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Full Length Research Paper

Antioxidant and antigenotoxic activities of different parts of persimmon (*Diospyros kaki* cv. Fuyu) fruit

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This study was conducted to determine the antioxidant activities and the antigenotoxic effects of acetone, ethanol, methanol and water extracts from four different parts (calyx, seed, peel and flesh) of persimmon fruits. Antioxidant activities were evaluated by DPPH (1,1-diphenyl-2-picryllhydrazyl) radical scavenging activity (RSA), ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammononium salt) RSA and reducing power (RP). Seed and calyx extracts showed significantly ($p < 0.05$) higher antioxidant activities and phenolic contents than peel and flesh extracts. This study also showed that ethanol was more effective on the extraction of antioxidant compounds from persimmon compared to other solvents. The antigenotoxic effects of the persimmon extracts on DNA damage induced by H_2O_2 in human leukocytes was evaluated by Comet assay. All persimmon extracts inhibited DNA damage induced by 200 µM of H_2O_2. Especially, calyx and seed extracts showed stronger inhibition activity than peel and flesh extracts. Therefore, these results suggest that persimmon extracts may have beneficial effects on oxidant and protective effect against oxidative DNA damage.

Key words: Persimmon extract, antioxidant activities, antigenotoxic effect.

INTRODUCTION

Oxidative stress, caused by the imbalance of reactive oxygen species (ROS) and antioxidative defense systems, is considered as a major etiological and/or pathogenic agent of most degenerative diseases such as cancer, Alzheimer's, diabetes and aging (Datta et al., 2000). The antioxidants are of interest in the treatment of several cellular degenerations, and they inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions (Behera et al., 2006). Antioxidants interfere with the production and the activation of free radicals (Machlin and Bendich, 1987), resulting in protecting the human body from free radicals that may cause some chronic diseases including cancer and cardiovascular diseases (Kinsella et al., 1993). They are also used to preserve food by retarding rancidity, discoloration or deterioration due to auto-oxidation (Huang et al., 2003). Though synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxyltoluene and propyl gallate, have been commonly added to food products to retard lipid oxidation, the demand for natural antioxidants has increased because of questions and negative perception of consumers about the long-time safety of synthetic antioxidants (Yu et al., 2002). Regular consumption of fruit and vegetables containing natural antioxidants is correlated with the decreased risk of diseases such as cancer and cardiovascular diseases (Michels et al., 2000).

Persimmon (*Diospyros kaki* L.), which belongs to the Ebenaceae family, is cultivated in a wide area, including Eastern Asia, Spain and Israel. Persimmon fruit is known to have many bioactive compounds such as polyphenols,

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carotenoids, dietary fiber and minerals. For example, persimmon contains 70 mg of vitamin C, while apple contains only 4 mg in 100 g of its flesh part (New Food Composition Table Editing Committee, 2009). Persimmon has been traditionally used for many medicinal purposes such as blood pressure-lowering effect, diuretic effect and coughs, and it is probably involved in the reduction of degenerative human diseases (Steinmetz and Potter, 1996). Although there are some reports regarding antioxidant activity of fruits and peels of persimmon (Fukai et al., 2009; Lee et al., 2008; Jung et al., 2005), little work has been done on the comparative research about the functional activity of different parts of persimmon fruit. Thus, the aim of this study was to evaluate the antioxidant and antigenotoxic activities of four different parts (calyx, flesh, peel and seed) of persimmon extracted by four different solvents (acetone, ethanol, methanol and water).

MATERIALS AND METHODS

Materials

Persimmon (*D. kaki* cv. Fuyu) fruits were provided from Sweet Persimmon Research Institute (Gimhae, Korea). L-Ascorbic acid (Vitamin C), 2,2-azino-bis (3 - ethylbenzothiazoline-6-sulfonic acid) diammononium salt (ABTS), butylated hydroxyltoluene (BHT), dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrogen peroxide, potassium chloride, potassium ferricyanide and potassium phosphate were also purchased from Sigma Chemical Co. (St. Louis, MO). All the other organic solvents and chemicals used in this study were of analytical grade.

Preparation of extracts from persimmon fruits

Fresh persimmon fruits were divided into four parts (flesh, peel, calyx and seed), and each parts were freeze-dried with freeze dryer (Ilshin Lab., Seoul, Korea). The dried persimmon fruit parts were ground in a mill and passed through a 25-mesh sieve. Each 5 g of dried parts (flesh, peel, calyx and seed) was extracted with 100 ml of four different solvents (acetone, ethanol, methanol and water) overnight at room temperature and filtered through a Whatman No. 1 filter paper. Solvents were then removed by evaporation *in vacuo*, and the dried extracts were obtained. Each dried extract was re-dissolved in DMSO with concentration of 50 mg/ml for the experiments, and diluted with DMSO when needed. All samples were placed in a glass bottle and stored at 4℃ until used. Extraction yield was calculated by the following formula:

Extraction yield (%) = [dried extract weight/dried sample weight] × 100

DPPH radical scavenging activity (RSA)

The DPPH RSA of each extract was determined according to the method of Jeong et al. (2004). After a 0.1 ml aliquot of extract dissolved in DMSO was mixed with 0.9 ml of 0.041 mM DPPH in ethanol for 30 min, the optical density (OD) of the sample was measured at 517 nm using a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan). RSA was expressed as a percentage inhibition and it was calculated by the following formula:

DPPH RSA (%) = [1 – (sample OD/control OD)] × 100

ABTS radical scavenging activity (RSA)

The ABTS RSA was evaluated with the method of Muller (1985). Each extract (0.1 ml), potassium phosphate buffer (0.1 ml, 0.1 M, pH 5.0) and hydrogen peroxide (20 µl, 10 mM) were mixed and pre-incubated at 37℃ for 5 min. After pre-incubation, ABTS (30 µl, 1.25 mM, in 0.05 M phosphate-citrate buffer, pH 5.0) and peroxidase (30 µl, 1 unit/ml) were added to the mixture, and then it was incubated at 37℃ for 10 min. The OD level was obtained with a multiplate reader (Sunrise RC/TS /TS Color-TC/TW/BC/6Filter, Tecan Austria GmbH, Grödig, Austria) at 405 nm, and the ABTS RSA was calculated by the following formula:

ABTS RSA (%) = [1 – (sample OD/control OD)] × 100

Reducing power (RP)

The RP of each extract was determined according to the method of Oyaizu (1986). The extract (1.0 ml), sodium phosphate buffer (1.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide (1.0 ml, 10 mg/ml) were mixed and incubated at 50℃ for 20 min. Trichloroacetic acid (1.0 ml, 100 mg/ml) was added to the mixture and then the mixture was centrifuged at 13,400×*g* for 5 min. The supernatant (1.0 ml) was mixed with distilled water (1.0 ml) and ferric chloride (0.1 ml, 1.0 mg/ml), and then its absorbance was measured at 700 nm.

Total phenolic contents (TPC)

TPC in the each extract were determined according to the method of Gutfinger (1981). Each extract (1.0 ml) at concentration of the 1,000 µg/ml was mixed with 1.0 ml of 2% Na_2CO_3 after standing 3 min and 0.2 ml of 50% Folin-Ciocalteu reagent added to the mixture after standing for 30 min. The mixture was centrifuged at 13,400×*g* for 5 min. The absorbance was measured at 750 nm and TPC were expressed as gallic acid equivalents.

DNA damage by alkaline Comet assay

Leukocytes were isolated as a fraction of mononuclear cells (containing lymphocytes and monocytes) from anonymous buffy coat preserves by gradient centrifugation with HISTOPAQUE®-1077 (Sigma, St. Louis, MO, USA). Leukocytes were incubated with each extracts in DMSO (50 µg/ml) for 30 min at 37℃ in a dark incubator. For oxidative stimulus, they were then resuspended in PBS with 200 µM H_2O_2 for 5 min on ice. After each treatment, samples were centrifuged at 250×*g* for 5 min and washed with PBS. One percent of DMSO without oxidative stimulus was treated for negative control. The leukocytes were then mixed with 75 µl of 0.7% low melting agarose, and added to the slides precoated with 0.5% agarose. The slide was then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 hr at 4℃. The slides were then placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min. For electrophoresis of the DNA, an electric current of 25 V/300 ± 3 mA was applied for 20 min at 4℃. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4℃, and then treated with ethanol for another 5 min before staining with 50 µl of ethidium bromide (20 µg/ml). Measurements were carried out by an image analyzer

(Kinetic Imaging, Komet 4.0, U.K) and a fluorescence microscope (LEICA DMLB, Germany) to determine the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

Statistical analysis

All measurements were performed in triplicate, and analysis of variance was conducted by the General Linear Model using SAS software (1995), Student-Newman-Keul's multiple range tests were used to compare the significant differences of the mean values among treatments ($p < 0.05$). The data for Comet assay were the means of three determinations and was analyzed using the SPSS package for Windows (Ver. 12). The mean values of the DNA damage (tail intensity) from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A p-value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Extraction yields

The extraction yields of four different parts of persimmon were presented in Table 1. Relatively higher extraction yields were obtained from flesh and peel parts than those of calyx and seed parts. Among solvents, methanol was a most effective solvent on the extraction, exhibiting the highest extraction yields at most parts, except for seed. The highest extraction yield was found in the methanol extract from the flesh part with 91.02%, while the water extract of seed part (2.02%) had the lowest extraction yield. These results showed that the extraction yield varied by solvents and persimmon parts, indicating that each part consists of different components.

DPPH radical scavenging activity (RSA)

The scavenging activity on DPPH free radical is a common method to evaluate the antioxidative activity of antioxidants. The DPPH RSA of extracts from persimmon fruits was evaluated by comparing with ascorbic acid and BHT, which are well-known natural and synthetic antioxidants, respectively. As shown in Table 2, extracts from seed and calyx parts had relatively strong DPPH RSA (low IC_{50} value), exhibiting high antioxidant capacity compared to extracts from peel and flesh parts. Ethanol extract of seed and acetone extract of calyx showed the lowest IC_{50} values with about 48 μg/ml, which was only 1.78 fold lower than that of vitamin C (IC_{50} = 26.90 μg/ml) and 6.46 fold higher than that of BHT (IC_{50} = 310.16 μg/ml). Except for water, most solvents were effective to extract antioxidants from calyx and seed. Extract from peel part showed higher DPPH RSA than that from flesh part. Similar with our results, Choi et al. (2006) reported that 70% ethanol extract of persimmon peel showed significant higher DPPH RSA than that of flesh. Ahn et al. (2002) demonstrated that persimmon seed extract showed higher DPPH RSA than grape seed extract. The present results showed that extract from persimmon calyx by acetone had the strongest DPPH RSA. This might be due to the difference on (1) the amount and the form of tannin which is the main phenolic compound of persimmon fruit (Gu et al., 2008), (2) the amount of antioxidant carotenoid compounds (Veberic et al., 2010) and (3) the amount of vitamin C (Hosotani et al., 2004).

ABTS radical scavenging activity (RSA)

Like the results of DPPH RSA, the scavenging effect of extracts from four parts of persimmon fruit on the ABTS radicals also followed this order; calyx > seed > peel > flesh (Table 3). The highest ABTS RSA was detected in ethanol extract of calyx (IC_{50} = 18.16 μg/ml), exhibiting similar IC_{50} value compared with ascorbic acid and BHT (IC_{50} = 18.81 and 15.75 μg/ml, respectively). The ABTS method is suitable for monitoring of lipophilic antioxidants such as carotenoids and lipophilic extracts of nutritional components (Nicoletta et al., 1999). This fact can explain why water (most polar solvent) extracts from calyx, seed and peel parts exhibited the lower ABTS RSA compared with other solvent extracts. In flesh part, however, water extract showed the highest ABTS RSA, indicating that antioxidant compounds differ by parts of persimmon fruits.

Reducing power (RP)

The antioxidant ability of certain compounds is associated with their RP (Jayaprakasha et al., 2001), thus the RP may serve as a significant indicator of potential antioxidant activity (Meir et al., 1995). RP was determined by measuring the reduction of the Fe^{3+} form of Fe^{3+}/ferricyanide complexes to the ferrous (Fe^{2+}) form. The RP of extracts was evaluated and listed in Table 4. Extracts from calyx and seed parts showed significantly ($p < 0.05$) higher RP than peel and flesh parts, regardless of solvents. For example, ethanol extract from seed had the highest RP (IC_{50} = 72.41 μg/ml), and methanol extract from flesh showed the lowest RP (IC_{50} = 21111.50 μg/ml). Overall trend was very similar with other antioxidant activities described before.

Total phenolic contents (TPC)

Table 5 shows TPC of each extract at concentration of 1 mg/ml. Extracts from the calyx and the seed of persimmon fruits showed significantly ($p < 0.05$) higher TPC than extracts from peel and flesh parts. The highest TPC was found in acetone extract from seeds with 97.92 mg GAE/g, probably due to high amount of antioxidant compounds including phenolic compound such as tannin (Gorinstein et al., 1994). For solvents, acetone was a most effective solvent to extract phenolics from calyx, seed and peel, whereas water extract showed the highest

Table 1. Extraction yields from each part of persimmon by solvents.

(Unit: %)

Solvents	Parts of persimmon			
	Calyx	Seed	Peel	Flesh
Methanol	26.68^{cw}	7.87^{dx}	69.82^{bw}	91.02^{aw}
Ethanol	23.00^{cx}	6.80^{dy}	58.86^{by}	75.94^{ax}
Acetone	9.68^{bz}	2.02^{dz}	3.48^{cz}	11.64^{az}
Water	18.92^{by}	9.12^{dw}	67.41^{ax}	17.60^{cy}

Different letters within a row (a-d) and a column (w-z) are significantly different ($p < 0.05$).

Table 2. DPPH radical scavenging activity of extracts from different parts of persimmon by solvents

(Unit: IC_{50}[1)]).

Solvents	Parts of persimmon				Positive controls	
	Calyx	Seed	Peel	Flesh	Vit-C	BHT
Methanol	58.76 ± 2.14^{ay}	68.90 ± 0.52^{by}	1993.38 ± 59.76^{cy}	17308.18 ± 1823.21^{dz}	26.90 ± 0.15	310.16 ± 3.98
Ethanol	53.92 ± 1.93^{bx}	47.92 ± 0.72^{aw}	2388.19 ± 62.58^{cz}	15812.26 ± 562.60^{dy}		
Acetone	48.86 ± 1.19^{aw}	61.23 ± 1.55^{bx}	479.41 ± 7.77^{cw}	8768.91 ± 48.35^{dx}		
Water	196.12 ± 11.53^{az}	1354.56 ± 22.26^{cz}	1133.33 ± 48.35^{bx}	1590.87 ± 10.81^{dw}		

All measurements were done in triplicate, and all values are means ± standard deviation.
Different letters within a row (a-d) and a column (w-z) are significantly different ($p < 0.05$), n=3.
[1)] IC_{50} (μg/ml): Concentration for scavenging 50% of DPPH radicals.

Table 3. ABTS radical scavenging activity of extracts from different parts of persimmon by solvents

(Unit:IC_{50}[1)]).

Solvents	Parts of persimmon				Positive controls	
	Calyx	Seed	Peel	Flesh	Vit-C	BHT
Methanol	19.45 ± 0.37^{ax}	29.36 ± 0.96^{bx}	263.21 ± 27.44^{cx}	7137.59 ± 853.67^{dz}	18.81 ± 0.57	15.75 ± 1.12
Ethanol	18.16 ± 0.38^{aw}	36.63 ± 0.56^{by}	822.89 ± 36.39^{cy}	2398.40 ± 638.65^{dx}		
Acetone	22.42 ± 0.86^{ay}	34.86 ± 2.09^{by}	63.84 ± 3.56^{cw}	3184.71 ± 568.58^{dy}		
Water	43.51 ± 1.73^{az}	1055.77 ± 18.65^{bz}	3005.31 ± 360.91^{dz}	1765.30 ± 227.93^{cw}		

All measurements were done in triplicate, and all values are means ± standard deviation.
Different letters within a row (a-d) and a column (w-z) are significantly different ($p < 0.05$), n=3.
[1)] IC_{50} (μg/ml): Concentration for scavenging 50% of ABTS radicals.

Table 4. Reducing power of extracts from different parts of persimmon by solvents

(Unit: IC_{50}[1)]).

Solvents	Parts of persimmon				Positive controls	
	Calyx	Seed	Peel	Flesh	Vit-C	BHT
Methanol	127.13 ± 12.51^{by}	84.44 ± 0.67^{ay}	2119.50 ± 6.03^{cy}	21111.50 ± 36.26^{dz}	24.01 ± 0.15	52.32 ± 0.22
Ethanol	96.25 ± 2.60^{bx}	72.41 ± 0.37^{ax}	4094.00 ± 26.23^{cz}	21152.00 ± 97.93^{dz}		
Acetone	120.18 ± 3.17^{by}	88.89 ± 0.47^{ay}	683.17 ± 74.69^{cx}	7012.00 ± 791.36^{dy}		
Water	370.70 ± 6.15^{az}	1389.00 ± 9.62^{bz}	4361.00 ± 207.27^{cz}	6162.57 ± 13.31^{dx}		

All measurements were done in triplicate, and all values are means ± standard deviation.
Different letters within a row (a-d) and a column (x-z) are significantly different ($p < 0.05$), n=3.
[1)] IC_{50} (μg/ml): Concentration for increasing 0.500 value in optical density.

TPC in flesh part. The order of TPC of extracts from four different parts of persimmons nearly coincided with that of them, indicating that there was a correlation between the TPC and other antioxidant properties of extracts.

Table 5. Total phenolic contents of extracts from different parts of persimmon by solvents

Unit: mg GAE[1)]/g).

Solvents	Parts of persimmon			
	Calyx	Seed	Peel	Flesh
Methanol	79.20 ± 1.17^{by}	82.43 ± 0.84^{ay}	6.10 ± 0.13^{cz}	4.54 ± 0.13^{dy}
Ethanol	81.81 ± 0.91^{by}	87.15 ± 0.37^{ax}	6.92 ± 0.36^{cy}	4.27 ± 0.13^{dz}
Acetone	91.30 ± 1.06^{bx}	97.92 ± 0.19^{aw}	17.99 ± 0.29^{cw}	5.29 ± 0.21^{dx}
Water	42.04 ± 0.56^{az}	12.28 ± 0.32^{bz}	9.35 ± 0.11^{cx}	8.60 ± 0.24^{dw}

All measurements were done in triplicate, and all values are means ± standard deviation.
Different letters within a row (a-d) and a column (w-z) are significantly different ($p < 0.05$), n=3.
[1)] GAE: gallic acid equivalents

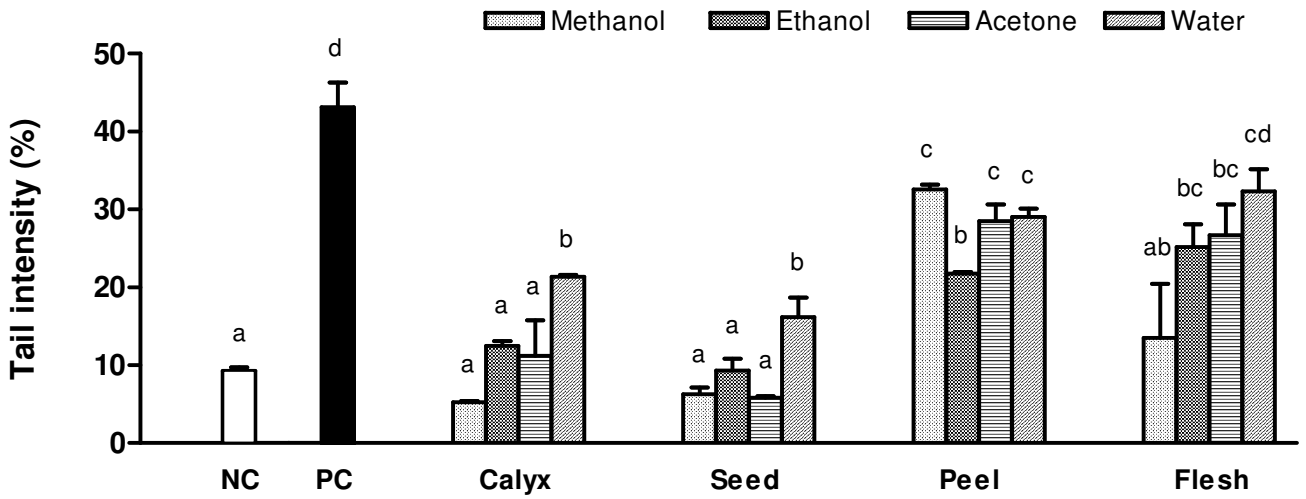

Figure 1. The effect of extracts (50 μg/ml) from persimmon calyx, seed, peel and flesh by methanol, ethanol, acetone and water on 200 μM H_2O_2–induced DNA damage in human leukocyte. Values are mean with standard deviation of triplicate experiments. NC, 1% DMSO treated negative control; PC, 200 μM H_2O_2 treated positive control. Values not sharing the same letter are significantly different from one another ($p < 0.05$) by Duncan's multiple range test.

Protective effect of persimmon on oxidative DNA damage in human leukocytes

The genotoxic effects of H_2O_2 and the protective ability of each extract were assessed in normal human leukocytes by the comet assay (Figure 1). At concentration of 50 μg/ml, the solvents (acetone, ethanol and methanol) extracts of calyx and seed, and methanol extract of flesh exhibited the greatest protective effect on H_2O_2 induced DNA damage, showing insignificant difference ($p > 0.05$) between extracts and DMSO treated negative control. The extracts from peel and flesh had relatively low protective effect compared to the extracts from calyx and seed.

Active oxygen species or free radicals, such as singlet oxygen ($^1O_2$), super oxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) are considered to cause oxidative damage to DNA, which bring about a variety of diseases, as well as aging (Sohal and Weindruch, 1996). Persimmon contains many bioactive compounds, such as polyphenols, flavonoids, and carotenoids (New Food Composition Table Editing Committee, 2009). Carotenoids, the most potent biological quenchers of singlet O_2, act as chain-breaking antioxidants (Liebler, 1993), and flavonids inhibit the enzymes responsible for $O_2^{\bullet-}$ production (Hanasaki et al., 1994). β-Carotene and flavonoids have been reported to prevent DNA damage against H_2O_2 in human lymphocytes assessed by the Comet assay (Astley et al., 2004; Noroozi et al., 1998). Likewise, numerous studies have demonstrated that polyphenolic compounds reduce DNA damage in human lymphocytes when assessed with the Comet assay (Kapiszewska et al., 2005). However, though flesh extracts showed the lowest TPC in every solvent (Table 5), they exhibited higher antigenotoxic activity than calyx and seed extracts (Figure 1). Therefore, it may be stated that antigenotoxic effect of persimmon extracts may be due to presence of some other constituents like β-carotene (Takahashi et al., 2006), tannin (Lee et al., 2007) and ascorbic acid (New Food Composition Table Editing Committee, 2009) instead of only polyphenolic compounds.

Conclusion

This study was the first attempt to show the difference in the functional activities of persimmon calyx, seed, peel and flesh. The results showed that extracts from calyx and seed had higher antioxidant activities and antigenotoxic activity compared to extracts from peel and flesh, regardless of solvents. In addition, this study revealed that ethanol was better solvent to extract antioxidant compounds from persimmon compared with other solvents. Therefore, this study suggests the potential use of persimmon extracts by ethanol as a good antigenotoxic compounds and a natural antioxidant by modulating oxidant metabolism by stimulation of antioxidant activity.

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