the comet assay to assess DNA damage, AEAP was more effective than EAP in protecting the DNA (Fig. 3B). As DNA is a major target of oxidative damage, oxidative stress often results in DNA strand breaks [30]. The comet assay is a sensitive technique that detects DNA breakage at the single cell level even at an early stage [31].

Fig. 3 Effect of EAP and AEAP treatment on protein carbonyl level (**A**) and DNA damage (**B**) in Caco-2 cells stimulated with H₂O₂. DNA damage was analyzed using a comet assay. Bars represent the mean $\pm$ SD ($n = 3$). * $P < 0.05$, versus H₂O₂-treated cells as analyzed by ANOVA followed by Dunnett's test. CON: treated with

vehicle only, H₂O₂: treated with vehicle and H₂O₂, EAP: treated with 70% ethanol extract of astringent persimmon (at the indicated concentrations) and H₂O₂, AEAP: treated with 70% ethanol with 1% HCl extract of astringent persimmon (at the indicated concentrations) and H₂O₂

Values of all DNA-damage parameters, such as tail length, intensity, and moment, increased after H₂O₂ challenge in Caco-2 cells; the damage to DNA was dose-dependently suppressed by EAP and AEAP administration. Based on the tail length and moment, AEAP more strongly suppressed DNA damage than EAP.

Among various bioactive compounds contained in astringent persimmon, highly condensed tannins are known as major antioxidant compounds [32]. High-molecular-weight condensed tannin from persimmon was characterized to be composed of myricetin terminal units, along with flavan-3-ols and a high amount of prodelphinidin [33]. A previous study reported that prodelphinidin, having a high number of reactive hydroxyls, increased the antioxidant capacity of proanthocyanidins [34]. On the contrary, a recent study reported that soluble tannins had superior antioxidant capacity compared with that of insoluble tannins [13]. The present results demonstrated that astringent persimmon fruit extracts, regardless of tannin content, were biologically active and protected against H₂O₂-induced oxidative stress in Caco-2 cells. These results led us to the conclusion that tannin extracts obtained from astringent persimmon could be developed as functional ingredients to protect from oxidative stress in the gut environment. Comparing the antioxidant activity of EAP and AEAP, the latter was slightly superior in preventing oxidative DNA damage and maintaining cellular GSH levels. However, our study did not reveal whether the antioxidant effects of EAP and AEAP involved enhancing the cellular defense system or the radical scavenging activity [9]. The use of ethanol, instead of methanol, was confirmed to produce AEAP with a high antioxidant capacity. The acid used in this preparation was not suitable for food manufacturing; to use these extraction processes for food manufacturing, organic acids should be considered instead of HCl. Furthermore, the identity of the compounds that contributed to the antioxidant activity was not clarified. The mechanisms of action of the astringent persimmon extracts and the active compounds responsible for the antioxidant effects will be investigated in future studies.

Acknowledgements This study was funded by the “Cooperative Research Program for Agriculture Science & Technology Development (No. PJ01169502)” of the Rural Development Administration, Republic of Korea.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Circu ML, Aw TY. Intestinal redox biology and oxidative stress. *Sem. Cell Dev. Biol.* 23: 729–737 (2012)
2. Da Silva M, Jagers GK, Verstraeten SV, Erlejan AG, Fraga CG, Oteiza PI. Large procyanidins prevent bile-acid-induced oxidant production and membrane-initiated ERK1/2, p38, and Akt activation in Caco-2 cells. *Free Radic. Biol. Med.* 52: 151–159 (2012)
3. Smeriglio A, Barreca D, Bellocchio E, Trombetta D. Proanthocyanidins and hydrolysable tannins: occurrence, dietary intake and pharmacological effects. *Br. J. Pharmacol.* DOI: [10.1111/bph.13630](https://doi.org/10.1111/bph.13630) (2016)
4. Candir EE, Ozdemir AE, Kaplankiran M, Toplu C. Physico-chemical changes during growth of persimmon fruits in the East Mediterranean climate region. *Sci. Hortic. (Amsterdam)*. 121: 42–48 (2009)
5. Del Bubba M, Giordani E, Pippucci L, Cincinelli A, Checchini L, Galvan P. Changes in tannins, ascorbic acid and sugar content in astringent persimmons during on-tree growth and ripening and in response to different postharvest treatments. *J. Food Comp. Anal.* 22: 668–677 (2009)
6. Schmitzer V, Slatnar A, Mikulic-Petkovsek M, Veberic R, Krska B, Stampar F. Comparative study of primary and secondary metabolites in apricot (*Prunus armeniaca* L.) cultivars. *J. Sci. Food Agric.* 91: 860–866 (2011)
7. Bilal S, Khan AL, Waqas M, Shahzad R, Kim ID, Lee IJ, Shin DH. Biochemical constituents and in vitro antioxidant and anti-cholinesterase potential of seeds from native Korean persimmon genotypes. *Molecules*. 21 (2016)
8. Matsumoto K, Yokoyama S, Gato N. Bile acid-binding activity of young persimmon (*Diospyros kaki*) fruit and its hypolipidemic effect in mice. *Phytother. Res.* 24: 205–210 (2010)
9. Jang IC, Jo EK, Bae MS, Lee HJ, Jeon GI, Park E, Yuk HG, Ahn GH, Lee SC. Antioxidant and antigenotoxic activities of different parts of persimmon (*Diospyros kaki* cv. Fuyu) fruit. *J. Med. Plants Res.* 4: 155–160 (2010)
10. Li CM, Leverence R, Trombley JD, Xu SF, Yang J, Tian Y, Reed JD, Hagerman AE. High molecular weight persimmon (*Diospyros kaki* L.) proanthocyanidin: a highly galloylated, a-linked tannin with an unusual flavonol terminal unit, myricetin. *J. Agric. Food Chem.* 58: 9033–9042 (2010)
11. Serrano J, Puupponen-Pimia R, Dauer A, Aura AM, Saura-Calixto F. Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Mol. Nutr. Food Res.* 53: S310–S329 (2009)
12. Matsumoto K, Kadowaki A, Ozaki N, Takenaka M, Ono H, Yokoyama S, Gato N. Bile acid-binding ability of Kaki-tannin from young fruits of persimmon (*Diospyros kaki*) in vitro and in vivo. *Phytother. Res.* 25: 624–628 (2011)
13. Shin YJ, Shon MS, Kim GN, Lee SC. Antioxidant and anti-adipogenic activities of persimmon tannins. *Food Sci. Biotechnol.* 23: 1689–1694 (2014)
14. Oshida M, Yonemori K, Sugiura A. On the nature of coagulated tannins in astringent-type persimmon fruit after an artificial treatment of astringency removal. *Postharvest Biol. Technol.* 8: 317–327 (1996)
15. Barceloux DG, Bond GR, Krenzelok EP, Cooper H, Vale JA, Hoc AACTA. American Academy of Clinical Toxicology practice guidelines on the treatment of methanol poisoning. *J. Toxicol. Clin. Toxicol.* 40: 415–446 (2002)
16. Kim L KO, Kim JY. Comparison of protective effects of young and ripened persimmon extracts against inflammatory stress induced by deoxycholic acid in small intestinal cells. *J. Korean Soc. Food Sci. Nutr.* 44: 1583–1587 (2015)
17. Celep E, Aydin A, Yesilada E. A comparative study on the in vitro antioxidant potentials of three edible fruits: Cornelian cherry, Japanese persimmon and cherry laurel. *Food Chem. Toxicol.* 50: 3329–3335 (2012)

18. Akerboom TP, Sies H. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods Enzymol.* 77: 373–382 (1981)
19. Johansson LH, Borg LA. A spectrophotometric method for determination of catalase activity in small tissue samples. *Anal. Biochem.* 174: 331–336 (1988)
20. Schiller C, Frohlich CP, Giessmann T, Siegmund W, Monnikes H, Hosten N, Weitschies W. Intestinal fluid volumes and transit of dosage forms as assessed by magnetic resonance imaging. *Aliment. Pharmacol. Ther.* 22: 971–979 (2005)
21. Matsumura Y, Ito T, Yano H, Kita E, Mikasa K, Okada M, Furutani A, Muroto Y, Shibata M, Nishii Y, Kayano S. Antioxidant potential in non-extractable fractions of dried persimmon (*Diospyros kaki* Thunb.). *Food Chem.* 202: 99–103 (2016)
22. Yang S, Jiang Y, Xu L, Shiratake K, Luo Z, Zhang Q. Molecular cloning and functional characterization of DkMATE1 involved in proanthocyanidin precursor transport in persimmon (*Diospyros kaki* Thunb.) fruit. *Plant Physiol. Biochem.* 108: 241–250 (2016)
23. Kim YJ, Ahn YH, Lim Y, Kim JY, Kim J, Kwon O. Daily nutritional dose supplementation with antioxidant nutrients and phytochemicals improves DNA and LDL stability: a double-blind, randomized, and placebo-controlled trial. *Nutrients.* 5: 5218–5232 (2013)
24. Kaushik S, Kaur J. Chronic cold exposure affects the antioxidant defense system in various rat tissues. *Clin. Chim. Acta.* 333: 69–77 (2003)
25. Guemouri L, Perrin R, Artur Y, Minn A, Siest G. Automated methods for clinical assay of superoxide-dismutase and glutathione-peroxidase in human-plasma and erythrocytes using a Cobas Bio Centrifugal Analyzer. *Clin. Chem.* 35: 1126 (1989)
26. Wijeratne SSK, Cuppett SL, Schlegel V. Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. *J. Agric. Food Chem.* 53: 8768–8774 (2005)
27. Kim JK, Jang HD. 6-shogaol attenuates H₂O₂-induced oxidative stress via upregulation of Nrf2-mediated gamma-glutamylcysteine synthetase and heme oxygenase expression in HepG2 cells. *Food Sci. Biotechnol.* 25: 319–327 (2016)
28. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell.* 48: 158–167 (2012)
29. Weber D, Davies MJ, Grune T. Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions. *Redox Biol.* 5: 367–380 (2015)
30. Lourenco JI, Pereira RO, Silva AC, Morgado JM, Carvalho FP, Oliveira JM, Malta MP, Paiva AA, Mendo SA, Goncalves FJ. Genotoxic endpoints in the earthworms sub-lethal assay to evaluate natural soils contaminated by metals and radionuclides. *J. Hazard. Mater.* 186: 788–795 (2011)
31. Azqueta A, Collins AR. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch. Toxicol.* 87: 949–968 (2013)
32. Gu HF, Li CM, Xu YJ, Hu WF, Chen MH, Wan QH. Structural features and antioxidant activity of tannin from persimmon pulp. *Food Res. Int.* 41: 208–217 (2008)
33. Souquet JM, Cheynier V, Brossaud F, Moutounet M. Polymeric proanthocyanidins from grape skins. *Phytochemistry.* 43: 509–512 (1996)
34. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 20: 933–956 (1996)